Analysis of Beta-agonists in Animal Feeds by Liquid Chromatography-Tandem Mass Spectrometry and Health Risk Assessment

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***Abstract*—**Contamination of beta-agonists (salbutamol, clenbuterol and ractopamine) were measured in a total 74 collected animal feed samples including 33 swine, 18 cattle and 23 chicken by using liquid chromatography-tandem mass spectrometry. The most often found were salbutamol, clenbuterol and ractopamine representing of 95, 74 and 53 percent, respectively and most found in cattle feeds followed by swine and poultry feeds, respectively. The result detected that there was a clenbuterol substance in high concentration of each kinds of samples. Clenbuterol which was found in high concentration the range from 0.35-3.94 ng/g on swine feeds. Salbutamol and ractopamine were found in the similar range of high concentration the range from 0.06-2.95 ng/g on cattle feeds. This illegal usage may harm to animal, human and environment. Therefore, monitoring of drugs in feed has become important to ensure feed and food safety.

***Keywords*—**Beta-agonists, animal feeds, LC-MS/MS.

I. Introduction

RECENTLY, trend of food safety become more concerned, especially for drugs resistance and antibiotic residue in humans and environment as well. Mixing of feed additives in food animals is critically important for not only for economy, but also included, importance of the health of humans and animals. Especially in terms of accelerating growth of animal. However, the abuse and overuse of these substances may be accumulated in animal organs potentially causing toxic effects to human health [1].

Beta-agonists are synthetic phenethanolamine compound is used for treatment of asthma and bronchial diseases in humans. It is also used in livestock for growth promotion for meat production. In Thailand some farmers use beta-agonists such as salbutamol, clenbuterol and ractopamine as feed additives to improve product performance and reduce carcass fat accretion [2]. For example, salbutamol that improves carcass quality of finishing pigs because it decreases the total fat (20.39%) and increases lean meat (10.74%) [3]. In addition, clenbuterol and salbutamol were used in broiler chicken diet, to increase growth and decrease abdominal fat deposition rate [4], [5]. However, there is no study report stated negative effect of using these compounds in animals and consumers. If the amount of beta-agonist exceeds the therapeutic doses 5-10 fold, these compounds, significant residual amount, accumulate these compounds in animal edible tissues. Because these drugs are stable chemical, and cannot be destroyed by heat [6]. For example, salbutamol is still stable after boiling in 100°C water temperature for about 45 min. Nonetheless, when a person eats the animal tissue (liver, kidney or meat) their residues in animal edible tissues can cause toxicity to human health. Thus, beta-agonists are banned from using in food-producing animals in many countries, especially in Thailand, the European Union and China.

The aim of this study was to investigate the beta-agonists contamination in animal feeds [Fig. 1] by using liquid chromatography-tandem mass spectrometry (LC-MS/MS) and to assess the human health risk from consumption of meat containing residues of veterinary in animal feed.

II. Materials and Methods

A. Sampling locations and collection

The samples of animal feeds were collected from swine, cattle and poultry farms in some parts of Thailand from June to September 2014. Altogether 74 samples assisted by Provincial Livestock Offices were collected in the North, the Central, and the Northeast of Thailand. The samples were blended then stored in zip-lock under dark and room temperature until analyzed.

B. Extraction and Clean up

About 2 g of homogenized animal feed was weighed into a 50 mL polypropylene centrifuge tube. Addition 20 mL of Milli-Q water and 2 mL 1M HCl into the tube. The mixture was shaken for 1 h. After that, sonicated it for 15 min, and centrifuged at 4,500 rpm for 20 min at 5°C. The supernatant was collected into another 50 mL polypropylene centrifuge tube for further clean up step.

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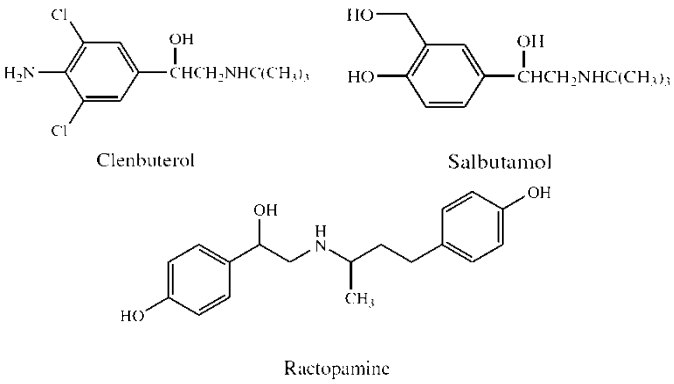

Fig. 1 Chemical structure of clenbuterol, salbutamol and ractopamine

Solid Phase Extraction (SPE) of each sample was performed by Bond Elut SCX cartridges (500 mg, 3 mL, Vertical Chromatography Co., Thailand). Cartridges were preconditioned with 3 mL methanol and 3 mL water sequentially. The sample supernatant was loaded into the cartridge with flow rate 1 mL/min. Then the cartridge was washed with 2 mL water and 2 mL 2% formic acid in water, cartridge was then dried using vacuum manifold for 45 min. The analytes were eluted with 5 ml 5% ammonia solution in methanol. The eluent was dried under nitrogen gas at 40°C and reconstituted to 1 mL with water/acetonitrile containing 0.1% formic acid (90/10, v/v). The sample was vortex and sonicated 2-3 min and filtrated using a 0.2 µm nylon membrane filter into an auto sampler vial for analysis LC-MS/MS.

C. Quantitative analysis using LC-MS/MS

Quantitative analysis was performed using Agilent 1200 SL high performance liquid chromatography (HPLC) coupling with an Agilent 6410 triple quadruple mass spectrometer (MS/MS). HPLC columns were consisting of a series of protective guard column Agilent ZORBAX Eclipse Plus C18, (12.5 x 4.6 mm, 5 µm) and an analytical column Agilent ZORBAX Eclipse Plus C18, (2.1 x 100 mm, 1.8 µm). The column temperature was set at 40°C, the flow rate was at 0.25 mL/min, and injection volume was 10 µL. The mobile phase were used (A): water+0.1% formic acid+2 mM Ammonium acetate and (B): Acetonitrile+0.1% formic acid, using a gradient was 0min: 50% B, 0.5min: 20% B, 4min: 10% B, 5min: 98% B, 6.5min: 98% B, 7min: 50% B. The mass spectrometry conditions were as follows: positive electrospray ionization mode (ESI), capillary voltage 3.4 kV, desolvation temperature 300°C, desolvation gas 10 L/min, and nebulizer 50 psi.

D. Health risk assessment

The human health risk was calculated using amount of beta-agonists in percentage that remained in animal products, referred from researches [7]-[9]. The obtained results are hereafter being identified for typing of animal feeds for finding the amount of beta-agonists that residue in animal product, by compare between the proportion in term of percentage and the residue in each kind of animal product. As the results, the amounts of beta-agonists residue in animal products were compared with Codex reference Maximum Residue Limit (MRL) to assess health risk.

III. Results & Discussion

A. Beta-agonists contaminated in animal feeds

Total 74 collected animal feed samples were contaminated by beta-agonists about 75 percent, in all categories sample, as shown in Table I. It was found that the percentage of sample contaminated with beta-agonists from the North, the Central and the Northeast of Thailand were 85, 80 and 59 percent respectively. As described above, it may assume that farmers in the North and the Central are a majority farmer in Thailand, who owning a large livestock farms so they may have their own ability to buy drug for using as a supplementary to increase the growing rates of their animals regardless of the animals' health, including the effect that may occur from consuming these contaminated animal product.

TABLE I
Beta-agonists found separated by regional

Region	Total number of sample	Number of sample found Beta-agonists			Total (%)
		Rac	Sal	Clen	
North	20	16	17	18	85
Northeast	27	5	17	26	59
Central	27	18	21	26	80
Total	74	39	55	70	75

In addition, the most beta-agonists contamination was found in cattle feeds, representing of 91 percent. Whereas the beta-agonists contamination in swine and poultry feeds were similar, representing of 69 and 68 percent, respectively as shown in Table II.

The highest amount of contaminated beta-agonists in cattle feeds, probably cause by the use orientation of cattle had been changed from labor animal to be meat production and become increasing every year not only for internal consumption but also for international market as well. From all of the above, it can assume that, usages of beta-agonists may be related to the cattle farming objective. For swine and poultry, more over 70 to 80 percent of feed production factory in Thailand, they produce swine and poultry feeds together in the same production line, meaning that in case of beta-agonists contaminated to either swine or poultry production, it may be possible to cause a cross contaminate. Salbutamol was mostly detected in animal feeds followed by clenbuterol and ractopamine, representing as 95, 74 and 53 percent in contaminated feeds, respectively. These results were indicated that salbutamol maybe commonly use in animal feeds. Probably there are easily to find and also registered. Annual report from the Department of Livestock Development (2010-2012) stated that the beta-agonists as salbutamol and ractopamine were prohibited in animal feeds. In the present time, salbutamol is more widely used because of local availability and it has been used to treat the asthma disease in human. The effect of these drugs causes the increase of heart rate, the decrease of fat in animal body and the increase of the amount of red meat [10]. Therefore a trend is to be widely

used and its made beta-agonists usage compounds are increase relatively in farm production.

TABLE II
BETA-AGONISTS RESIDUE FOUND IN ANIMAL FEEDS

Kinds of sample	Region	Number of sample found Beta-agonists			Total (%)
		Ractopamine	Salbutamol	Clenbuterol	
Swine	North	11/13	13/13	12/13	
	Northeast	2/11	11/11	2/11	
	Central	4/9	9/9	4/9	
	Total	17/33	33/33	18/33	69
Cattle	North	2/2	2/2	2/2	
	Northeast	3/6	5/6	6/6	
	Central	9/10	10/10	10/10	
	Total	14/18	17/18	18/18	91
Poultry	North	3/5	3/5	3/5	
	Northeast	0/10	10/10	9/10	
	Central	5/8	7/8	7/8	
	Total	8/23	20/23	19/23	68
Total		39/74 (53)	70/74 (95)	50/74 (74)	76

Contaminate experiment of beta-agonist compounds, it totally showed in Fig. 2, the average concentration of clenbuterol is the highest value, which the average concentration is 1.32 ng/g, followed by salbutamol and ractopamine, which the average concentration are 1.07 ng/g and 0.94 ng/g respectively.

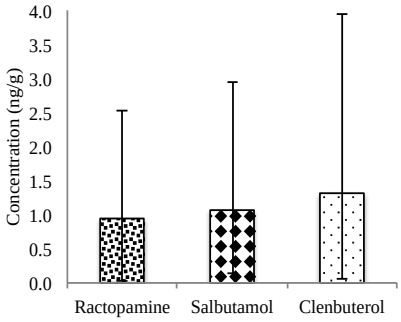

Fig. 2 Beta-agonists concentration in each drug compound

Table III showed the concentration of each contaminated residues, which clenbuterol was the most contaminant with concentration value of 3.94 ng/g and this found at swine feeds. According to the study report from of Vietnam, which study on the matter of the beta-agonists in swine feeds. It was found that the level of clenbuterol with an average of 124.82 ng/g is higher than salbutamol the average of 57.71 ng/g [11]. Ractopamine was detected with the highest concentration of 2.54 ng/g follow by poultry and swine feeds at the concentration of 2.02 and 1.57 ng/g respectively. This result is consistent with Harintharanon [10] who said that ractopamine was more effective with cattle than swine and poultry. Salbutamol was detected with the highest concentration in cattle feeds, with the value of 2.95 ng/g followed by poultry and swine feeds at the concentration of 2.51 and 2.17 ng/g respectively. And a detected amount of salbutamol in each feed showed no significant difference.

The contaminations of three types of beta-agonists as mentioned above were detected in all examined samples. The propose of using beta-agonist substances were to increase the amount of red meat by being affected to β2 receptors on muscle cells, structure and fat cells. It has been reducing protein synthesis which caused muscle cells growth bigger [2]. Therefore, it was found that there were the uses a group of beta-agonist substances within livestock processes.

TABLE III
RANGE CONCENTRATION OF BETA-AGONISTS IN ANIMAL FEEDS

Kinds of sample	Concentration of Beta-agonists (ng/g)		
	Ractopamine	Salbutamol	Clenbuterol
Swine	0.03-1.57	0.13-2.17	0.35-3.94
Cattle	0.06-2.54	0.18-2.95	0.30-3.73
Poultry	0.04-2.02	0.21-2.51	0.06-2.93

In this study, ractopamine was not found in poultry feeds in the Northeast region as shown in Fig. 3. It may present in low concentrations; on the other hand, the uses of ractopamine is to improve growth and performance of the poultry meat production, including the ability of stimulating protein and changes a trend of lipid metabolism [12], [13]. The average outstandingly high concentration value is clenbuterol in swine feeds, because swine farmers in the Northeast region were mostly use their own mixing materials. Clenbuterol were commonly widely and effectively used to feed their animals [2], [14], [15]. Thus, the average concentration is outstandingly higher than are the other regions.

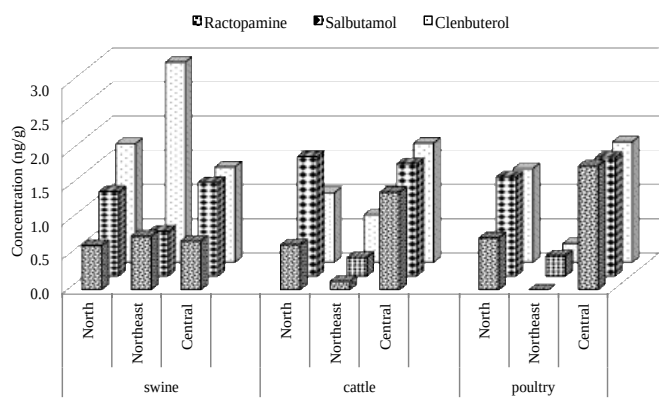

Fig. 3 Differentiation of average concentrative of beta-agonists found in animal feeds separated by regional

B. Health risk assessment

Ractopamine, salbutamol and clenbuterol contaminated in animal feeds were used to assess human health risk by referred to the references [7]-[9] of residues in animal products, as shown in Table IV. And the highest concentrations of ractopamine, salbutamol and clenbuterol contaminated in those samples, to calculate the amount of residues in animal products.

TABLE IV
PROPORTION IN PERCENTAGE AND THE RESIDUE IN EACH KIND OF ANIMAL PRODUCTS

Compounds	Target species	Sample type	Amount residue (%)	Reference
Clenbuterol	Swine	Liver	8.00	Pleadin et al., 2010
		Muscle	22.00	
Ractopamine hydrochloride	Swine	Muscle	0.03	Qiang et al., 2007
Salbutamol	Swine	Muscle	0.10	Hung et al., 2010
		Lung	0.87	
		Kidney	1.06	
		Liver	2.35	

These results were then compared to the analytical data of animal feeds. The results of residue in muscle of ractopamine, clenbuterol and salbutamol were 0.0002 ng/g, 0.3000 ng/g and 0.0010 ng/g, respectively. Subsequently, the results were compared with Codex reference Maximum Residue Limit (MRL) to assess health risk, as shown in Table V. It was found that all the values of residue substances exceeded the level permitted by Codex reference, except ractopamine, which was below from the MRL standard, meaning that the samples were not harmful to human health. However, if this residue accumulated for a long time, it may affect consumers' health.

TABLE V
CALCULATED HEALTH RISK ASSESSMENT OF BETA-AGONIST IN ANIMAL TISSUES

Compound	Target species	Sample type	Calculated health risk assessment (ng/g)	Codex MRL(ng/g)
Clenbuterol	Swine	Liver	0.1000	-
		Muscle	0.3000	-
Ractopamine hydrochloride	Swine	Muscle	0.0002	10
Salbutamol	Swine	Muscle	0.0010	-
		Lung	0.0100	-
		Kidney	0.0100	-
		Liver	0.0200	-

IV. CONCLUSION

This study was determined the contamination of beta-agonists in animal feeds, whereas ractopamine, clenbuterol and salbutamol could be concluded as follows;

1. All sampling sites, beta-agonists were widely used in the North of Thailand followed by the Central and the Northeast.

2. Beta-agonists were detected in all of animal feed samples; cattle feeds were found the highest amount of beta-agonists, while swine and poultry feeds are similar.

3. A result from all of samples salbutamol was detected in mostly sample; followed by clenbuterol and ractopamine respectively.

4. High concentration of beta-agonists, the highest value detected on clenbuterol at a concentration of 3.94 ng/g in swine feeds and the lowest concentration value detected on ractopamine, as well in swine feeds. Therefore, the further usages of beta-agonists have to be reconsidering of beneficial and its risks, including the effects of Public health issues and feeds industry.

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