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RESEARCH PAPER

Development of an ELISA to detect clenbuterol in swine products using a new approach for hapten design

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Abstract This research outlines the application of an enzyme-linked immunosorbent assay (ELISA) for the analysis of clenbuterol in animal products. Our assay showed good sensitivity for clenbuterol (0.4 ng/g or 0.4 ppb) and low detection limit (0.09 ng/g or 0.09 ppb). A low cross-reactivity for other β_2-agonist drugs such as salbutamol, terbutaline, and epinephrine led to formatting an ELISA kit considered to have a high specificity for clenbuterol. A survey of Ho Chi Minh City pork market was conducted as part of the validation of our ELISA. ELISA results showed a surprisingly high value of contamination. However, it will be necessary to conduct a more statistically valid replicated survey with evaluation by other instrumental methods to obtain a definite conclusion. This ELISA kit will be used to monitor growth promoter residues in Vietnam's animal products.

Keywords ELISA · Clenbuterol · Hapten synthesis · β_2-agonist

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Introduction

Clenbuterol (CLEN) is structurally a derivative of adrenaline. In addition to β_2-adrenoceptor effects of relaxing smooth muscle, clenbuterol improves nitrogen retention, induces a reduction of body fat, and promotes muscle growth [1]. Hence, farm animals can be produced more economically. On the other hand, residues of CLEN have been reported to accumulate in edible animal tissues [2]. Its residues can have adverse action on the human respiratory and cardiac system [3]. Therefore, the use of CLEN to fatten cattle and other livestock is a potential health hazard for consumers.

As a consequence, CLEN is not allowed to be used as a growth promoter in many countries such as the USA, Europe, China, and more recently Vietnam [2]. Therefore, monitoring for CLEN residues in animal-derived products is an important food contaminant preventive method. To detect the use of illegal xenobiotic drugs and to prevent the use of inappropriate therapeutic doses, liquid chromatography-mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS) have been recommended as the suitable detection methods [1, 3, 4]. However, due to the shortage of trained technicians and expensive instruments, these methods are less available in rural area where CLEN is suspected to be frequently used. From a public health point of view, it is essential to have an accurate, economic, and simple operating assay for screening CLEN residues.

Immunoassays are considered to be an effective analytical screening method because they have high sensitivity, are simple to run, and are cost-effective [5, 6]. Hence, we decided to develop an enzyme-linked immunosorbent assay (ELISA) test to improve the sensitivity and selectivity compared to previous research products. We also chose pork samples collected in Vietnam as the most appropriate samples to evaluate the performance of our test kits. Besides, this study was to investigate the occurrence of CLEN residue contamination in pork in Vietnam, and then establish a quality assurance system to help minimize the risk of food poisoning from CLEN residues.

Materials and methods

Materials and instruments

- Bovine serum albumin (BSA) horseradish peroxidase (HRP) was purchased from Roche, France. Keyhole limpet hemocyanin (KLH) and dialysis tubing, Tween 20, secondary goat anti-rabbit antibody, all of organic chemicals, and Freund incomplete adjuvants were obtained from Sigma-Aldrich, USA. Sample of clenbuterol was obtained from Jianfeng Pharmaceutical Company, Beijing, China. Maxisorp polystyrene 96-well microplates were from Nunc, Denmark. Silica gel H was obtained from Merck, Darmstadt, Germany.
- ^{1}H Nuclear magnetic resonance (NMR) was recorded on a Varian Gemini-300 instrument (300 MHz) using $CDCl_3$ as a solvent and tetramethylsilane (TMS) as internal reference. Chemical shifts were reported in δ (ppm) relative to the internal reference. Silica gel $60F_{254}$ pre-coated plates with visualization under exposure to either UV light or iodine vapor. Flash column vacuum chromatography was carried out on Merck silica gel H (TLC grade). Evaporation of organic solvents was carried out on a Buchi rotary evaporator under water pump reduced pressure.

Methods

Hapten synthesis

Synthesis of 1-(4-amino-3, 5-dichlorophenyl)-2-[tert-butyl-(2-mercapto-ethyl)-amino]-ethanol (hapten 1) A free thiol group was attached to CLEN in order to bind to a maleimide group of the modified carrier protein. This hapten was synthesized via the reaction between CLEN and ethylene sulfide [6] (Fig. 1).

Ethylene sulfide (0.5 g; 0.84 mmol) was added to a stirred suspension of clenbuterol (0.58 g; 0.21 mmol) in toluene (3 mL) in a sealed reaction vessel. The reaction was heated to 70 °C and followed by TLC. The reaction was shown to be almost complete after 48 h. The toluene was removed by distillation under reduced pressure, and the residue was dissolve in ethyl acetate, washed three times with water, and dried by sodium sulfate. After evaporation of the ethyl acetate, the residue was purified through flash column using ethyl acetate: acetone 9.5:0.5. This hapten was obtained as a smelly oil with 60 % crude yield.

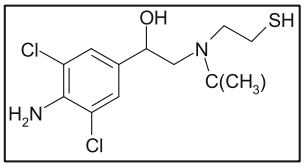

Fig. 1 Chemical structure of hapten 1

Synthesis of 6-maleimidohexanoic acid Amino caproic acid (5.0 g; 38 mmol) and maleic anhydride (3.74 g; 38 mmol) were added to acetic acid (90 mL). The reaction mixture was stirred and refluxed in an oilbath at 120° under nitrogen for 8 h. The acetic acid was removed under reduced pressure. The crude reaction mixture was extracted three times with ethyl acetate. 6-Maleimidohexanoic acid was isolated by flash chromatography using ethyl acetate: acetone 95:5 as eluting solvent.

Yield: 4.5 g (75 %)

^{1}H NMR δ($CDCl_3$): 6.69 (s, 2H, maleimide), 3.52 (t, 2H, J = 7.2 Hz, NCH_2-CH_2), 2.35 (t, 2H, J = 7.5 Hz, $COCH_2$-), 1.63 (m, 6H, CH_2-CH_2-CH_2-).

Synthesis of 4-maleimidobutanoic acid 4-Maleimidobutanoic acid was made using a protocol similar to that above, except that amino butyric acid replaced the amino caproic acid.

Yield: 4.0 g (72 %)

^{1}H NMR δ($CDCl_3$): 6.71 (s, 2H, maleimide), 3.60 (t, 2H, J = 7.0 Hz, NCH_2-CH_2), 2.35 (t, 2H, J = 7.2 Hz, $COCH_2$-), 1.93 (m, 2H, CH_2-).

Synthesis of 6-(2,5-dioxo-2,5-dihydro-pyrrol-1-yl)-hexanoic acid [2-(4-amino-3,5-dichloro-phenyl)-2-hydroxy-ethyl]-tert-butyl-amide (Hapten 2) Ethyl chloroformate (108 μL; 1.26 mmol) was added with stirring to 6-maleimidohexanoic acid (253 mg; 1.20 mmol) dissolved in dry THF (10 mL) and triethylamine (304 μL) at 0 °C. After 60 min, clenbuterol (550 mg) was added to the mixture and stirring was continued overnight while the mixture was allowed to reach room temperature. The THF was evaporated under reduced pressure, and the reaction residue was dissolved in ethyl acetate. The organic layer was washed with water and extracted with ethyl acetate. The residue was

purified by flash column chromatography using ethyl acetate: dichloromethane 1:3. Hapten 1 was obtained as a yellow viscous liquid (Fig. 2).

Yield: 400 mg (65 %).

^{1}H NMR δ ($CDCl_3$): 7.19 (s, 2H, Ar-H), 6.68(s, 2H, maleimide), 5.57 (dd, $J_{AX} = J_{BX} = 8.4$ Hz, 1H, Ar-C<u>H</u>-O), 4.46 (s, 2H, NH_2), 3.49 (t, $J = 7.2$ Hz, 2H, N<u>C</u>H_2CH_2-), 2.95 (dd, 1H, $J_{AB} = 12$ Hz, $J_{BX} = 4$ Hz), 2.76 (dd, 1H, $J_{AB} = 12$ Hz, $J_{AX} = 7.2$ Hz), 2.35–2.29 (m, 2H, COC<u>H</u>$_2$), 1.67–1.23 (m, 6H, −C<u>H</u>$_2$C<u>H</u>$_2$CH$_2$-), 1.08 (s, 9H, tBu).

Immunogens and antibody preparation

Preparation of essential proteins and immunogens In order to prepare immunogens for antibody production, the clenbuterol haptens were conjugated to bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH). For hapten 1, the proteins were activated for reaction with the thiol group by introduction of a maleimide group with BMPS. For hapten 2, the proteins were activated by reaction with 2-iminothiolane which increased reactivity with the thiol-reactive maleimide group on the hapten.

Diazonium conjugates method Clenbuterol hydrochloride (10 mg) was dissolved in 1.33 mL deionized water (7.5 mg/mL), then the pH was adjusted to 2.5 by adding 1 mL of 1 N HCl, and 0.8 mL 10 % sodium nitrate solution was added with stirring, followed by incubation at 0 °C for 30 min. Then, ammonium sulfate (50 mg/mL) was added until no more nitrogen bubbles were given off. The diazonium salt was then reacted with proteins (BSA and KLH) in PBS buffer (pH 7.5). The reaction mixture was kept stirred at 4 °C overnight, then dialyzed in PBS pH 7.2 and stored at 4 °C [7, 8].

Preparation of protein conjugates for hapten 2 Hapten 2 (thiol-reactive hapten) was attached to proteins through modified sulfhydryl groups in proteins. However, due to the low level of cysteine residues (containing free thiol group) contained in BSA or KLH, these proteins have to be modified by Traut's reagent (2-iminothiolane) to yield more thiol groups. Traut's reagent is fully water-soluble and reacts with a primary amine at pH 7 to 10. The reaction can be monitored by 2-iminothiolane's absorbance at 248 nm. Protein modification with 2-iminothiolane is very efficient and proceeds rapidly at slightly basic condition [6].

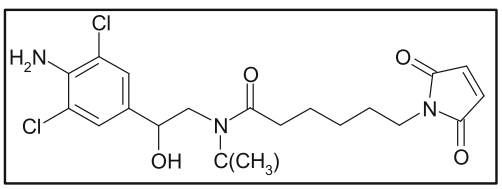

Fig. 2 Chemical structure of hapten 2

Coupling protocol A sample of 0.1 mL 2-iminothiolane solution was added to 0.7 mL of 0.5 mM solution of protein (if coupling to KLH, use 0.25 mM) in 25 mM sodium borate buffer. The reaction mixture was kept at 23 °C for 1 h and the excess reagent was removed by dialysis against the same buffer solution at 4 °C. The sample was dialyzed with this buffer overnight, and then dialyzed with PBS buffer pH 6.6 for 1 h.

A sample of 0.3 mL of 23 mM hapten (dissolved in 0.1 mL DMF, 0.2 mL PBS pH 6.6) was added to 0.7 mL of thiolated protein above. The reaction mixture was left overnight with stirring at 25 °C in darkness. Then the reaction mixture was dialyzed against PBS pH 6.6.

Preparation of protein conjugates for hapten 1 In order to attach hapten 1, proteins (BSA, KLH, and HRP) had to be modified by 3-maleimidopropionic acid *N*-hydroxysuccinimide (BMPS). The NHS ester end of this reagent can react with the primary amine groups on proteins to form stable amide bonds. The maleimide end of BMPS is specific for coupling to sulfhydryl groups of hapten 1 when the reaction pH is in the range of 6.5 to 7.5 [6].

Protocol Ten milligrams of protein (BSA, KLH, or HRP) was dissolved in 1 mL of PBS, pH 7.2. BMPS (2 mg) was added to the above solution. The reaction was left for 1 h at room temperature with periodic mixing. The maleimide-activated protein was then purified immediately by applying the reaction mixture to a Sephadex G-25 desalting column. The solution was not dialyzed because the maleimide activity was lost over the time course of the reaction. Hapten 1 was then added to the maleimide-activated protein with a molar ratio of 20:1 (BSA, KLH) or 40:1 (HRP). This reaction was run for 24 h at room temperature, and then dialyzed at 4 °C in PBS.

Antibody production Six female white New Zealand rabbits were used for obtaining antibodies, and were immunized with a hapten conjugate (immunogen) by intradermal and intramuscular injection. Blood was collected from the marginal ear vein on a monthly basis, isolated by centrifugation, and persevered with sodium azide. The antiserum was purified using affinity chromatography on Protein A-Sepharose [6] and stored at 4 °C.

Formatting and optimizing ELISA test

Indirect competitive ELISA This method is based on a competitive colorimetric ELISA assay. A microplate was coated with CLEN-BSA (0.3 ng in 100 μL per well in 0.5 M carbonate buffer) followed by incubation at 4 °C overnight. The plate

was washed three times with PBS containing 0.05 % Tween 20 (PBST), then blocked with 5 % skimmed milk powder in PBS by incubation at room temperature for 1 h. The reaction was stopped with stopping solution (2 M sulfuric acid). The absorbance at 490 nm was read on a microplate reader.

Direct competitive ELISA This method is based on the recognition of the target compound by a specific antibody. A microplate was coated with the purified antibody (10 ng in 100 μL per well in 0.5 M carbonate buffer). The plate was incubated overnight at room temperature and washed three times with PBST, followed by blocking with 100 μL blocking solution (5 % skimmed milk). The reaction was stopped with 50 μL per well of stopping solution (2 M sulfuric acid). The absorbance at 490 nm was read on a microplate reader.

The sensitivity and limit of detection of the assay

Sensitivity of the assay was expressed as the IC_{50}, which was the concentration of the analyte required to reduce the color development by 50 %. The limit of detection (LOD) for the assay referred to the concentration that reproducibly provides 20 % inhibition of color development.

Colorimetric assay using gold nanoparticles in the presence of melamine

In order to compare the sensitivity, selectivity, and portable property, a new portable detecting method was applied to detect clenbuterol. Colorimetric assay using gold nanoparticles (AuNPs) in the presence of melamine [9] can detect clenbuterol at field application. The methodology used previous research [9, 10]. The stock solution of different clenbuterol was prepared in Milli-Q distilled water. PBS buffer at pH 7.2 was chosen as neutral condition to optimize the adsorbent of melamine to AuNPs surface. First of all, 40 μL of 20 μg/L melamine and 100 μL of different concentration of CLEN were mixed and shaken for 30 min to get stable solution. Then, 70 μL of the above mixture was added to 0.5 mL of 0.4 ppt AuNPs solution and diluted with phosphate buffer solutions (0.01 M, pH 7.2) to 1.8 mL. The solution was transferred to record the absorbance. The absorbance ratio (A_{670}/A_{520}) was used to quantify the concentration of CLEN.

The specificity of the assay The specificity of the immunoassay is determined by the cross-reactivity of the antibody for compounds with similar structure to the analyte of interest. In our research, in order to assess the specificity of the CLEN ELISA, competitive standard solution of salbutamol, terbutaline, epinephrine, and standard solutions of CLEN prepared at concentrations from 0.046 to 1000 ppb were added to the microplate wells. The IC_{50} of each compound was determined and the cross-reactivity was calculated using the following formula in section:

$$\%\ \text{Cross reactivity} = \frac{IC_{50}\ \text{of reference compound}}{IC_{50}\ \text{of cross reactant}} \times 100\,\%$$

Validation of ELISA test

Sampling and pork sample extraction A survey was conducted using market samples, obtained by Quoc Anh Bui on a field trip, to detect the presence of CLEN residues. Twenty pork samples were collected randomly from Ho Chi Minh City markets. Six blank sample were fortified with CLEN up to 0.5, 1.0, 3.0, 5.0, 10.0, and 30.0 ppb, Fortified samples were prepared freshly prior to analysis to establish the ELISA standard curve for real sample

Swine meat samples were minced, and then 5 g was diluted with 20 mL acetate buffer (pH 5.2) and 50 μL β-glucuronidase enzyme and vortexed for 5 min. These samples were incubated at 37 °C for 24 h followed by centrifugation (5000 rpm) for 10 min. The supernatant was adjusted to pH 11–12 with sodium hydroxide (1 M) and then transferred to a separatory funnel for partition with ethyl acetate (10 mL × 3). The organic layer was collected and dried under gentle nitrogen stream. The residue was dissolved in 1 mL mixture of methanol phosphate buffer pH 7.4 (2:8).

Spike and recovery experiments The recovery of this method was determined by spiking with a standard solution into pork samples at concentrations of 0.5, 1.0, 3.0, and 5.0 ppb. These spiked samples were extracted following the above extraction protocol, and then analyzed for clenbuterol by GC-MS and the ELISA method. The recovery was calculated by comparison of the peak areas of the spiked samples with the standard solutions at the same concentration. The samples were analyzed in the Centre for Training and Development of Chromatography (Ho Chi Minh City) for CLEN by GC-MS.

Results and discussion

Hapten selection and synthesis

Based on the principles of hapten design for ELISA, the synthesized hapten should be a near perfect mimic of the target structure in size, shape, and electron density [6]. The hapten spacer arm should not elicit antibody recognition and should be of an appropriate length (the sensitivity is improved with some increase in spacer length). Although a hapten for

clenbuterol may be prepared by directly coupling clenbuterol to a carrier protein through a diazonium salt formed from the aniline NH_2, other methods of hapten preparation involving the use of a spacer arm were preferable. From the previous discussion, the preferred length of a spacer arm would be three to six carbons.

In order to raise antibodies which have low cross-reactivity with other β-agonist compounds, the attachments to aliphatic amine of clenbuterol was chosen. Because the aromatic ring has a significant β_2-agonist pharmacological activity, linking of the spacer arm to the aliphatic amine end of the molecule should not block the important clenbuterol identification group. Specificity could therefore be increased.

According to research conducted by Zhang et al. [7], CLEN was conjugated to a carrier protein via the diazonium bridge from the primary amine. The immunoassay developed by using the antibody raised against this immunogen resulted in the half maximum inhibition (IC_{50}) at 1.8 ng per mL of pork sample, but showed high cross-reactivity with other β_2-agonists. Therefore, thio-reactive hapten (hapten 2) and maleimide-reactive hapten (hapten 1) were synthesized with long spacer arms joining the protein (three-carbon chain).

As a result, different strategies were investigated, firstly by producing an amide linkage to the basic nitrogen of CLEN, and secondly by taking advantage of the ability of ethylene sulfide to alkylate a basic nitrogen under mild conditions.

Haptens 1 and 2 were designed with longer linker arms: hapten 1 using a two-carbon bridge in addition to the BMPS attachment to the protein; and hapten 2 using a five-carbon bridge in addition to the 2-iminothiolane attachment to the protein.

Antibody characteristic

As seen from the above results, the antibody from rabbit hapten 1 exhibited superior sensitivity for clenbuterol (0.4 ppb) in an indirect ELISA. Therefore, this antibody was selected to format and optimize for the prototype ELISA test kit production.

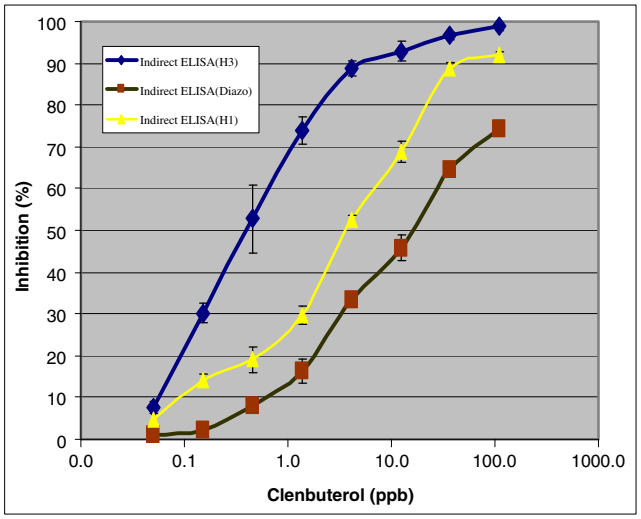

Fig. 3 ELISA standard curve from two kinds of immunogens

Standard curve and range of detection

From the standard curves of clenbuterol from Fig. 3, the detection limit (LOD), which was calculated as the concentration of standard solution causing 20 % inhibition of color development, was about 0.09 ng per milliliter while the relative standard deviation (RSD of the replicate test on the same micro plate) obtained at the concentration in the range of 0.051 to 100 ppb and was between 0.8 and 18.3 %.

Indirect competitive ELISAs were used in this study to format and validate our ELISA test kit. Different combinations of antibodies and hapten conjugates were investigated, indicating that the heterologous assays were more sensitivity than the homologous assays. The antibody from the hapten 1 provided the highest sensitivity among antibodies raised from other hapten protein conjugates.

Assay specificity

Therefore, three β_2-agonist, namely salbutamol, terbutaline, and epinephrine, were used to check the specificity of our ELISA. We will conduct more experiments with other compounds in the future. The antibodies raised from hapten 3

Table 1 Recovery of CLEN from three different methods

Spike pork (ppb)	GCMS		ELISA		AuNPs with melamine	
	CLEN (ppb)	Recovery (%)	CLEN (ppb)	Recovery (%)	CLEN (ppb)	Recovery (%)
0	0.13		0.15		0	
0.5	0.55	84	0.60	90	0.25	50
1.0	1.23	110	1.31	116	0.70	70
3.0	3.83	123	4.00	128	4.29	143
5.0	5.87	115	6.18	121	7.34	146.8

Table 2 Clenbuterol residues in pork samples collected randomly from Ho Chi Minh City markets. Residues are urgently needed to reduce impacts of such residues on human health

No.	Sample code	Clenbuterol residues (ppb)
1	TD1	7.4
2	TN2	34.1
3	TD4	33.7
4	VT2	14.1
5	AS2	9.9
6	VT1	25.0
7	TD3	83.5
8	AS1	3.6
9	VS1	<1.0
10	TN3	71.1
11	TD2	38.9
12	VT3	<1.0
13	TN1	7.1
14	VS2	<1.0
16	PV1	<1.0
17	PV3	<1.0
18	PV2	<1.0
19	MT1	1.3
20	MT2	<1.0

TD random sample from Tan Dinh market, Ho Chi Minh City, *TN* random sample from Thi Nghe market, Ho Chi Minh City, *VT* random sample from Van Thanh market, Ho Chi Minh City, *AS* random sample from An Suong market, Ho Chi Minh City, *PV* random sample from Pham Van Hai market, Ho Chi Minh City, *MT* random sample from Metro supermarket, Ho Chi Minh City, *VS* random sample from Vissan food store, Ho Chi Minh City

immunogen demonstrated lower cross-reactivity than almost all the structurally related compounds tested. Therefore, this antiserum was selected for further development.

Assay optimization

Effect of pH The effect of pH on assay performance was investigated to optimize the pH for extraction. Generally to prepare a test sample, clenbuterol hydrochloride was neutralized to pH 8–9.

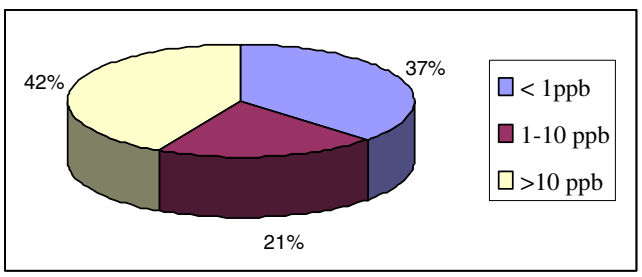

Fig. 4 ELISA results of real pork samples ($n = 20$)

In this investigation, CLEN standard curves in phosphate buffer at pH 5.0, 6.0, 7.4, 9.0 were evaluated for the effect of pH on assay performance. Our assay was not affected by pH in the range of 5 to 9. Hence, at lightly basic pH (8–9) of pretreated samples (adjusted to basic condition), the test results should be reliable.

Sample extraction

Enzymatic hydrolysis was chosen to further investigate sample preparation for the ELISA [11]. Coupling this method with a twofold sample dilution assay buffer showed good recoveries of CLEN in pork samples. The effect of methanol and pH with the pork sample extract on the assay performance was investigated. As of the initial optimization, 20 % methanol in PBS 7.4 was confirmed as the best condition for ELISA analysis of CLEN, and this protocol was used for the validation study and pilot survey of potential CLEN pork contamination. In addition, pH 8–9 was chosen for CLEN diluent.

Validation of the ELISA test kit by analysis of CLEN residues in pig meat

Test of recovery Pork samples were collected randomly from various markets in Ho Chi Minh City and they were analyzed for CLEN residues by GC-MS, ELISA, and colorimetric sensing using gold nanoparticles in the presence of melamine. The samples were extracted following the extraction method described above.

From Table 1, it can be seen that the recoveries of spiked pork at concentration between 0 and 5 ppb were between 90 and 128 %. The correlation of test results obtained from ELISA and GC-MS is shown in Fig. 5. These results demonstrated that our ELISA can reliability detect and quantify CLEN in pork. The recoveries of CLEN from colorimetric detection of AuNPs were not stable, change from 50 to 150 %. Moreover, AuNPs colorimetric methods is very sensitive with pH and urine sample; therefore, it is difficult to

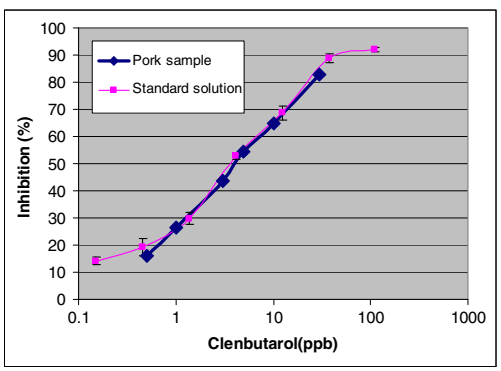

Fig. 5 Correlation between the ELISA standard curve and CLEN spiked pork sample

apply for conducting fields studies. It follows that the ELISA methods may be suitable for semi-quantifying CLEN in urine and pork tissue sample, but AuNPs colorimetric methods may be considered as screening method to detect CLEN in pork without quantification.

Real sample analysis The validation tests on real pork samples were conducted by Bui Quoc Anh on a field trip to the laboratory of Southern-sub Institutes of Agricultural Engineering and Post Harvest Technology (SIAEP) in Ho Chi Minh City. Samples ($n = 20$) were collected randomly from various traditional markets in Ho Chi Minh City. The samples were extracted following the extracting protocol described earlier. In order to establish use of the standard curve on real samples, six blank samples were fortified with CLEN at concentration of 0.5, 1.0, 3.0, 5.0, 10, and 30 ppb,. Based on this standard curve, the clenbuterol contamination level was detected in the real samples. The CLEN residue values were obtained from the equation of the linear line between two points of the real sample standard curve.

Figure 5 showed the similarity between the real sample standard curve and standard solution standard curve. This demonstrates that the sample matrix does not interfere with the test results and the standard curve can be used to determine CLEN residues in pork sample.

From the results presented in Table 2 and Fig. 4, about 60 % of the pork samples were clenbuterol positive. These results have been obtained in validating the ELISA and should be confirmed by further surveys. The concentration of the positive samples ranged from 1 to 70 ppb. Hence, it is suspected that clenbuterol had been abused in piggeries, and establishing a quality assurance system for animal feed and animal products for growth promotion (Fig. 5).

Conclusions

Based on the results of this research, two conclusions can be obtained regarding to the influence of hapten design and hapten heterology on the sensitivity and specificity of the ELISA for CLEN. First of all, the demonstration of hapten design is a significant factor to the development of a high sensitive and specific immunoassay for CLEN. Antibodies with different specificities can be obtained by using different hapten design strategies. The position of the spacer arm attachment is important for the specificity. Two different haptens (maleimide-reactive and thiol reactive) were successfully synthesized and showed high sensitivity and high cross-reactivity to CLEN when compared with the diazo method previously reported by other authors.

Secondly, the use of heterologous system was a valuable approach to improve the immunoassay sensitivity for CLEN. Our ELISA test is able to detect levels below MRLs, so it should be used in combination to other analytical methods to monitor for the presence of CLEN and its derivatives. The small pilot survey of pork collected in HCMC called for urgent attention of misuses of CLEN in swine product in Vietnam. Further application of this clenbuterol ELISA kit developed from this research is anticipated in screening of animal feeds and animal products in Vietnam. In combination with other sophisticated instrumental methods, it would provide a powerful monitoring tool for detection of clenbuterol in animal products.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Statement on animal ethical care All procedures related to the antibody production involving animals followed were in accordance with the ethical standards approved by the animal ethics committee at the University of New South Wales.

Informed consent Informed consent was obtained from all individual participants included in this study.

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