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1. Cover Letter

Dear Manager Tanya Devanny,

Attached is the revised manuscript "Screening of Salbutamol Residues in Swine Meat and Animal Feed by an Enzyme Immunoassay in Taiwan" (Manuscript ID: ACA-09-00165) resubmitted to Analytica Chimica Acta for possible publication. We thank you very much for perceptive review on our manuscript. Now, we have done our best to thoroughly revise and correct the manuscript.

In Taiwan, the analysis of salbutamol in swine serum, meat and feed are the more practical basis for large scale surveillance programs. Revised regulation for the maximal tolerances of the animal drug residues was enacted by DOH (Department of Health), Taiwan. A maximum residue limit of 0.2 μg kg^{-1} for clenbuterol in the meat of cattle and horses; however, salbutamol can not be detected. In the majority of commercially available competitive enzyme-linked immunosorbent assay (ELISA) kits were specific to β-agonist clenbuterol. Deficient cross-reactivity was seen with salbutamol in commercial β-agonist test kits. As a consequence, the veterinary and the national authorities are required to develop extensive monitoring and screening programs. We then, undertook this current research project.

This is an original experiment-applied study by using a new immunoassay for routine examination for extensive monitoring and screening programs for the residues of salbutamol in swine meat and animal feed, and to compare simultaneously with a commercial kit in field test screens. The work described has not been submitted elsewhere for publication and all the authors listed have approved the manuscript that is enclosed.

Thank you very much for your attention and consideration.

Best regards.

Sincerely yours

Tzong-Fu Kuo

Response to Reviewers #1:

(1) In order to "fight" against illegal use of beta-agonists, not only the presence of salbutamol should be detected but also other beta-agonists. How can this kit play a role in a full monitoring plan?

Answer: Salbutamol and clenbuterol are commonly used illegally as repartitioning agents in food producing animals. The specificity experiments of homemade ELISA kit indicated 100% cross-reactivity with salbutamol, 70% with clenbuterol, 90% with terbutaline. A high specific test gives few false-positive results and identifies individual drugs and/or their metabolites. However, an immunoassay obtained from Tecna of Italy, shows 100% cross-reactivity with clenbuterol, and 20% with salbutamol. With the assay RIDASCREEN manufactured by R-Biopharm of Germany, shows 100% cross-reactivity with clenbuterol, but 11% with salbutamol. Comparatively, our kit may play a role in a full monitoring plan more than the others.

(2) The introduction must be more extended, more references to literature must be made and aspects should be described more in detail.

Answer: Yes, the introduction has been extended. We talk more extensively about; the use, in human and in animals, the potential consumer health hazards including the clinical profile; the regulation for the maximal tolerances of the animal drug residues in Taiwan. The unsuitable commercially available kits specific to clenbuterol, would cause salbutamol false positive test results, and the potentially positive samples would then require confirmation, therefore not ideal for screening large numbers of samples. Our recent report has shown that the dilution of serum decreased and eliminated the matrix effect. The number of false-positives reported by the Randox assay was nearly 10 times higher than the assay we developed. We could decrease the number of samples submitted to time-consuming sample cleanup, and chromatography analysis would be

considerably reduced.

(3) When two systems are compared, both the systems must be described in detail.

Answer: The distinction to be made between the two systems: including sample preparation (Table 2) and the cross-reactivities of the antibody with the different compounds. Within the manual of Randox, tissue sample preparation must be processed through the post immunoaffinity column and there are no instructions for feed samples.

(4) No GC-MS details were described. However, they were used to confirm the results received with ELISA.

Answer: Samples for the further confirmation testing were analyzed by GC-MS, which were provided by the Technical service center, National animal industry foundation, Pingtung, Taiwan. The column and MS conditions are described in Table 3; the masses of the ions, and the ion ratios used for identification criteria are summarized in Table 4.

(5) The advantages of the home-made kit must be pointed out more.

Answer: Yes, we have completed our comparison and do our best to point out more, the advantages of the home-made kit.

Response to Reviewers #2:

(1) What is the novelty or originality of the homemade ELISA? Hapten for antibody preparation or the obtained antibody itself may be original. If it is true, reproducibility for the antibody preparation should be shown, because binding ability and specificity of antibody is sometimes different in the different lot.

Answer: (a) All commercially β-agonist kits were specific to clenbuterol, the deficient cross-reactivity was seen with salbutamol. Due to the analysis of salbutamol in swine serum, meat and feed they were the more practical basis for large scale surveillance programs in Taiwan. Therefore we undertook this current research project. (b) The immunogen preparation was described in more detail in section 2.5. According to the optimum antiserum which was determined previously by chequerboard titration, using auricular arteriae blood sampling in rabbits for attaining immune serum of polyclonal antibodies was simple and caused so slight an injury that the rabbits would still be alive after being sampled, while more volume and better quality blood can be obtained continuously and the serum acquired was perfect. While the binding ability and specificity of antibody is sometimes different in the different lot, we could titrate again the dilution fold of tracer, or to modify the molar ratio between hapten and HRP.

(2) Why was the homemade ELISA superior to other commercialized systems?

Answer: In Taiwan, the analysis of salbutamol in swine serum, meat and feed were the more practical basis for large scale surveillance programs. Revised regulation for the maximal tolerances of the animal drug residues was enacted by DOH (Department of Health), Taiwan. A maximum residue limit of 0.2 $\mu g\ kg^{-1}$ for clenbuterol in the meat of cattle and horses; however, salbutamol can not be detected.

The commercially available ELISA kits were specific to β-agonist clenbuterol. The specificity experiments of the homemade ELISA kit indicated 100%

cross-reactivity with salbutamol, 70% with clenbuterol, 90% with terbutaline. A high specific test gives few false-positive results and identifies individual drugs and/or their metabolites. However, an immunoassay obtained from Tecna of Italy, shows 100% cross-reactivity with clenbuterol, and 20% with salbutamol. With the assay RIDASCREEN manufactured by R-Biopharm of Germany, clenbuterol shows 100% cross-reactivity with clenbuterol, but 11% with salbutamol. Deficient cross-reactivity was seen with salbutamol in commercializing β-agonist test kits. In consequence, the veterinary and the national authorities are required to develop extensive monitoring and screening programs. We then undertook this current research project.

In addition, the unsuitable commercially available kits specific to clenbuterol, would cause salbutamol false positive test results, and the potentially positive samples would then require confirmation, therefore not ideal for screening large number of samples. Our recent report has shown that the dilution of serum decreased and eliminated the matrix effect. The number of false-positives reported by the Randox assay was nearly 10 times higher than the assay we developed. We could decrease the number of samples submitted to time-consuming sample cleanup, and chromatography analysis would be considerably reduced.

Furthermore, the distinction to be made between the two systems: including sample preparation (Table 2) and the cross-reactivities of the antibody with the different compounds. Within the manual of Randox, tissue sample preparation must be processed through the post immunoaffinity column, which is complex and cost lots of time, and has no instructions for feed samples.

From the practical point of view, the kit could be advantageously utilized for the screening of large groups of field samples, and the kit employed has good reliability even in routine application for the control of the illegal use of the drug.

(3) 'slabtamol' should be salbtamol in page 3, line 2. Comma after 'Immunoassay' should be deleted.

Answer: Yes, we have made those changes.

Thank you very much for your attention and consideration.

* 2. Reviewer Suggestions

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Analytica Chimica Acta

Novelty Statement

Please provide a **Novelty Statement** for your manuscript below (one or two sentences). This statement should provide information as to what is new and novel in the manuscript, and is provided to referees for consideration when reviewing manuscripts.

Residues of β-agonists (salbutamol, clenbuterol and terbutaline etc.) could present a potential risk for public health safety. The effective surveillance for the illicit use of β-agonists requires sensitive and satisfactory analytical techniques for their detection in serum or tissues from treated animals. The screening procedure is a powerful means to detect and track; several conventional immunoassay methods that are commonly used. All commercially available competitive enzyme-linked immunosorbent assay (ELISA) kits are specific to β-agonist clenbuterol, such as Randox (UK), r-Biopharm (Germany), Tecna (Italy) etc. However, the analysis of salbutamol in the swine serum and meat is the more practical basis for large scale surveillance programs in Taiwan. As a consequence, we undertook this current research project, to investigate the use of a new immunoassay for the determination of salbutamol residues in swine meat and animal feed samples, and to compare with a commercial kit in field test screens.

222 field samples of swine meat and 120 samples of animal feed that were taken from local meat markets, auction markets and feed mills for further confirmation, all samples that showed to be ELISA positive for salbutamol residues were analyzed by GC-MS. Adopting 2 $\mu g\ kg^{-1}$ salbutamol as a cut-off value for swine meat, the commercial β-agonist ELISA had a sensitivity of 85.3% and a specificity of 95.2%

versus GC-MS, at a cut-off of 2 μg kg^{-1}. The homemade salbutamol ELISA had a sensitivity of 100% and a specificity of 90.9% and gave no false-negative rate results. Furthermore, adopting 20 μg kg^{-1} salbutamol as a cut-off value for animal feed, both the commercial and homemade ELISA showed 100% sensitivity and 100% specificity.

The described enzyme-linked immunosorbent assay kit for the quantitative determination of β-agonist salbutamol in swine meat and animal feed showed appreciable accuracy and precision. The sample preparation procedures are simple and rapid. From the practical point of view, the kit could be advantageously utilized for the screening of large groups of filed samples, and the kit employed has good reliability even in routine application for the control of the illegal use of the drug. Improper use of illegal bronchodilator drugs, such as β-agonist, may result in undesirable residues in food supplies that could be hazardous to consumers and become a serous health problem. We work hard developing extensive monitoring and screening programs for the residues of these drugs in the serum, feed, meat and meat-related products destined for human consumption.

* 6. Manuscript
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Screening of Salbutamol Residues in Swine Meat and Animal Feed by an Enzyme Immunoassay in Taiwan

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Abstract

An ELISA was developed for routine examination for extensive monitoring and screening programs for the residues of salbutamol in swine serum, animal feed, meat, and meat-related products destined for human consumption in Taiwan. Objectives of the study were to investigate the use of a new immunoassay for the detection of salbutamol residues in swine meat and animal feed samples, and to compare with a commercial kit in field test screens. A simple and reliable ELISA to monitor the presence of β-agonist, salbutamol, in 222 field samples of swine meat and 120 samples of animal feed that were taken from local meat markets, auction markets and feed mills. The application and the results of two ELISA kits (a homemade and a commercial kit) for the screening of salbutamol were presented. Adopting 2 μg kg^{-1} salbutamol as a cut-off value for swine meat, the commercial β-agonist ELISA had a sensitivity of 85.3% and a specificity of 95.2% versus GC-MS at a cut-off of 2 μg kg^{-1}. The homemade salbutamol ELISA had a sensitivity of 100% and a specificity of 90.9% and gave no false-negative rate results. Furthermore, adopting 20 μg kg^{-1} salbutamol as a cut-off value for animal feed, both the commercial and homemade ELISA showed 100% sensitivity and 100% specificity. In conclusion, a sensitive, specific salbutamol polyclonal antibody-based ELISA has been developed that could serve as a rapid screening assay, and the detection of positive samples at the place of sampling can result in more effective control of the illegal use of β-agonists.

Keywords: Immunoassay; β-agonists; salbutamol; meat; feed; residues

1. Introduction

β-agonists are extensively used as bronchodilatators in asthmatic patients in human being [1]; in animals, applications of β-agonists have been restricted to the treatment of chronic obstructive pulmonary disease in horses and to control of parturition in cattle and sheep [2]. It has been apparent that the drug shows a repartitioning activity resulting in an increase in muscle tissue accretion and reduced fat deposition making it an important veterinary drug as a growth promoter and fattening agent in cattle, sheep, pigs and poultry, with the resulting substantially increased economic profit [3,4].

There is appreciable concern about the potential consumer health hazards associated with the illegal use of sabutamol and clenbuterol as growth promoting agents. There is a strong likelihood of danger to the consumer, especially to patients with pre-existing cardiac deficiencies and those in the late stages of pregnancy [2]. The clinical profile of β-agonists poisoning in humans is characterized by headache, nausea, dizziness, nervousness, palpitation, tachycardia, peripheral vasodilatation, tremors, fever, chills, and in acute cases, breathing interruptions [1,2].

Within the European Union the use of any drug to improve animal growth for mass therapy, such as bronchodilators, is banned [5]. In Taiwan, the Bureau of Food Sanitation at the Department of Health (DOH) collaborates with the Council of Agriculture (COA) closely to ensure food safety. Revised regulation for the maximal tolerances of the animal drug residues was enacted by DOH Food No.0960400187, promulgated on January 25, 2007. A maximum residue limit of 0.2 μg kg^{-1} for clenbuterol in the meat of cattle and horses was enacted; however, salbutamol can not be detected.

For reasons of public health and safety, measures for monitoring are focused on live animal sampling on the farm and at the market place, to enable more effective checking for the possession of prohibited substances, illegal treatment and withdrawal period compliance [6]. In slaughterhouses, body fluids (plasma and urine), liver, kidney, muscle and eye tissues can be sampled. In Taiwan, serum testing allows immediate detection which can be taken in auction markets, is the most frequently analyzed material for the detection of growth promoters; salbutamol is repeatedly discovered during

routine control by regulatory authorities. A larger surveillance programs is extremely helpful to document positive serum, muscle and feed cases; it must be cheap for routine analysis.

To monitor for misuse or illegal use, the particular challenge in developing analytical methods for salbutamol screening is derived from the rapid rate of elimination from the body and the low maximum residue limit (MRL). The value of immunoassays for salbutamol residue analysis in a variety of matrices has been widely reported [7-10]. Effective surveillance for the illicit use of β-agonists requires sensitive and satisfactory analytical techniques for their detection in serum and tissues from treated animals, and even includes the animal feed detection, with strengthening management of feed resource and realizing the security limit of drug residue. Immunoassays offer the requisite degree of sensitivity, although samples may need to be extracted and concentrated prior to analysis, such procedures may be used in conjunction with GC-MS to provide some degree of confirmation.

The majority of commercially available competitive ELISA kits were specific to β-agonist clenbuterol. Deficient cross-reactivity was seen with salbutamol in commercial β-agonist test kits: An immunoassay RIDASCREEN manufactured by r-Biopharm of Germany, showed an 11% cross-reactivity toward salbutamol; the assay obtained from Tecna of Italy, showed a 20% cross-reactivity toward salbutamol, etc. (Table 1). Kits for the comparative testing were purchased from Randox and showed an 86% cross-reactivity toward salbutamol. However, the importance and necessity to strengthen control programs was emphasized by the illegal use of salbutamol by farms, so the analysis of salbutamol in swine serum, meat and feed were the more practical basis for large scale surveillance programs in Taiwan. The unsuitable commercially available kits specific to clenbuterol, would cause salbutamol false positive test results, and the potentially positive samples would then require confirmation [3], therefore not ideal for screening large number of samples.

(Insert Table 1)

We evaluated the performance of such a β-agonist ELISA kit from Randox of the UK. It is recommended for the detection of the following (% of cross-reactivity): clenbuterol (100%), salbutamol (86%), terbutaline (50%) (Table 1). The analysis of salbutamol in swine meat and animal feed, which can be taken in food processing plants and slaughter houses, feed mills and/or inspection of import feed or feed additives in practice. In the present study, we investigated the use of homemade ELISA, a new immunoassay for the determination of salbutamol residues in swine meat and animal feed samples, and compared it with a commercial kit in field test screens simultaneously.

2. Materials and methods

2.1. Reagents

Salbutamol free base, salbutamol hemisulfate salt, clenbuterol hydrochloride, terbutaline hemisulfate salt, isoproterenol hydrochloride, succinic anhydride, *N*-hydroxysuccinimide (NHS), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC), ovalbumin (OVA), porcine thyroglobulin (TG), bovine serum albumin (BSA), Freund's complete/incomplete adjuvant and *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) were commercially available from Sigma (USA). Salbutamol, VETRANAL, analytical standard was from Riedel-de Haen (Germany), and Salbutamol-*tert*-butyl-d_9 was from Aldrich (USA). Paylean was kindly provided by Eli Lilly and Company (Taiwan), Inc. Horseradish peroxidase (HRP) was from Roche (Switzerland). TMB, ready-to-use substrate was obtained from Kem-En-Tec (Denmark). Methanol and acetonitrile were of liquid chromatographic grade. All other chemicals were of analytical-reagent grade and were used as obtained. Deionized water was purified on a Milli-Q system (Millipore, MA, USA).

2.2. Buffers

a. Coating buffer: 0.05 M carbonate/bicarbonate buffer solution, pH 9.6.

b. Washing buffer: phosphate buffered with Tween 20, pH 7.4 were prepared with 0.01 M phosphate buffer, 0.0027 M KCl, 0.14 M NaCl, and 0.05% Tween.

c. Dilution buffer: the same as washing buffer.

2.3. Materials

Ninety-six-well plates were obtained from Costar (Cambridge, MA, USA). Bond-Elut Certify columns were purchased from Varian Diagnostics (Harbor City, CA, USA). GC capillary column HP-1 was from Agilent (Santa Clara, CA, USA).

2.4. Instrumentation

A High Speed Refrigerated Centrifuge and a Tabletop Centrifuge (Kubota 6900 and 5400, Tokyo, Japan) were used. The antibody was dispensed in microtiter plates using a μFill microplate dispenser (Bio-Tek, Winooski VT, USA). The microtiter plates were washed with the washing solution to remove unbounded antibodies using a 96PW microplate washer (Tecan, SLT., Salzburg, Austria). The absorbances of each well were measured with the EMax microplate reader (Molecular. Devices, Sunnyvale, CA). The sample extraction and cleanup for GC-Mass confirmation was performed using solid phase extraction vacuum manifold 12 Port (Supelco, USA) and TurboVap LV Concentration workstations (Caliper, Hopkinton, MA, USA). The Gas chromatograph−mass spectrometer used for analyzing the β-agonists was a 6890 GC/5973 MSD (Agilent, USA).

2.5. Immunogen Preparation

a. Preparation of Salbutamol Succinate. Salbutamol free base (2.39 g, 10 mmol) was dissolved in dry ethanol (20 mL) using a magnetic stirrer. While stirring, 1.2 g (12 mmol) of succinic anhydride was added. A cloudy white suspension appeared, and the formation of the salbutamol succinate was monitored by thin layer chromatography (R_f salbutamol = 0.51, R_f succinyl derivative = 0.21). After 30 min at room temperature, the reaction appeared complete, the stirring was stopped, and the suspension

was centrifuged at 3,000 g for 15 min. The solid phase was washed three times with diethyl ether and dried in vacuum at 50℃ for 5 h.

b. Preparation of Immunogen by Coupling of Salbutamol Succinate to OVA. After dissolution of 1.7 mg of salbutamol succinate (5 μmol), 0.7 mg of *N*-hydroxysuccinimide (6 μmol) and 1.4 mg of *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (7.5 μmol) in 1 mL dry DMF were added. The mixture was stirred for 1 h at room temperature. OVA solution (16 mg in 4 mL of 0.1 N phosphate buffer, pH 8.0) was added by drop to the above hapten mixture. The resultant solution was stirred for 1 h at room temperature, and the conjugate was then dialyzed against 3 L of 0.01 M phosphate buffer solution containing 0.14 M NaCl for 1 day with 2 changes of buffer. The dialyzed solution of immunogen was frozen at −20 °C until use.

2.6. Immunization of Rabbits

Five rabbits (New Zealand breed) were immunized by sc injection with salbutamol succinate conjugated to OVA. Primary immunizations were composed of 500 μL of PBS containing 1 mg of immunogen emulsified in 500 μL of Freund's complete adjuvant. Subsequent immunizations, at 2−5 week intervals, were of the same volume, with complete adjuvant replaced by incomplete adjuvant. The sera titer was detected by an indirect ELISA.

2.7. Antibody Preparation.

The salbutamol-specific antibodies were purified from high titer rabbit serum by affinity chromatography on a Protein A-Sepharose column. The antibodies were concentrated using Centriprep (Amicon Ultra, 50,000 MWCO; Millipore). Antibody concentration was measured with Bio-Rad protein assay. According to the optimum antiserum which was determined previously by chequerboard titration, using auricular arteriae blood sampling in rabbit for attaining immune serum of polyclonal antibodies

was simple and exerted so light an injury that the rabbit would be alive after being sampled, while more volume and better quality blood can be obtained continuously and the serum acquired was perfect.

2.8. ELISA Kit

A competitive enzyme immunoassay method for β-agonists has been developed using an antiserum raised in rabbits (New Zealand breed) by immunization against salbutamol derivative coupled to ovalbumin. The same derivative of the hapten was used, prepared by coupling to horseradish peroxidase to synthesize the enzymatic tracer. The 96-well microtiter plate was precoated with 0.9 μg/well antisalbutamol polyclone antibody overnight at 4 °C in coating buffer. The wells were blocked with 3% bovine serum albumin (BSA) in PBS for 2 h at room temperature the second day after washing them three times with washing buffer. The microtiter plates were dried at 20 °C, 25% RH for 4 h. The quantitative β-agonist ELISA kit contains the following components: a 96-well microtiter plate (spilt into 12 strips of 8 wells each), precoated with antisalbutamol antibody, salbutamol−horseradish peroxidase enzyme conjugate, six salbutamol standard solutions (0, 0.05, 0.1, 0.25, 0.5, and 1 ng/mL), chromogen [3,3',5,5'-tetramethylbenzidine (TMB)], dilution buffer, washing buffer, and stop solution.

2.9. Sample Preparation for ELISA Screening

An amount of 4 g of the homogenized meat samples was accurately weighted into a centrifuge tube and 6 ml hydrochloric acid (0.01 N) was added with vortex mixing for 1 min. The preparation was incubated at room temperature for 30 min, afterward, the resulting mixture was centrifuged at 3,000 rpm for 10 min and the upper clear solution was diluted with dilution buffer (1:3, v/v) for further immunoassay determination. The feed was ground using an agate mortar, and then 1 g was weighed into a centrifuge tube. Hydrochloric acid (0.01 N, 5 ml) was added to the samples followed by vortex mixing for 1 min and centrifugation for 10 min at 3,000 rpm at room temperature. The supernatant was diluted with dilution buffer (1:9, v/v) for immunoassay determination.

The two kits that may be employed to quantitatively measure the levels of β-agonists in different sample matrices are presented in Table 2. Within the manual of Randox, tissue sample preparation must be processed through the post immunoaffinity column and had no instructions for feed sample.

(Insert Table 2)

2.10. Immunoassay Procedure

A commercially β-Agonist ELISA kit obtained from Randox Laboratories, (Antrim, U.K.) was used for the analysis of salbutamol and the cross-reactivities of the antibody with the different compounds were given in Table 1 (Manufacturer's data). The homemade direct ELISA test was carried out using the following procedure: 100 μL of standard or sample and 50 μL of tracer solution were added to each well. Unbound compounds were removed by washing solution after incubation for 1 h at room temperature (19−25 °C). Developing substrate solution (100 μL) was then added to each well in the dark and the enzyme reaction was stopped after 20 min incubation at room temperature. Absorbance values were measured at 450 nm and the concentration of the analyte in a sample was calculated from the calibration curve. To get the final result in μg kg^{-1}, the concentration read from calibration curve was multiplied by a dilution factor. For meat and feed samples, the real concentration were calculated as follows: result (μg kg^{-1}) = result of ELISA × 10 and 50 respectively.

2.11. ELISA Validation

The blank meat samples taken from swine free from salbutamol treatment and the blank feed samples had previously been proved to be free of salbutamol and other β-agonists using GC-MS determinations. The blank samples were assayed using the ELISA, as described above, to demonstrate the range of blank matrix effects in the assay. Intra-assay (within day) variation was assessed for 5 replicates by the analysis of one meat sample fortified with salbutamol at 1, 2, and 4 μg kg^{-1}. To

measure inter-assay (between days) variation, the concentration of 1, 2, and 4 μg kg^{-1} salbutamol was determined for 5 replicates on each of 5 different days. Similarity, the intra- and inter-assay variations were measured of 5, 10, and 20 μg kg^{-1} of the salbutamol spiked feed. The recovery (%) of the fortified salbutamol at the levels, 1, 2, and 4 μg kg^{-1} in meat samples and 5, 10, and 20 μg kg^{-1} in feed samples, for 3 different analysis performed, was calculated using the equation: conc. measured/conc. fortified × 100.

2.12. Field Trial Measurements

The preliminary survey of the local market to detect the presence of salbutamol residues was conducted. The samples included 222 swine meats and 120 animal feeds that were collected from the local meat markets and processed to detect any salbutamol residues. The performances of two such ELISA kits for the screening of salbutamol were evaluated. Samples for the further confirmation testing were analyzed by GC-MS and were kindly provided by the Technical Service Center, National Animal Industry Foundation, Pingtung, Taiwan.

2.13. GC-MS Analysis

GC-MS of the β-agonists was performed according to Chinese National Standards (CNS) General No.14619, Catalog No.N4187 [11-12]. The column and MS conditions are described in Table 3. For the purpose of confirmation of the identity, additional (fragments) ions must be monitored. TMS derivatives of the appropriate β-agonists (salbutamol-3TMS, terbutaline-3TMS, and clenbuterol-TMS) fragments, the masses of the ions, and the ion ratios used for identification criteria are summarized in Table 4.

(Insert Table 3)

(Insert Table 4)

3. Results and Discussion

3.1. Analytical Performance of Homemade ELISA kit

The typical standard curve obtained using the homemade ELISA method was presented in Figure 1. The result of the salbutamol-ELISA produced linear ranges from 0.05 to 1 μg kg^{-1}. The average IC_{50} value (concentration of salbutamol resulting in 50% binding inhibition in optimized assays) was 0.20 μg kg^{-1}. Specificity of the assay was estimated by measuring cross-reactivity, as calculated at 50% relative binding, with the following β-agonists (% of cross-reactivity): salbutamol (100%), clenbuterol (70%), terbutaline (90%), isoproterenol (29%) and Paylean (0.05%) as shown in Table 1.

(Insert Figure 1)

Sample matrices often adversely affect many assays, including immunoassay. To minimize matrix effects, two common routes are often taken, dilution or sample cleanup such as liquid or solid phase extraction. Our recent report has shown that the dilution of serum decreased and eliminated the matrix effect [10]. Obviously, the more the meat extract is diluted the smaller the matrix effect one would expect, but with concomitant lowering of the assay sensitivity. Consequently, we elected to use the dilution factor as follows: serum (5×), meat (10×), and feed (50×) in this analysis. By running the standard curve with blank meat and blank feed of the same concentration as the samples, problems with accuracy and precision will be minimized.

The sensitivity of the assay was evaluated by examining meat and feed matrices. It appears that the limit of detection (mean of 20 blank samples + 3 times standard deviation) [13-14] of this assay for meat and feed matrix was estimated to be 0.30 and 1.50 μg kg^{-1}, and the limit of determination (mean of 20 blank samples + 10 times standard deviation) [15] for meat and feed samples was 0.60 and 3.0 μg kg^{-1} respectively.

Recoveries were investigated by adding increasing amounts of salbutamol to swine meat (1, 2, and 4 μg kg^{-1}) and animal feed samples (5, 10, and 20 μg kg^{-1}). The results indicated a recovery ranging from 89 to 116% of the targeted value with coefficients of variation of 11% or less (Table 5). In this study both methods gave satisfactory results in recovery studies from the meat and feed samples containing salbutamol. Mean recovery thus indicated a reasonable parallelism and accuracy of the assay when applied to real samples.

(Insert Table 5)

Precision of the assay was assessed by replicate measurements of three known swine meat samples with a final salbutamol concentration of 1, 2, and 4 μg kg^{-1}. Moreover, the precision was also measured of 5, 10, and 20 μg kg^{-1} of the salbutamol spiked feed. The obtained mean values ± SD and CV (%) by replicate analyses (n = 5) in the same run (intra-assay) and in separate runs (inter-assay) are shown in Table 6. The CV % values were below 11%, demonstrating an acceptable level of precision. The use of an automated dispensing system helps improve the quality of the assay.

(Insert Table 6)

3.2. Application of Homemade and Commercial β-Agonist Test Kits in Field Trial Measurements

Field trials with 222 swine meat and 120 animal feed samples from local meat markets were analyzed. The qualitative immunoassay reports each sample as either positive or negative, on the basis of predetermined cut-off concentrations. In the ideal diagnosis, results would be positive if the animal took the drug (true positive) and negative if the drug was not taken (true negative). However, false-positive or false-negative results can occur; therefore, it is imperative to interpret the results carefully. In context, the sensitivity of a test is the ability to detect a class of drug, while the specificity is the ability

to identify a particular drug. A high specific test gives few false-positive results and identifies individual drugs and/or their metabolites. High sensitivity is due to the test's ability to detect the drug and/or its metabolite(s) and to reach the cut-off concentration for a positive report. This method was found to be suitable for surveillance programs in Taiwan, and a cut-off limit of 2 μg kg^{-1} salbutamol swine meat and 20 μg kg^{-1} animal feed was set by the national authorities, Council of Agriculture, Executive Yuan, Taiwan, R.O.C., and administered by the Bureau of Animal and Plant Health Inspection and Quarantine (BAPHIQ, Council of Agriculture, Executive Yuan, Taiwan). According to the recommended cut-off concentration of salbutamol, results were determined to be positive or negative. The sensitivity is the proportion of true positives, and the specificity is the proportion of true negatives [10,16].

Table 7 demonstrated the performance of the ELISA in comparison with a confirmatory GC-MS method for the determination of salbutamol in swine meats and animal feeds. All 34 swine meat samples tested positive using the homemade ELISA, demonstrating a consistent qualitative performance (no false-negatives) in the analysis of these incurred samples that had confirmed concentration ranging from 2.0 to 13.1 μg kg^{-1}. The homemade salbutamol ELISA had a sensitivity of 100% and a specificity of 90.9%, while the commercial β-agonist test kit had a sensitivity of 85.3% and a specificity of 95.2% versus GC-MS at a cut-off of 2 μg kg^{-1} for swine meat detection. The five false-negatives results in Randox kit probably reflected the matrix effects and extraction efficiencies of the ELISA method with acid-treatment, practically the muscle sample preparation protocol in Randox's manual should be processed according to the post immunoaffinity column, which is a complex and time-consuming procedure. For animal feed determination, homemade ELISA showed 100% sensitivity and 100% specificity versus GC-MS (20 μg kg^{-1} salbutamol cut-off in animal feed), as well as the same with commercial test kits (Table 7).

(Insert Table 7)

Laboratories involved in residue analysis of veterinary drugs and growth-promoting agents must analyze a variety of compounds in biological samples, serum, tissue and animal feed. Objectives of the study were to develop a new assay and to compare it with a commercial kit in field test screening. The Randox β-agonist ELISA was evaluated for the screening of swine meat and animal feed for salbutamol residues in field samples, and the tests were described as sufficiently specificity and reproducible. Our concern for the analysis of salbutamol is for the routine examination for extensive monitoring and screening programs for the residues in serum, feed, meat, and meat-related products destined for human consumption in Taiwan. The homemade salbutamol ELISA showed a sensitivity of 100% and a specificity of 90.9%, and gave no false-negative rate results for swine meat tests. Furthermore, animal feeds were subjected to such analysis to show 100% sensitivity and 100% specificity. In this article, we present findings on the developed assay with regard to excellent sensitivity and specificity of the salbutamol measurements. We could decrease the number of samples submitted to time-consuming sample cleanup, and chromatography analysis would be considerably reduced.

When exploiting a commercial ELISA, Watanabe et al. have characterized the analytical performances that must be investigated as follows: (1) the fundamental parameters (sensitivity, dynamic range and limit of detection), (2) the cross-reactivity toward structurally related compounds, (3) the influence of the matrix interference on the reliability of the ELISA, (4) the accuracy and precision, and (5) the method comparison with the well-established chromatographic techniques [17]. The described enzyme-linked immunosorbent assay kit for the quantitative determination of β-agonist salbutamol in swine meat and animal feed showed appreciable accuracy and precision. The meat and feed sample preparation procedures are simpler and quicker than that of the Randox kit (Table 2). From the practical point of view, the kit could be advantageously utilized for the screening of large groups of field samples, and the kit employed has good reliability even in routine application for the control of the illegal use of the drug. This salbutamol ELISA kit is now commercially available (Taiwan Advance Bio-Pharm Inc., Taiwan).

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References

[1] I.D. Prather, D.E. Brown, P. North, J.R. Wilson, Med. Sci. Sports Exerc. 27 (1995) 1118−1121.

[2] A. Prezelj, A. Obreza, S. Pecar, Curr. Med. Chem. 10 (2003) 281-290.

[3] J.P. Hanrahan, Beta-Agonists and Their Effects on Animal Growth and Carcass Quality, Elsevier, New York, 1987.

[4] C.F. Kearns, K.H. McKeever, K. Malinowski, M.B. Struck, T. Abe, J. Appl. Physiol. 91 (2001) 2064−2079.

[5] Council Directive 96/22/EC. Official J. Eur. Commun. L125 (1996) 3−9.

[6] H.A. Kuiper, M.Y. Noordam, M.M. van Dooren-Flipsen, R. Schilt, A.H. Roos, J. Anim. Sci. 76 (1998) 195−207.

[7] G. Degand, A. Bernes-Duyckaerts, G. Maghuin-Rogister, J. Agric. Food Chem. 40 (1992) 70−75.

[8] G. Degand, A. Bernes-Duyckaerts, P. Delahaut, G. Maghuin-Rogister, Anal. Chim. Acta 275 (1993) 241−247.

[9] L. Howells, M. Sauer, R. Sayer, D. Clark, Anal. Chim. Acta 275 (1993) 275−278.

[10] Y.C. Lei, Y.F. Tsai, Y.T. Tai, C.Y. Lin, K.H. Hsieh, T.H. Chang, S.Y. Sheu, T.F. Kuo, J. Agric. Food Chem. 56 (2008) 5494−5499.

[11] Chinese National Standards (2002) CNS 14619, N4187. Bureau of Standards, Metrology and Inspection, Taipei, Taiwan.

[12] T.F Tai, H.T. Hua, L.T. Chien, C.K Liu, Taiwanese J. Agri. Chem. Food Sci. 38 (2000) 42−50.

[13] H. Kaiser, Z Anal. Chem. 209 (1965) 1−18.

[14] European Community comments on the Proposed Draft Guidelines on Analytical Terminology for Codex Use. (Agenda item 4, CX/MAS 08/29/5)

[15] D. MacDougall, W.B. Crummett, Anal. Chem. 52 (1980) 2242−2249.

[16] M.C. Yang, J.M. Fang, T.F. Kuo, D.M. Wang, Y.L. Huang, L.Y. Liu, P.H. Chen, T.H. Chang, J. Agric. Food Chem. 55 (2007) 8851−8856.

[17] E. Watanabe, S. Miyake, Anal. Chim. Acta 583 (2007) 370−376.

Table 1

Cross-Reactivities of Antibodies with Different Compounds (Manufacturer's Data)

	Cross reactivity (%)					
compound	Randox [a]	r-Biopharm	Tecna	Euro	Neogen	Homemade
salbutamol	86	11	20	30		100
clenbuterol	100	100	100	100	100	70
terbutaline	50	10		10		90
isoproterenol		4				29
Paylean						0.05
carbuterol	90	4.5				
methyl-clenbuterol	75					
brombuterol	53			100		
mabuterol	45	71	60	70		
mapenterol	32			80		
pirbuterol	25	0.9				
cimaterol	10	5.5		10		
ractopamine	< 0.2					
feneterol	< 0.2					
adrenaline		< 0.01				
noradrenalin						
tulobuterol					1.85	
cimbuterol					60	
fenfluramine					0.03	
propranolol					0.47	
pindolol					0.06	
fenspiride					0.04	
alprenolol					0.04	
methylene blue					0.01	
penbutolol					0.01	
propafenone					0.01	

[a] Manual Revised 02/01/02

Table 2

Different Methods Used for the Determination of β-Agonist in Serum, Muscle and Feed Samples

Matrix	Randox [a]	Homemade
Serum	Non-provided	- Dilute 1 + 4 with diluent buffer - Centrifuge for 5 min at 3,000 rpm - Ready for immunoassay determination
Muscle	① Pre-immunoaffinity column extraction: - 5 g sample (finely chopped) + 15 mL diluted extraction buffer + 5 mg Pronase E type XIV - Homogenize for approx. 30 sec - Incubate homogenate at 55℃ for 2 h with frequent shaking (10-15 min) - Cool to 4℃ and then vortex for 30 sec - Centrifuge at 4,000 rpm for 20 min - Pour off supernatant and filter - To 12 mL of filtered supernatant add 1 mL of protease inhibitor solution - Adjust the pH of treated supernatant to 8.8-9.0 with 1 M NaOH - Make volume up to 16 mL with H_2O - Ready for immunoassay determination ②Immunoaffinity chromatography clean-up of extracted tissue samples ③Post immunoaffinity column tissue sample preparation	- Homogenize an appropriate amount of the sample in a blender - Weight 4 g of homogenized sample into a centrifuge tube - Add 6 mL of hydrochloric acid (0.01N) - Vortex for 1 min - Incubate at room temperature for 30 min - Centrifuge for 10 min at 3,000 rpm - Dilute the supernatant 1 + 3 with diluent buffer - Ready for immunoassay determination
Feed	Non-provided	- Approximately 50 g of sample is ground and pulverized into a fine powder - Weight 1 g of homogenized sample into a centrifuge tube - Add 5 mL of hydrochloric acid (0.01N) - Vortex for 1 min - Centrifuge for 10 min at 3,000 rpm - Dilute the supernatant 1 + 9 with diluent buffer - Ready for immunoassay determination

[a] Manual Revised 02/01/02

Table 3

Apparatus and GC-MS Conditions Used for Salbutamol Analysis

GC-MS	Agilent 6890 series
column	HP-1 fused-silica capillary column
	length 30 m, 0.25 mm I.D., film thickness 0.25 µm
injection pressure	2.9 psi
column gas flow rate	He, 0.56 mL/min
injector temperature	280 °C
detector temperature	310 °C
oven program	initial 70 °C (remain 1 min)
	program 30 °C/min
	middle 230 °C (remin 5 min)
	program 30 °C/min
	final 300 °C (remain 6 min)
injection mode	splitless
injection volume	1 µL
detection mode	selective ion monitoring (SIM)
electron energy	70 eV

Table 4

Ions and Ratios for GC-MS Analyses of β-Agonists-TMS Derivatives [a]

compound	N [b]	M_0 [c]	M_d [d]	ions			
salbutamol	3	239	455	86	350	369	371
				(100)	(2.3)	(94.4)	(16.2)
terbutaline	3	225	442	86	336	356	358
				(100)	(2.8)	(67.0)	(10.9)
clenbuterol	1	277	348	86	243	262	264
				(100)	(8.1)	(16.5)	(10.9)

[a] The number in parentheses indicates the relative peak intensity.

[b] N = number of TMS groups.

[c] M_0 = molecular mass.

[d] M_d = molecular mass after derivatization.

Table 5

Recovery Values Obtained in Matrix Samples for the Determination of Salbutamol by Homemade ELISA

matrix	fortified conc. (μg kg^{-1})	recovery	
		mean (n=3)%	CV (%)
swine meat	1.0	106.5	8.8
	2.0	95.8	7.9
	4.0	115.6	9.6
animal feed	5.0	102.0	8.2
	10.0	113.1	9.5
	20.0	89.5	10.1

Table 6

Inter- and Intra-assay Variations of Matrix Samples Spiked with Salbutamol by Homemade ELISA

matrix	fortified conc. (μg kg^{-1})	inter-assay			intra-assay		
		n	measured (μg kg^{-1})	CV (%)	n	measured (μg kg^{-1})	CV (%)
swine meat	1.0	5	1.10 ± 0.10	8.8	5	1.06 ± 0.08	7.5
	2.0	5	1.92 ± 0.20	10.5	5	1.91 ± 0.17	8.7
	4.0	5	4.24 ± 0.42	9.8	5	4.61 ± 0.32	6.9
animal feed	5.0	5	5.06 ± 0.44	8.7	5	5.11 ± 0.39	7.6
	10.0	5	10.56 ± 1.06	10.0	5	11.3 ± 0.89	7.8
	20.0	5	19.6 ± 2.07	10.6	5	18.0 ± 1.46	8.1

Table 7

Comparison of Homemade and Commercially Available ELISA with 222 Swine Meat Samples and 120 Animal Fees Samples from Local Meat Markets

swine meat (222 samples)				animal feed (120 samples)			
cut-off conc. 2 μg kg^{-1}		number of samples		cut-off conc. 20 μg kg^{-1}		number of samples	
screen-ing	confirm-ation	home-made	commercially available	screen-ing	confirm-ation	home-made	commercially available
+ [a]	+ [a]	34	29	+ [a]	+ [a]	6	6
- [b]	+ [a]	0	5	- [b]	+ [a]	0	0
+ [a]	- [b]	17	9	+ [a]	- [b]	0	0
- [b]	- [b]	171	179	- [b]	- [b]	114	114
sensitivity [c]		100%	85.3%	sensitivity [c]		100%	100%
specificity [d]		90.9%	95.2%	specificity [d]		100%	100%

[a] Positive test result.

[b] Negative test result.

[c] % Sensitivity = (True positives – false negatives)/(true positives)×100%.

[d] % Specificity = (true negatives – false positives)/(true negatives)×100%.

Figure 1

Calibration curve for salbutamol ELISA. B = absorbance of each standard or sample, B_0 = absorbance of standard = control