VALIDATION OF ELISA TEST KITS FOR DETECTION OF BETA-AGONIST RESIDUES IN MEAT

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Abstract. Three ELISA test kits, the Randox ELISA beta-agonist test kit, Euro-Diagnostica test kit, and Ridascreen beta-agonist test kit, were evaluated for screening of meat and liver for beta-agonist residues in fortified and field-incurred samples. It was found that the Randox beta-agonist test kit was more suitable as a screening tool due to its accuracy, ease of use, and lower cost. The tests were able to detect beta-agonist residues at the minimum level of detection, as claimed by the suppliers. The performance of the method as assessed through recovery rates of beta-agonists in fortified samples was satisfactory with a low coefficient of variation (1-3%). Repeatability, as measured through the coefficient of correlation was also satisfactory. For field-incurred positive samples, the test kit showed a sensitivity of 100% and a low rate of false positives for goat and cow tissues. However, a high rate of apparent false positives was obtained for tissues of swine.

INTRODUCTION

Veterinary drug residues in meat pose a hidden but serious threat to public health. The monitoring of raw meat and poultry for drugs and chemical residues is necessary to ascertain that approved compounds are not misused and are not presenting a danger to consumers. Beta-agonists are a group of veterinary drugs that have been used illegally in some countries as they have a similar action to anabolic steroids in altering body composition (Reeds *et al*, 1988). Beta-agonists act by impeding the uptake of adrenal hormones by nerve cells and stimulation of the cardiovascular system. When the treatment is prolonged, they also induce a redistribution of fat to muscle tissues (Watson, 1993). The beta-agonist clenbuterol has been implicated in several food poisoning cases in European countries (Martinez-Navarro,1990; Pulce *et al*, 1991). In the Asian region, nine people fell ill in Hong Kong after consuming pig lungs tainted with traces of clenbuterol.

The Ministry of Health of Malaysia had been alerted to the possibility of beta-agonist residues being used to produce leaner meat among swine and began taking enforcement samples in 1998. The potential risk for human health posed by the presence of beta-agonists in meats is high, due to the severity of the possible adverse effects of this contaminant, as well as the importance of meat in the total diet of Malaysians. Analysis of meats for beta-agonist residues are presently carried out at surveillance laboratories using gas chromatography mass spectrometry. The procedures for this analytical method are labor-intensive, tedious, and time-consuming. Thus, the usage of validated rapid methods that enable screening of samples for adulteration would increase the possibility of detecting meats that violate the Foods Act 1983 and the Food Regulations, 1985. It would also permit the examination of more samples and reduce the costing of analysis for non-violating samples of meat at the laboratories.

Thus, the objective of this study was to compare the effectiveness and accuracy of three commercial rapid screening tests for detecting beta-agonist residues in fortified and field-incurred tissue samples.

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MATERIALS AND METHODS

Chemicals

Sodium hydroxide (Ajax),methanol (HPLC

Grade; Ajax); acetic acid (Univar); pronase E (Sigma); UHQ Grade 1 water; hydrochloric acid (Univar); *Helix pomatia* (edible snail) juice (Merck); sodium carbonate (Ajax); isobutanol; clenbuterol hydrochloride (Sigma), salbutamol hemisulphate (Sigma) and terbutaline hemisulphate (Sigma), were used in the study.

Materials

Tests validated in this study were the Randox β-agonist ELISA test kit SU1248 (supplied by Randox Laboratories Ltd, Ardmore, Diamond Road, Crumlin Co, UK); Euro-Diagnostica β-agonist EIA 5016BAG test kit (supplied by Euro-Diagnostica BV, NL6082, the Netherlands); Ridascreen β-agonist ELISA test kit (supplied by R-Biopharm Gmbh, Darmstardt, Germany).

Food samples

For the fortified sample study, residue-free samples of meat and liver from pigs, cows and goats were used for preparation of fortified samples. For the field-incurred study, eleven samples of meat and liver that had tested positive for beta-agonist residues when analysed using gas chromatography-mass spectrometry were used for the study. Field samples that tested negative by gas chromatography-mass spectrometry were also used to study the occurrence of false-positive results.

Fortified meat standard preparation

The beta-agonists used were salbutamol hemisulphate (Sigma S-5013), terbutaline hemisulphate (Sigma T-2528) and clenbuterol hydrochloride (Sigma C-5423). The reference standards for the three beta-agonist drugs used are summarized in Table 1. These drugs were stored and handled according to the manufacturer's instructions before use. Reference drugs were adjusted for purity (correction factor) to provide the correct amount of active ingredients, and dissolved in HPLC grade methanol. The detailed preparations and serial dilutions to the final concentration of the reference drugs are summarized in Table 2. Five grams of meat and liver were weighed in triplicates directly into polypropylene centrifuge tubes, and then spiked with beta-agonists. Final concentrations of drug-fortified meat and liver samples were achieved based on the minimum detection limits claimed by the suppliers of the various test kits. Table 3 shows the minimum detection levels claimed by the suppliers of the test kits.

Field-incurred meat preparation

Table 4 shows the types and quantities of beta-agonist residues detected initially in meat and liver from a previous prevalence study, which

Table 1
Reference standards of drugs used for the validation of beta-agonist residue.

Drug	Source	Form	Lot No.	Correction factor (%) purity
Salbutamol-S5013	Sigma	Hemisulphate salt	29H0630	95
Clenbuterol-C5423	Sigma	Hydrochloride	21K1528	95
Terbutaline-T2528	Sigma	Hemisulphate salt	59H0844	95

Table 2
Preparation of drugs for spiking of tissues.

Drug	Drug weight (g)	Active ingredient (g)	Concentration of stock standard solution (ppm)	Serial dilution factors	Concentration of stock mixed standard solution
Salbutamol hemisulphate	0.0122	0.01	1,000	1:100, 1:100 1:100 (1ppm)	50 µl of 1ppm of all three standards + 850 µl digest buffer (50 ng/ml)
Clenbuterol hydrochloride	0.0113	0.01	1,000	1:100, 1:100 1:100 (1ppm)	
Terbutaline hemisulphate	0.0121	0.01	1,000	1:100, 1:100 1:100 (1ppm)	

Table 3
Minumum detection level of EIA test-kits claimed by supplier.

Test kit	Clenbuterol	Salbutamol	Terbutaline
Randox			
Tissue	0.5 ng/g	0.6 ng/g	1 ng/g
Euro-Diagnostica			
Muscle	0.2 ng/g	0.2 ng/g	0.5 ng/g
Liver	0.5 ng/g	0.5 ng/g	1.2 ng/g
Rida-screen			
Muscle	0.4 ng/g	*4.0 ng/g	*4.0 ng/g
Liver	0.4 ng/g	*4.0 ng/g	*4.0 ng/g

* = Not specified

Table 4
Type and concentration of beta-agonist residues in edible tissues of swine obtained by multi-residue analysis using immunoaffinity column gas chromatography-mass spectrometry.

Food Beta-agonist detected	Mean (ng/g)	SD	N	CV (%)
Pork				
Terbutaline	2.9633	0.175	3	2.85
Terbutaline	6.1377	0.107	3	4.71
Terbutaline	5.5702	0.077	3	3.57
Clenbuterol	2.2670	0.066	3	1.18
Clenbuterol	2.1440	0.062	3	2.08
Pig liver				
Terbutaline	1.6630	0.033	3	5.00
Salbutamol	9.3190	0.315	3	3.37
Terbutaline	6.7000	0.111	3	1.66
Terbutaline	1.0290	0.097	3	9.45
Salbutamol	6.4850	0.122	3	1.88
Clenbuterol	1.2210	0.097	3	7.91

were used as field-incurred tissues.

Randox β-agonist ELISA test kit

Five grams of macerated sample was weighed directly into polypropylene centrifuge tubes. Fifteen milliliters of digest buffer (Randox) with protease enzyme (Sigma P-1547) was added and the contents were homogenized with a vortex mixer (Vortex-Genie 2). The mixture was incubated in a waterbath (Memmert, Schwalaxh, Germany) at 55±2C for 2 hours with frequent mixing. The sample was then cooled to 4ºC in an ice bath before being centrifuged at 4,000rpm for 20 minutes in a refrigerated centrifuge (IBC Centra MP4R). The supernatant was filtered using a glass fiber filter paper (GC 50 90mm Advantec Toyo). One milliliter of protease inhibitor solution (Randox) was added to 12ml of filtered supernatant. The pH of the treated supernatant was adjusted to 8.8-9.0 with 5M sodium hydroxide (Analar) before the volume was made up to 16ml with UHQ water. This was followed by removal of interferences (clean-up) by immunoaffinity column (Randox) which had been brought to room temperature. The column eluate, which was collected in 5ml reaction vials (Supelco), was then evaporated to dryness under a stream of nitrogen with an Analytical Nitrogen Evaporator Model III (N-Evap Organomation Associates Inc, Berlin) at 65±5ºC. The dried extract was resuspended in 0.8ml diluted diluent prewarmed to 50ºC and vortexed.

Fifty microliters of standards and control were pippeted into the wells in triplicate while samples were pippeted in replicates of twenty, 50 µl of conjugate was added. The microtiter plate was covered with adhesive film, gently tapped from side to side, and incubated for one hour at room temperature. The plate was inverted and the liquid was tapped out. The microtiter plate was washed 6 times with diluted diluent over a 10-15 minute period. After the final wash, it was tapped onto a tissue paper. Immediately after washing, 125 µl of one-shot substrate solution was pippeted into each well. The microtiter plate was gently tapped and incubated for 20 minutes at room temperature in the dark. The color reaction was stopped by addition of 100 µl of 0.2M hydrochloric acid per well. A color change of blue to yellow was evident, and the optical density was measured at 450nm within 10 minutes.

The mean absorbance of the standards, controls, and samples were calculated. A graph displaying absorbance *vs* $\log_{10}$ standard was plotted. The sample and control absorbances were compared with the standard curve.

Euro-Diagnostica β-agonist ELISA test kit

One gram of homogenized meat was weighed directly into a polypropylene tube. Four milliliters of pronase containing Tris buffer were added. After an overnight incubation at 55ºC and centrifugation at 1,500 rpm for 10 minutes, 2 ml of the supernatant was pippeted into a test tube. The pH was adjusted to 9.5 by adding a few drops of sodium hydroxide (10M). Four milliliters of isobutanol was added, then the solution was vortexed and left for 5 minutes. Subsequently, 2 ml of the upper layer was pippeted into a glass tube and evaporated at 60ºC under a stream of nitrogen. The residue was dissolved in 250 µl of dilution buffer.

One hundred microliters of dilution buffer was pippeted into the first well in triplicate (A1,A2,A3). Fifty microliters of standards and control were pippeted into the wells in triplicates while samples were pippeted in 20 replications. Twenty-five microliters of conjugate was added to all wells except A1,A2,A3. The microtiter plate was sealed, gently tapped from side to side and incubated for one hour at room temperature in the dark. The plate was inverted and the liquid was tapped out. The microtiter plate was washed 3 times with rinsing buffer. Immediately after washing, 100 µl of substrate solution was pippeted into each well. The microtiter plate was gently tapped and incubated for 30 minutes at room temperature in the dark. The color reaction was stopped by addition of 100 µl of stop solution per well. The optical density was measured at 450nm within 10 minutes.

The optical density values of the six standards and samples were divided by the mean optical density value of the zero standards, and multiplied by 100. The % maximal absorbance of the standards, controls, and samples were calculated. A graph displaying maximal absorbance *vs* $\log_{10}$ standard was plotted. The sample and control absorbances were compared with the standard curve.

Ridascreen β-agonist ELISA test kit

Five grams of well-minced sample was homogenized with 25 ml of 50 mM tris buffer pH8.5 for 30 minutes. Fifteen milliliters of n-heptane was added and shaken for 5 minutes. The mixture was then centrifuged for 5 minutes at 4,000*g* at 10-15ºC. The upper heptane layer and thin intermediate layer of fat was removed using a Pasteur pippet. This was repeated with another 15 ml of heptane. 0.5 ml of half-concentrated hydrochloric acid was added to the aqueous meat homogenate, and shaken for one hour. Six grams of meat homogenate was weighed and centrifuged for 15 minutes at 4,000*g*. The supernatant was transferred into another centrifugeable vial, and 300 µl of 1M sodium hydroxide was added and mixed for 15 minutes. Four milliters of potassium dihydrogen phospate buffer was added, mixed briefly and stored at 4ºC for $1^{1}/_{2}$ hours or overnight. This underwent centrifugation at 4000rpm for 15 minutes at 10-15ºC. The entire supernatant was allowed to reach room temperature and then underwent clean-up with RIDA C18 column. The eluant was evaporated completely at 50-60ºC under a stream of nitrogen, and redissolved in 0.4 ml of distilled water.

A sufficient number of wells was inserted into the microwell holder. One hundred microliters of diluted antibody solution was added to each well and incubated at 2-8ºC for 15 hours overnight. The liquid was poured out of the wells and the microwell holder was tapped upside down vigorously thrice against absorbent paper. The wells were filled with 250 µl wash solution and rinsed thrice. Twenty

microliters of standards and control were pippeted into the wells in triplicate while samples were pippeted in replicates of twenty. One hundred microliters of conjugate was added. The microtiter plate was covered with adhesive film. The microtiter plate was gently tapped from side to side and incubated for one hour at room temperature. The plate was inverted and the liquid was tapped out. The microtiter plate was washed 3 times with 250 µl wash solution. After the final wash, it was tapped onto a tissue paper. Immediately after washing, 50 µl of one-shot substrate solution was pippeted into each well. The microtiter plate was gently tapped and incubated for 20 minutes at room temperature in the dark. The color reaction was stopped by addition of 100 µl of stop solution per well. A color change of blue to yellow was evident, and the optical density was measured at 450 nm within 10 minutes.

The optical density of the standards, controls, and samples were calculated. A graph displaying absorbance *vs* $\log_{10}$ standard was plotted. The sample and control absorbances were compared with the standard curve.

Statistical analysis

Data collected was statistically analyzed for one-way analysis of variance, Student's *t*-test, and variance ratios or F-ratios were performed.

RESULTS

The three test kits were compared in terms of accuracy, repeatability, ease of use, and the cost involved. Table 5 shows the reactivity pattern of antibodies within each test kit to the beta-agonists. Here, it can be seen that the Randox and Euro-Diagnostica test kits were able to detect a large proportion of the beta-agonists of interest compared with the Ridascreen test kit, which appeared more specialized for the detection of clenbuterol.

The calibration curves for all three test-kits were satisfactory, with the coefficient of determination (R^2) exceeding 0.99. In terms of sensitivity, all three test kits were able to detect the beta-agonists at the minimum level of detection, as claimed by the suppliers. Table 6 shows the recovery rate of the beta-agonists by the various test kits. The Euro-Diagnostica test kit showed the highest recovery of the beta-agonists of interest. Table 7 shows the coefficient of variation and the correlation coefficients for the various test kits for fortified samples.

It was found that all three test kits showed a sensitivity of 100% for field-incurred positive samples. Table 8 shows the coefficients of variation and correlations coefficient for the various test kits for field-incurred samples. It was found

Table 5
Cross-reactivities of antibodies in various test-kits to beta-agonist compounds.

Compound	% Cross-reactivity Randox	% Cross-reactivity Euro-diagnostica	% Cross-reactivity Ridascreen
Clenbuterol	100	100	100
Salbutamol	86	100	11
Terbutaline	50	40	10
Carbuterol	90	40	4
Methyl-clenbuterol	75	50	N.S.
Bromobuterol	53	100	200
Mabuterol	45	60	71
Pirbuterol	25	N.S.	N.S.
Mapenterol	32	70	N.S.
Cimaterol	10	10	5
Ractopamine	<0.2	N.S.	N.S.
Feneterol	<0.2	N.S.	N.S.
Cimbuterol	N.S.	75	N.S.
Tolubuterol	N.S.	50	N.S.

N.S. = Not specified

Table 6
Recovery rate of beta-agonist residues using the various test-kits.

	Randox	Euro-Diagnostica	Ridascreen
Clenbuterol	90 - 92%	114-132%	84-92%
Salbutamol	77 - 80%	121-123%	20-22 %
Terbutaline	46 - 48%	43-56%	19-21 %

Table 7
Coefficients of variation and coefficients of correlation (R) of various test kits for fortified samples.

	Randox	Euro-Diagnostica	Ridascreen
Coefficient of variation (C.V.) for fortified samples	1-2%	1-6%	1-3%
Repeatability: Coefficient of correlation (R) for fortified samples	0.89055	0.94458	0.9817

Table 8
Coefficients of correlation between GC-MS and test kit, and coefficents of correlation (R) of the various test kits for field-incurred samples.

	Randox	Euro-Diagnostica	Ridascreen
Coefficient of correlation (R) for positive field-incurred samples and GC-MS	0.996023	0.906087	0.24945
Repeatability: Coefficient of correlation (R) for field-incurred samples	0.99384	0.993014	0.98328

that the Randox test kit showed the greatest correlation between the gas chromatography-mass spectrometry readings and the ELISA readings, while a poor correlation was shown by the Ridascreen test kit. Table 9 shows the proportion of false positives shown by each test kit, while this information is repeated in terms of sensitivity and specificity in Table 10. It was found that all three test kits showed a low rate of false positives for cows and goats, but high rates of apparent false positives for swine.

DISCUSSION

Apparent false positives could be due to the presence of beta-agonists at levels lower than that which can be detected by gas chromatography-mass spectrometry; the presence of beta-agonists other than salbutamol, clenbuterol, and terbutaline, or the presence of other drugs or chemicals that trigger positive reactions from the ELISA test kit.

In terms of ease of use, the Ridascreen test kit was a little more tedious in that the diluted antibodies had to be embedded on the base of the walls by incubating 100 μl of the antibody solution in each well at 2-8ºC for 15 hours prior to testing. Otherwise the testing procedures of the three test kits were similar.

The test kits were also compared in terms of cost, to determine which was most economical. It was found that the Randox test kit incurred the lowest cost, while the Euro-diagnostica test kit was the most expensive.

In general, while both the Randox and Euro-diagnostica test kits were quite reliable and similar, the Randox ELISA test kit appeared to be the most suitable due to its appreciably lower cost.

Table 9
Proportion of false positives obtained by the various test kits.

	Randox		Euro-Diagnostica		Ridascreen	
	Resp +ve	Resp -ve	Resp +ve	Resp -ve	Resp +ve	Resp -ve
Goat meat	2	18	2	18	2	18
Goat liver	2	18	2	18	1	19
Pig meat	11	9	11	9	10	10
Pig liver	13	7	13	7	11	9
Cow meat	0	20	0	20	0	20
Cow liver	1	19	1	19	1	19

Table 10
Sensitivity and specificity of the various test kits for samples that tested negative by gas chromatography-mass spectrometry.

	Randox		Euro-Diagnostica		Ridascreen	
	Sens[a]	Spec[b]	Sens[a]	Spec[b]	Sens[a]	Spec[b]
Goat meat	10	90	10	90	10	90
Goat liver	10	90	10	90	5	95
Pig meat	55	45	55	45	50	50
Pig liver	65	35	65	35	55	45
Cow meat	0	100	0	100	0	100
Cow liver	5	95	5	95	5	95

[a]Sens = Sensitivity; [b]Spec = Specificity

Conclusion

It was found that the Randox beta-agonist test kit was suitable as a screening tool due to a few factors. The reactivity pattern of the antibodies within the Randox beta-agonist test kit, to the beta-agonists of interest which have been detected in this country, was good. The test kit was able to detect beta-agonists at the minimum level of detection claimed by the supplier. The performance of the method as assessed through recovery rates of the beta-agonists in fortified samples was satisfactory. The coefficient of variation, which gives a measure of the reproducibility of the method, was low (1-3%), indicating acceptable variation. Repeatability, as measured through the coefficient of correlation, was satisfactory. For field-incurred positive samples, the test kit showed a sensitivity of 100%.

As with the other two kits, it was found that the Randox ELISA test kit showed a low rate of false positives for goat and cow tissues, but high rates of apparent false positives for swine tissues. Despite the high rate of false positives for swine, samples testing negative can be confidently eliminated from undergoing confirmatory tests.

ACKNOWLEDGEMENTS

This study was funded by the Food Quality Control Division of the Ministry of Health under the Food Contaminant Monitoring Program.

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