

Effects of ractopamine hydrochloride on the toxicity, growth performances, body tissue compositions, residual and pharmacokinetics in tilapia (*Oreochromis mossambicus*)

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Abstract

The aim of this study focused on the study of the effects of the ractopamine hydrochloride (RAC) on the toxicity, growth performances, body composition, residual, and pharmacokinetics in tilapia (*Oreochromis mossambicus*). Total 198 tilapia were randomly separated into 3 groups as high dose RAC group (oral administration with 60 ppm RAC), low dose RAC group (oral administration with 30 ppm RAC), and the control group (oral administration with methanol that volume was the same as RAC groups). Tilapia in both RAC groups were fed with RAC-contained diets for 12 weeks, continuously. The detection condition settings of high-performance liquid chromatography (HPLC) included Cosmosil 5C₁₈-MS-II column and ultraviolet (UV) light detector as station phase. The mobile phase included acetonitrile : 7 mM ammonium acetate solution (15 : 85, pH 8) and 1.1 mL/min flow rate were established. The results showed the major peak of RAC was occurred at 194 nm wavelength. There was no significant gross and histo-pathologic lesions in the heart in both RAC groups. Both RAC groups were significantly higher on body weight (BW) gain compared with the control group. Additionally, there was the highest level of muscular protein in the high dose RAC group. Muscular lipid level was decrease following with the dietary RAC in a dose-dependent manner. After oral administration with 60 ppm BW / day of RAC, the pharmacokinetic parameters $t_{1/2}$ (half-lives), T_{max} (time to maximum concentration) C_{max} (maximum concentration), MRT (mean residence time), CL_{tot} (total body clearance), and AUC_{0-inf} (area under the concentration-time curve from 0 h to infinity) were 3.42 hr, 2.0 hr, 9.7 ppm, 4.74 hr, 964 mL hr⁻¹ kg⁻¹, and 337.8 hr × µg mL⁻¹, respectively. Taken all results together, we considered that tilapia were fed with diets supplemented with 30 and 60 ppm RAC had no toxicity in the heart, short withdraw time, and the positive effects on BW gain, and protein and lipid levels in muscle.

Keywords: Body tissue compositions and growth performances; High performance liquid chromatography; Ractopamine hydrochloride; Residue and toxicity; Pharmacokinetics; Tilapia

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1. Introduction

Taiwan is a tropical/subtropical country. The subtropical fish species is the majority of aquaculture production in Taiwan (Hung et al., 2018; Hung et al., 2019; Hung et al., 2020a; Chang et al., 2022). Tilapiine fishes of the cichlid family are native to Africa and 20-30°C is the optimal growth temperature for most tilapia according to their original life environment in Africa (Hung et al., 2020a). Tilapia is easily adaptable to the new environment and conditions as well as any kind of food source. They contain lots of good nutrition materials for human health. They does not only taste delicious but also quite affordable compared to another source of protein. Tilapia plays a major role in aquaculture as world production of this tropical species exceeds that of salmon. Tilapia are traditionally raised in earth ponds and fed with by-products from food-producing agriculture. Currently, a more intensive type of fish farming of tilapia has started to develop. During a long time of the genetic improvement and research, tilapia have gradually become a unique and high-quality strain in Taiwan. Current, tilapia's industry name in Taiwan was named "Taiwan tilapia" (Hung et al., 2020a). Therefore, tilapia farming is one of many good business prospects in the farming and agriculture fields.

According to the reports of Food and Agriculture Organization of the United Nations (FAO), due to tilapia's strong viability, they have been carried out in more than 140 countries around the world. In 2017, global tilapia aquaculture production reached 6.5 million metric tons, most of which came from aquaculture, and capture fishery catches are estimated to be less than 1 million metric tons. In 2018, the global sales of tilapia reached 12 billion United States (US) dollars, 95% of which were exported from Asia, with China and Taiwan as the main suppliers, and the US as the main importer. The consumption of tilapia is declining, and the Asian region is absorbing large quantities of tilapia from its domestic market (Hung et al., 2020a).

Ractopamine hydrochloride is a kind of veterinary drugs. Its pharmaco-function is beta-adrenoceptor agonist. The acceptable daily intake (ADI) and marker residue of ractopamine hydrochloride is 0-1 µg/kg body weight (BW) and ractopamine. Currently, ractopamine residual levels in various tissues of animal species were reported. Maximum residue limits (MRL) of ractopamine hydrochloride recommended by Joint FAO/WHO Expert Committee on Food Additives (JECFA) in cattle and pig' fat, kidney, liver, and muscle is 10 µg/kg BW, 90 µg/kg BW, 40 µg/kg BW, and 10 µg/kg BW, respectively (<https://inchem.org/documents/jecfa/jecmono/v53je08.htm>). Taiwan also conducted JECFA risk assessment for ractopamine and established MRLs for ractopamine in beef and pork in 2007. MRL of ractopamine in cattle' muscle is 0.01 ppm; in pigs' muscle / fat, liver, kidney, and other edible tissues are respectively 0.01 ppm, 0.04 ppm, and 0.01 ppm (<https://consumer.fda.gov.tw/Law/VeterinaryDrugList.aspx?nodeID=519&sf=Ractopamine>). Taiwan's government prohibited beta-adrenoceptor agonists such as salbutamol, terbutaline, clenbuterol, and ractopamine to manufacture, adjust, import, export, sell, or display for the use of food-producing animals, currently. However, Taiwan's amendments were taken effect on January 1, 2021 that Taiwanese livestock farmers still cannot add ractopamine to their feed even the import restrictions on United State-sourced pigs and cattle meats were eased.

Currently, the tilapia culture industry is important in Taiwan (Hung et al., 2020a). JECFA-recommended standards of ractopamine for aquaculture is still lack. Therefore, the evaluation of ractopamine on safe and pharmacokinetics is very an important issue. The goals of this study were to evaluate the toxicity, growth performances, body tissue compositions, residual, and pharmacokinetics of ractopamine hydrochloride after oral administration (p.o.) in tilapia (*Oreochromis mossambicus*).

2. Materials and methods

2.1. Preparation of chemicals

In this study, acetonitrile [high-performance liquid chromatography (HPLC)-grade, methanol (HPLC-grade), ractopamine hydrochloride (analytical grade) (C₁₈H₂₃NO₃; Fig. 1), Sodium hydroxide, boric acid, ammonium acetate, ammonia, glacial acetic acid, and selenium powder were ordered from Merck (Kenilworth, New Jersey, USA). Tricaine methanesulfonate (MS222, Sigma-Aldrich, St. Louis, MO, USA) was used for euthanasia.

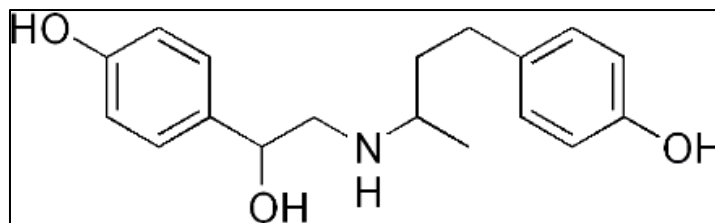


Figure 1 Chemical structure of ractopamine hydrochloride

2.2. Preparation of the standard solution and the extract solutions

Preparation of the ractopamine hydrochloride standard solution was conducted according to previously described procedures (Hung et al., 2018; Hung et al., 2019; Hung et al., 2020a,b). Ractopamine hydrochloride was dissolved in methanol to form 1,000 ppm ractopamine solution (stock solution). Ammonium acetate (0.54 g) was dissolved in deionized water (1,000 mL) to form 7 mM ammonium acetate solution. Later, 1,000 ppm ractopamine solution was well mixed with acetonitrile and 7 mM ammonium acetate solution to form 100, 50, 25, 10, 5, 1, 0.5, 0.1, 0.05, and 0.01 ppm standard solutions, were filtered via 0.45 μ m (pore size) filter membrane.

2.3. HPLC apparatus and set up

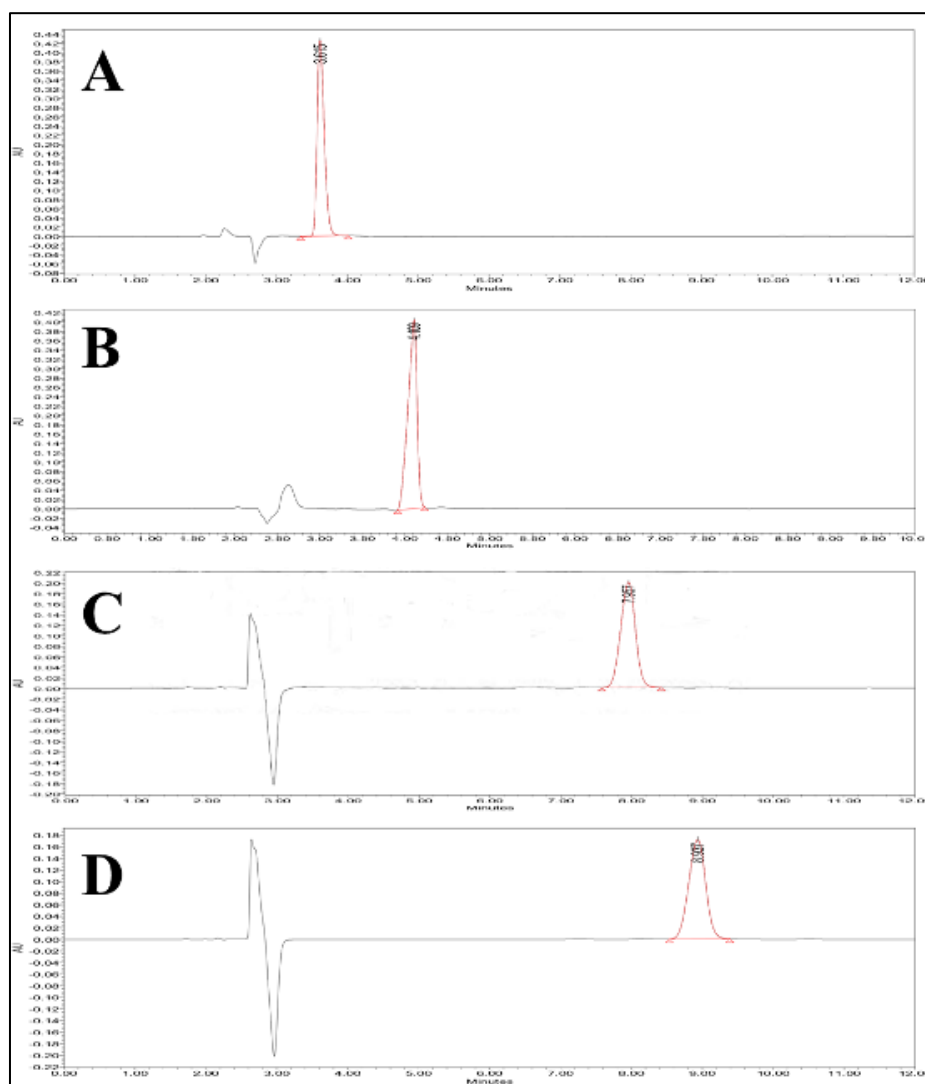


Figure 2 HPLC chromatographs for 100 ppm ractopamine standard solution with different mobile phases composited with double distilled water / acetonitrile (A) 50/50; (B) 40/60; (C) 30/70; (D) 15/85 used for testing, respectively.

The HPLC system and conditions set up were used according to previously described procedures (Hung et al., 2018; Hung et al., 2019; Hung et al., 2020a,b). An ultraviolet (UV) light detector with 194 nm detection wavelength and a Cosmosil 5C18-MS-II column (4.6 μ m, inner diameter 4.6 mm $\times$ 25 cm; Nacalaitesque, CA, USA) were applied. The various conditions of the mobile phase (7 mM ammonium acetate solution : acetonitrile) of HPLC respectively included 50 : 50 (v/v), 40 : 60 (v/v), 30 : 70 (v/v), and 15 : 85 (v/v) (Fig. 2).

2.4. Standardization and construction of calibration curves

Linearity were carried out according to previously described procedures (Hung et al., 2018; Hung et al., 2019; Hung et al., 2020a,b). Ractopamine hydrochloride solution (1,000 ppm) was well co-mixed at 1 : 1 (v : v) with 7 mM ammonium acetate solution and acetonitrile (pH 8.0). The 100, 50, 25, 10, 5, 1, 0.5, 0.1, 0.05, and 0.01 ppm standard solutions were analyzed via HPLC with an UV light detector (Photodiode Array Detector, Model 996, Water, Ajax, Ontario, Canada) and a Cosmosil 5C18-MS-II column (Nacalaitesque).

2.5. Limits of quantification and detection and specificity

The limits of detection (LOD), the limits of quantification (LOQ), and specificity of ractopamine hydrochloride were carried out according to previously describe procedures (Hung et al., 2018; Hung et al., 2019; Hung et al., 2020a,b).

2.6. Recovery assay

The 50 ppm ractopamine hydrochloride solution were respectively supplemented into the blank sera (5 g), blank muscles (5 g), and liver (5 g). After residue reconstitution with 7 mM ammonium acetate solution : acetonitrile (15 : 85, v/v; pH 8), the internal standard was finally added. The maximum absorption wavelength and flow rate was 194 nm and 1.1 mL min⁻¹, respectively.

2.7. Quantification and accuracy

Standardization and construction of calibration curves were carried out according to previously described procedures (Hung et al., 2018; Hung et al., 2019; Hung et al., 2020a,b). Samples of 5 g blank sera, 5 g blank muscle, and 5 g blank liver were respectively supplemented with known amounts of ractopamine hydrochloride and the internal standard and were analyzed as unknown samples.

2.8. Validation of intra-assay and inter-assay

The validation of intra-assay and inter-assay of ractopamine hydrochloride was conducted according to described previously (Ramos et al., 2003; Hung et al., 2018; Hung et al., 2019; Hung et al., 2020a,b). The concentration of ractopamine hydrochloride (20, 2, and 0.2 ppm) was used for the three repeat intra-assay. The values of SD (standard deviation), mean, and CV (coefficient of variation) were collected and evaluated the experimental repeat. On the other day, the concentration of ractopamine hydrochloride (20, 2, and 0.2 ppm) was used for the three repeat inter-assay and collected SD, mean, and CV values for evaluating the instrument precision.

2.9. Animal care and the experimental design

Healthy tilapia (*Oreochromis mossambicus*) (10.0 $\pm$ 0.8 g) were obtained from a commercial tilapia farm (Xuejia District, Tainan County, Taiwan) and were housed in three fiberglass reinforced plastic tank (length $\times$ width $\times$ depth is 120 cm $\times$ 60 cm $\times$ 45 cm) with a freshwater recirculation system. Experiments began after an acclimation period of 2 weeks.

Animal care in this study were carried out according to previously described the guideline of the Institutional Animal Care and Utilization Committee (IACUC). Tilapia (*Oreochromis mossambicus*) (total of No. is 198) were fed the laboratory diet [a standard laboratory diet (Table 1) or ractopamine hydrochloride-contained diets (30 ppm and 60 ppm ractopamine hydrochloride)] thrice a day and each feed weight was about 10% fish' BW. After feeding, the residual diet were cleaned. Fish' BW were detected weekly. Before the experiment, liver, sera, and muscle were collected from 6 fish per group to confirm no ractopamine residues in these samples. Three groups (n = 60) were divided in this experiment (12 weeks). Environment conditions were kept on a 12-h dark/light cycle and temperature was set at 20 $\pm$ 3°C and water pH was set at 6.8 $\pm$ 0.4.

Table 1 Basal diet ingredients and nutrition composition in this experiment

Crude protein	Crude fat	Crude fiber	Crude ash	Moisture content	HCl insoluble
> 45.5%	< 3%	< 1.2%	< 16.5%	< 11%	< 2%

2.10. Tissues collection of tilapia for the residual ractopamine detection

After 12 weeks experiment, six tilapia per group were randomly picked and performed euthanasia and sampling. The muscle (5 g) and liver (5 g) were collected and homogenized from each fish in each group. These homogenized tissues were used to detect the residual level of ractopamine hydrochloride.

2.11. Detection of the body protein and body fat in all groups

Detection of the body protein and body fat was according to the previous describe (Folch et al., 1957; Jiang et al., 2014). After 12 weeks experiment, six tilapia per group were randomly picked and performed euthanasia and sampling. The fat (5 g) was collected and homogenized from each fish in each group. These homogenized tissues were used to detect the residual level of ractopamine hydrochloride.

2.12. Sera, muscle, and liver sample clean-up, derivatization, and extraction

The sample clean-up, derivatization, and extraction were conducted according to previously described procedures (Ramos et al., 2003; Hung et al., 2018; Hung et al., 2019; Hung et al., 2020a,b).

2.13. Sample collection and process of the experimental tilapia for the pharmacokinetic analysis of ractopamine hydrochloride

The pharmacokinetic analysis progress of blood samples was carried out according to previously described procedures (Hung et al., 2018; Hung et al., 2019; Hung et al., 2020a,b). Adult tilapia (500 ± 20 g) per group were given single p.o. of ractopamine hydrochloride (60 ppm) by gavage, whereas age and size matched 6 control tilapia were p.o. of a standard diet (Table 1). Approximately, sera (3-4 mL) were collected, cleaned up, derivatized, extracted, and performed the pharmacokinetic analysis of ractopamine from each group on 1, 2, 4, 8, 12, 16, 18, and 24 hour after ractopamine hydrochloride (60 ppm) administration.

Pharmacokinetic data analysis by using WinNonlin professional software (version 1.0, Pharsight Corporation, NY, NY, USA) were carried out according to previously described (Hung et al., 2018; Hung et al., 2019; Hung et al., 2020a,b). Pharmacokinetic data analysis of ractopamine hydrochloride in sera of the experimental tilapia ($n = 6$ / the experimental point) was using a non-compartmental 202 model. The parameters evaluated for serum were T_{max} (time to maximum concentration, hr), $t_{1/2}$ (half-lives, hr), C_{max} (maximum concentration, $\mu\text{g mL}^{-1}$), MRT (mean residence time, hr), CL_{tot} (total body clearance, $\text{mL hr}^{-1} \text{kg}^{-1}$), and $AUC_{0-\infty}$ (area under the concentration-time curve from 0 h to infinity, hr mg L^{-1}). S-PLUS software (Insightful Corp., NY, NY, USA), SigmaStat software (version 3.1, Systat software Inc., San Jose, CA, USA) and SigmaPlot software (version 9.0, Systat software Inc.) were also applied in the pharmacokinetic data analysis.

2.14. Statistical analysis

Statistical analysis were carried out according to previously described (Hung et al., 2018; Hung et al., 2019; Hung et al., 2020a,b). The statistical significant difference was at $*p < 0.05$ by using Student's t -test.

3. Results

3.1. Gross and histo-pathologic examination under ractopamine hydrochloride administration

At the end of the experiment, tilapia (*Oreochromis mossambicus*) in all groups were randomly picked and performed the gross and histo-pathologic examinations by a senior pathologic veterinarian. According to the gross and histo-pathologic examinations for tilapia (*Oreochromis mossambicus*) in all groups, no significant lesions were found in all groups with or without ractopamine hydrochloride (30 ppm or 60 ppm) administration (Fig. 3).

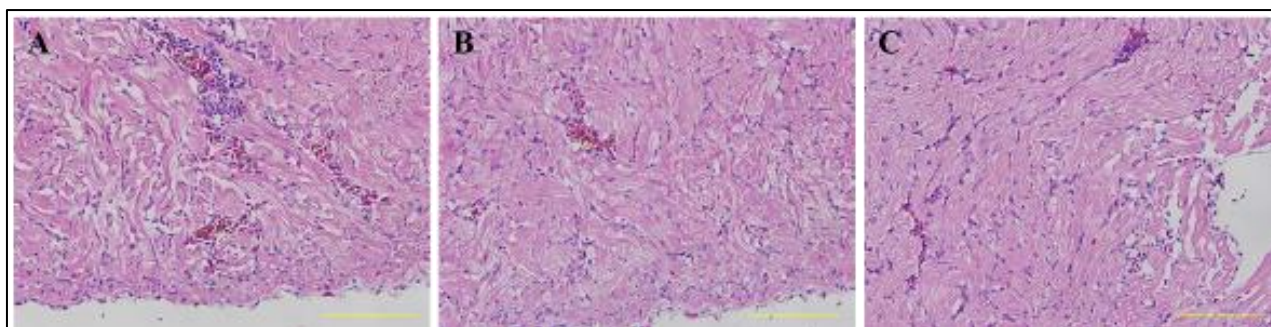


Figure 3 Toxicity effects of ractopamine hydrochloride in tilapia's heart. The regular histological section of myocardial cell. In all group, no lesions was found in myocardial cell. (A) the control group; (B) the low dose group; (C) the high dose group. The bar = 100 nm

3.2. Change of tilapia' BW and body protein and fat percentages in all groups with or without ractopamine hydrochloride administration

After feeding the laboratory diet [a standard laboratory diet or ractopamine hydrochloride-contained diets] thrice a day for 12 weeks and each feed weight was about 10% fish' BW. Tilapia' average of BW in the control group, low dose ractopamine group, and high dose ractopamine group were 150.6 g, 192.9 g, and 193.2 g, respectively. The tilapia' average of BW in the low dose ractopamine group and high dose ractopamine group was significantly increase about 28% (28.09%-28.29%) BW compared to the control group ($p < 0.05$) (Table 2). The tilapia' percentage (%) of body protein in the low dose ractopamine group and high dose ractopamine group was decrease (4.99% and 4.79%) compared to the control group (6.53%) (Table 3). The tilapia' percentage (%) of body fat in the low dose ractopamine group and high dose ractopamine group was increase (89.02% and 89.05%) compared to the control group (85.12%) (Table 3). Based on the results of body protein and body fat percentages, a trend was shown as ractopamine hydrochloride treatments decreased the percentage of body protein and increased the percentage of body fat in tilapia.

Table 2 Weight increase of tilapia fed 0, 30 or 60 ppm of ractopamine hydrochloride for 12 weeks

	W0	W2	W4	W6	W8	W10	W12
	(gram)						
Control group	10.0 ± 1.2	22.8 ± 0.9	39.7 ± 1.7	61.4 ± 3.1	92.7 ± 4.1	129.4 ± 7.1	150.6 ± 6.6
Low dose group	10.0 ± 1.4	23.9 ± 1.1	46.3 ± 0.7	75.2 ± 2.2	121.3 ± 3.7	172.0 ± 4.4	192.9 ± 7.1
High dose group	10.0 ± 1.7	23.7 ± 2.4	46.1 ± 1.4	75.5 ± 2.9	120.9 ± 2.9	172.3 ± 5.9	193.2 ± 5.4

Table 3 Muscle composition of tilapia fed 0, 30 or 60 ppm of ractopamine hydrochloride for 12 weeks

	Control group	Low dose group	High dose group
Body fat (%)	85.12	89.02	89.05
Body protein (%)	6.53	4.99	4.79

Control group: tilapia were fed with a standard diet; Low dose group: tilapia were fed with 30 ppm ractopamine hydrochloride-contained diet.

High dose group: tilapia were fed with 60 ppm ractopamine hydrochloride-contained diet.

3.3. The standard curve at various ractopamine hydrochloride concentrations

The results showed an obvious peak for 100 ppm ractopamine at 8.937 min under the UV light detector (the maximum absorption of the ractopamine standard was at 194 nm) (Fig. 4). The standard curve at various ractopamine hydrochloride concentrations (0.01, 0.05, 0.1, 0.5, 1, 5, and 10 ppm) was $Y = 1.9530 \times 10^4 X - 308.8$ (X: concentration; Y: area), $r^2 = 0.999$ (Fig. 5). LOD and LOQ were 0.01 ng/mL ($S / N \geq 3$) and 0.05 ng/mL ($S / N \geq 10$), respectively.

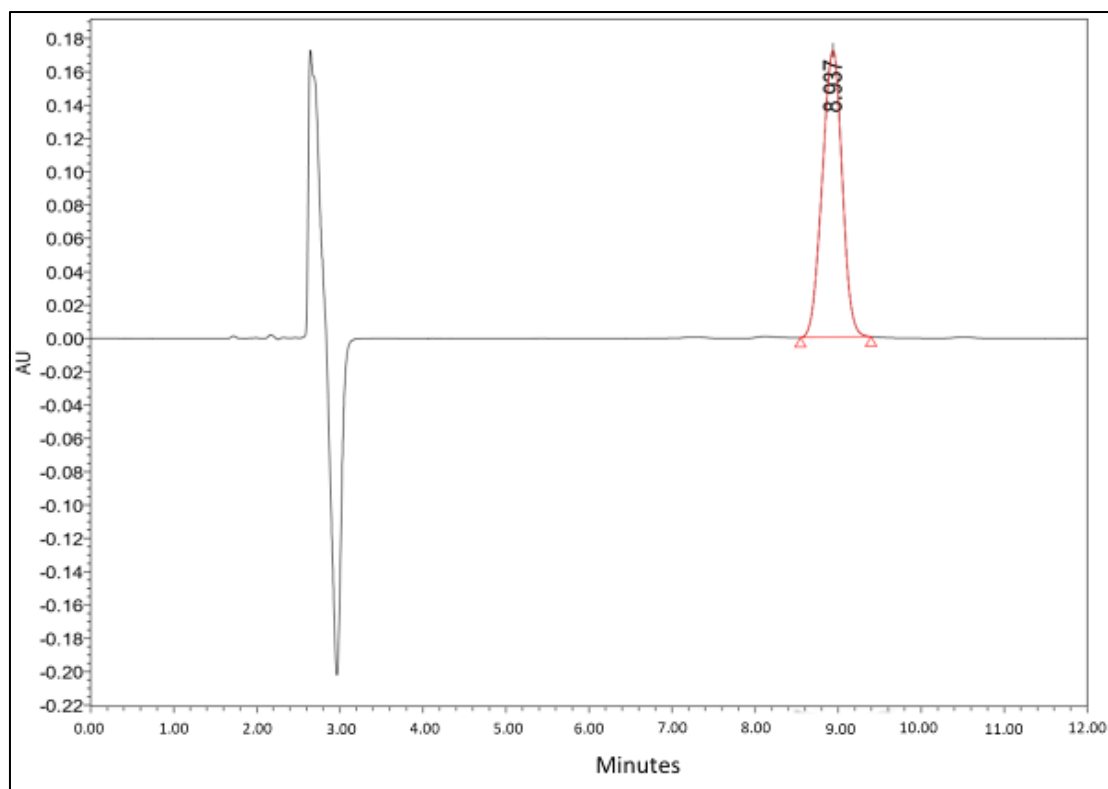


Figure 4 An obvious ractopamine hydrochloride standard solution (100 ppm) peak under HPLC chromatographs with UV detection (194 nm)

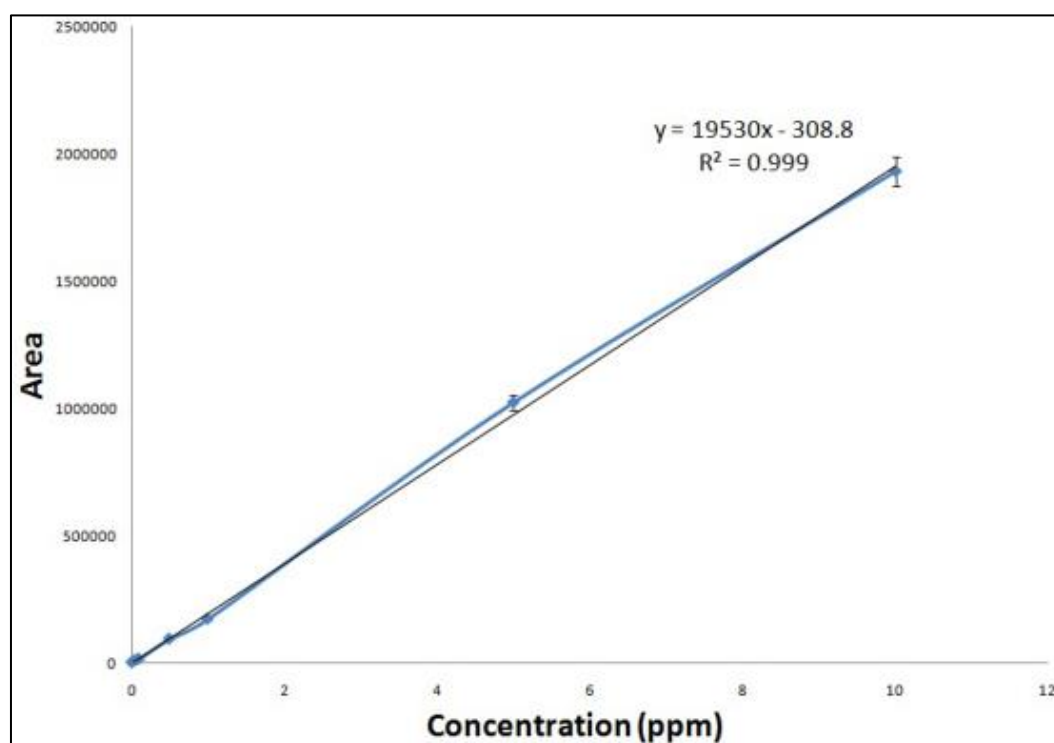


Figure 5 Standard curve of the standard ractopamine hydrochloride solution by HPLC with UV light detector. The standard curve at various ractopamine hydrochloride concentrations (0.01, 0.05, 0.1, 0.5, 1, 5, and 10 ppm) was $Y = 1.953 \times 10^4 X - 308.8$ (X: concentration; Y: area) and $r^2 = 0.999$

The coefficient of variation (CV) of intra-day precision was 0.01-0.09% (the average of CV is 0.033%) (Table 4). The CV of inter-day precision was 0.005-0.15% (the average of CV is 0.037%) (Table 4). The CV of precision was lower than the 10% that indicated the precision was acceptable in this study.

Table 4 Accuracy and precision for ractopamine hydrochloride determination using HPLC with a UV detector

Day	Intra/inter-day	Ractopamine (ppm)	Area (mean)	SD	CV (%)
1 st	Intra-day				
		20	3,353,393	91,365	2.7
		2	330,166	15,445	4.6
		0.2	39,630	1,904	4.8
	Inter-day				
1 st		20	3,518,680	131,766	3.7
		2	346,094	13,406	3.8
		0.2	39,980	1,001	2.5
2 nd		20	3,498,064	116,457	3.3
		2	397,110	19,471	4.9
		0.2	32,270	1,322	3.5
3 rd		20	3,564,806	87,228	2.4
		2	362,774	12,172	3.3
		0.2	39,937	1,076	2.6

n = 3 and the values are mean $\pm$ SD; Abbreviations: CV (coefficient of variation) and SD (standard deviation).

HPLC chromatographs of 50 ppm ractopamine hydrochloride were showed between 8.893-9.183 minutes in the spiked tissues (muscle, liver, and sera) compared to the spiked blank tissues (muscle, liver, and sera). Residual concentrations (ppm) and recovery (%) of 50 ppm ractopamine hydrochloride in the spiked tissues (muscle, liver, and sera) were 44.6 ± 1.3 ppm, 89.5%; 45.5 ± 1.5 ppm, 90.2%; 49.5 ± 1.3 ppm, 97.4% in muscle, liver, and serum, respectively (Table 5).

Table 5 Ractopamine hydrochloride residue concentrations in the liver, muscle, and serum of the experimental tilapia (n = 6) at various time points during the experiment

Tissues	Concentration (ppm)	Mean $\pm$ SD (ppm)	Recovery (%)
Muscle	50	44.6 ± 1.3	89.5
Liver		45.5 ± 1.5	90.2
Serum		49.5 ± 1.3	97.4

n = 3 and the values are mean $\pm$ SD; Abbreviations: SD (standard deviation).

3.4. Residue concentrations in the experimental tilapia' tissues p.o. with ractopamine hydrochloride (30 ppm or 60 ppm) at various time points

Detection of ractopamine hydrochloride concentrations of tilapia' muscle and liver were followed p.o. with 30 ppm or 60 ppm ractopamine for 12 weeks. After 12 weeks ractopamine hydrochloride administration, the detection of the residual ractopamine concentrations were performed in the experimental tilapia' tissues (muscle and liver) post 1, 2, 4, 8, 10, and 12 day ractopamine hydrochloride administration.

At day 1 after 60 ppm ractopamine hydrochloride p.o. to tilapia, ractopamine hydrochloride concentration in liver was the highest (90.2 ± 1.42 ppm). The ractopamine concentration in liver decreased gradually after ractopamine hydrochloride p.o. At day 12 after ractopamine p.o., ractopamine hydrochloride concentration in liver was below the LOD ($0.01 \mu\text{g mL}^{-1}$) (Table 6). At 1 day after 60 ppm ractopamine hydrochloride p.o. to tilapia, ractopamine

concentration in muscle was the highest (12.9 ± 0.83 ppm). At day 2 after 60 ppm ractopamine hydrochloride p.o. to tilapia, ractopamine concentration in muscle was 0.04 ± 0.01 ppm. At day 4 after ractopamine hydrochloride p.o., ractopamine hydrochloride concentration in muscle was below the LOD ($0.01 \mu\text{g mL}^{-1}$) (Table 6).

Table 6 Ractopamine hydrochloride residue concentrations in the liver and muscle of the experimental tilapia (n = 6) at various time points during the experiment

60 ppm ractopamine hydrochloride administration			
Time (day)	Liver (ppm)	Muscle (ppm)	Blank (ppm)
1	90.2 ± 1.42	12.9 ± 0.83	–
2	46.4 ± 1.37	0.04 ± 0.01	–
4	8.30 ± 1.01	–	–
8	3.80 ± 0.87	–	–
10	0.35 ± 0.04	–	–
12	–	–	–
30 ppm ractopamine hydrochloride administration			
Time (day)	Liver (ppm)	Muscle (ppm)	Blank (ppm)
1	78.4 ± 1.09	7.4 ± 0.50	–
2	34.3 ± 1.82	–	–
4	7.20 ± 0.70	–	–
8	3.10 ± 0.37	–	–
10	0.24 ± 0.09	–	–
12	–	–	–

n = 6 at the various time points during the experiment and the values are mean $\pm$ SD; '–' : Under LOD (0.01 ppm); Abbreviations: LOD (limit of detection) and SD (standard deviation).

At day 1 after 30 ppm ractopamine hydrochloride p.o. to tilapia, ractopamine hydrochloride concentration in liver was the highest (78.4 ± 1.09 ppm). After ractopamine hydrochloride p.o., the ractopamine concentration in liver decreased gradually. At day 12 after ractopamine hydrochloride p.o., ractopamine hydrochloride concentration in liver was below the LOD ($0.01 \mu\text{g mL}^{-1}$) (Table 6). At day 1 after 30 ppm ractopamine hydrochloride p.o. to tilapia, ractopamine hydrochloride concentration in muscle was the highest (7.4 ± 0.5 ppm). At day 2 after ractopamine hydrochloride p.o., ractopamine concentration in muscle was below the LOD ($0.01 \mu\text{g mL}^{-1}$) (Table 6).

3.5. Pharmacokinetic analysis of ractopamine hydrochloride in the experimental tilapia' sera

Pharmacokinetic analysis of ractopamine hydrochloride residue concentration in sera post p.o. with 60 ppm ractopamine hydrochloride at 1, 2, 4, 8, 12, 16, 18, and 24 hr. At each time point, 6 tilapia was used. At 1, 2, 4, 8, 12, and 16 hr after 60 ppm ractopamine hydrochloride p.o. to tilapia, ractopamine hydrochloride concentration in sera was 7.4 ± 0.87 ppm, 9.7 ± 0.63 ppm, 3.8 ± 0.12 ppm, 2.7 ± 0.74 ppm, 0.44 ± 0.02 ppm, and 0.02 ± 0.01 ppm, respectively. At 18–24 hr after ractopamine hydrochloride p.o., ractopamine hydrochloride concentration in sera was below the LOD ($0.01 \mu\text{g mL}^{-1}$) (Table 7). After p.o. with 60 ppm ractopamine hydrochloride, the pharmacokinetic analyses of ractopamine in the experimental tilapia' sera were showed that $t_{1/2}$ was 3.42 ± 0.03 hr; $T_{\max}$ occurred at 2.0 ± 0.0 hr; $C_{\max}$ was 9.7 ± 0.16 ppm; MRT was 4.74 ± 0.14 hr; CL_{tot} was 946 ± 17.2 mL hr $^{-1}$ kg $^{-1}$; $AUC_{0-\text{inf}}$ was 337.8 ± 10.2 hr $\mu\text{g mL}^{-1}$ (Table 8).

Table 7 Ractopamine hydrochloride residue concentrations in the sera of the experimental tilapia (n = 6) at various time points during the experiment

Time (hr)	Sera (ppm)	Blank (ppm)
1	7.4 ± 0.87	–
2	9.7 ± 0.63	–
4	3.8 ± 0.12	–
8	2.7 ± 0.74	–
12	0.44 ± 0.02	–
16	0.02 ± 0.01	–
18	–	–
20	–	–

n = 6 at the various time points during the experiment and the values are mean ± SD; '–' : Under LOD (0.01 ppm).

Abbreviations: LOD (limit of detection) and SD (standard deviation).

Table 8 Pharmacokinetic parameters of ractopamine hydrochloride in sera of the experimental tilapia (n = 6) after 60 ppm ractopamine treatment

Parameters	Sera
$t_{1/2}$ (hr)	3.42 ± 0.03
T_{max} (hr)	2.0 ± 0.0
C_{max} (ppm)	9.7 ± 0.16
MRT (hr)	4.74 ± 0.14
CL_{tot} (mL hr ⁻¹ kg ⁻¹)	964 ± 17.2
AUC_{0-inf} (hr × µg mL ⁻¹)	337.8 ± 10.2

n = 6; the values are mean ± SD; Abbreviations: $t_{1/2}$ (half-lives, hr), C_{max} (maximum concentration, µg mL⁻¹), MRT (mean residence time, hr), CL_{tot} (total body clearance, mL hr⁻¹ kg⁻¹), AUC_{0-inf} (area under the concentration-time curve from 0 h to infinity, hr mg L⁻¹), and SD (standard deviation).

4. Discussion

Since the late 1980s, it has been found that the addition of beta-adrenoceptor agonists to animal feeds can increase BW, reduce waste excretion, and improve feed meat exchange rates in addition to reducing fat and increasing lean meat production (Anderson et al., 1987; Bark et al., 1992; Beermann, 2002; Armstrong et al., 2004; Armstrong et al., 2005). Therefore, beta-adrenoceptor agonists have begun to be used in economic animals as feed additives in some countries. However, in the early 1990s, some food poisoning incidents caused by the ingestion of animal products with clenbuterol residues began to appear in Europe, which gradually led to the use of beta-adrenoceptor agonist and attention has also led many countries to prohibit the use of beta-adrenoceptor agonist in the production of economic animals (Walker et al., 2006; Williams et al., 1994). In recent years, there have been many studies on the use of ractopamine in economic animals such as pigs, cattle, and sheep etc., and the effects of drugs and the residual status of ractopamine in tissues such as muscle, fat, and hair have been discussed (Smith et al., 1993; Crome et al., 1996; DeCamp et al., 2001; Stahl et al., 2007; Culp et al., 2013). However, the most of the studies on the use of ractopamine in aquatic animals focus on the effects of promoting growth, and there is still no study on drug residues or pharmacokinetics. Taiwan has the very well developed fisheries, therefore, the more emphasis should be placed on drug residues of in aquatic products. This study is aimed at exploring the effect of ractopamine on tilapia as well as its pharmacokinetics and residues.

In recent years, the solid-phase extraction (SPE) has become more and more common. However, the SPE tube is through packing the extremely small pore size materials in the column for adsorbing substances. Generally, the complicated pretreatment steps for the sample processes were used in order to prevent the extract from plugging the extraction

tube to cause the large difference in flow rate and affect the recovery rate. At present, the strong acids and high-speed centrifuge were used to settle proteins and n-hexane was used to remove the most of the lipids for the better purification effect. The vacuum pumping device was used in the experiment process to operate several samples at a time, however, the experiment must be care taken not to make the column dry, flow rate should not be too fast, and each process step must be well matched for the decrease effects of the recovery and the variability of the analysis. Moreover, the eluent in solid-phase extraction is also one of the factors affecting the recovery rate. Most of the eluents were used as organic phases or mobile phases (Du et al., 2014; Tang et al., 2014; Wu et al., 2014).

The application of high performance liquid chromatography (HPLC) to detect drug residues of beta-adrenoceptor agonist in food, feed or animal tissue or the study of pharmacokinetics has been discussed (Smith et al., 1993; Shelper and Smith, 2002; Du et al., 2014; Tang et al., 2014; Wu et al., 2014). The detection of ractopamine by HPLC has the advantages of being cheap, fast, accuracy, and sensitivity. In recent years, the ultraperformance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) was applied to detect the ractopamine residual status of urine, blood, and hair in cattle after oral ractopamine administration. The ractopamine residue concentration in urine was about 1,631.1 ng/mL after 1st day of ractopamine administration, which decreased to 8.3 ng/mL on the 14th day of ractopamine administration. The blood level was approximately 11,796.7 ng/mL after 1st day of ractopamine administration and decreased to 39.7 ng/mL after 14th day of ractopamine administration. The LOD for ractopamine was 0.2 ng/mL (Tang et al., 2014). Liquid chromatography with tandem mass spectrometry (LC-MS/MS) was also used to detect the residual situation of ractopamine in pig hair and no ractopamine residual level in the hair was detected after 24 hours of ractopamine administration. The LOD for ractopamine was 10 pg/mg and the recovery rate was 99.47% (Wu et al., 2014).

Application of HPLC in the ractopamine residues in animal tissues was verified. HPLC with an ultraviolet (UV) light detection system to detect the ractopamine residues in pigs' muscle and urine. The LOD for ractopamine was 0.01 µg/mL. Recovery was 93.9%-109.2% in muscle and 93.4% in urine (Du et al., 2014). HPLC with fluorescence detection was used to detect the drug residual time of ractopamine in turkeys. Ractopamine in both urine and blood was below LOD (0.01 ppm) after 48 hours of ractopamine administration (Smith et al., 1993). HPLC with fluorescence detection was used to detect the ractopamine residues in liver, kidney, and urine of sheep. The ractopamine residues were lower than LOD (2.5 ppb) 7 days post-drug withdrawal (Shelper and Smith, 2002). HPLC tandem UV and fluorescence detectors were used to detect 5 kinds of beta-adrenoceptor agonist (included ractopamine) in pigs' feeds (Shishani et al., 2003).

Currently, the studies of pharmacokinetics of ractopamine are still few in aquatic animals. The pharmacokinetics of ractopamine after oral administration in male and female rats were even studied. When 20 ppm ractopamine was administered orally in male and female rats, T_{max} were 0.5 hours and 2 hours, $t_{1/2}$ were 4.4 hours and 7.5 hours, and the peak concentrations were 3.85 and 9.02 ppm in male and female rats, respectively (Dalidowicz and Babbitt, 1986). The pharmacokinetics of ractopamine after oral administration in male and female dogs were researched. When 5 ppm ractopamine was administered orally in male and female dogs, T_{max} were 2 hours and 4-8 hours, $t_{1/2}$ were 4.0 hours and 7.4 hours, and the peak concentrations were 0.58 and 0.27 ppm in males and female dogs, respectively (Williams, 1987). According to the above literatures (Dalidowicz and Babbitt, 1986; Williams, 1987), the highest concentration of ractopamine was within 4 hours after administration of ractopamine and $t_{1/2}$ was mostly 4-7 hrs in the rats and dogs. In this study, $t_{1/2}$ of ractopamine was 3.42 hr, the highest ractopamine concentration in blood was at the 2nd hour after oral administration. The highest ractopamine concentration in blood was 9.7 ppm and the average retention time of ractopamine was 4.74 hr. After oral administration of ractopamine for 3 months, the ractopamine residues in muscle and liver were respectively after drug withdrawal 2 and 10 days were below LOD (0.01 ppm).

Additionally, the experimental animal sex was a great relationship with the metabolism of ractopamine (Dalidowicz and Babbitt, 1986; Williams, 1987; Dunshea et al., 1993; Uttaro et al., 1993). The ractopamine was absorbed and metabolized more rapidly in males than in female. In this study, the experimental fish was male tilapia and also presented the characteristics of rapid absorption, rapid metabolism, and short residual time after oral administration with ractopamine hydrochloride in tilapia. However, the metabolism and respiration of tilapia will change following with the change of their body temperature. The body temperature of fish will change with the water temperature. The low water temperature (lower than 20°C) reduced the tilapia's food intake (Hung et al., 2020a). In this study, the experimental period was in winter (December-February), the air temperature was about 17-20°C. The ractopamine hydrochloride was mixed in the feed and the reduction of food intake would also affect the tilapia's food intake of ractopamine hydrochloride. Therefore, it is speculated that if this experiment is carried out in a higher temperature season, the metabolism of ractopamine may be accelerated due to the vigorous metabolism of the fish. The residual time of ractopamine in the tissue is shortened, the drug withdrawal period is shorter, and the effect of BW gain and body composition improvement is better.

In animal husbandry, the administration route of ractopamine is mostly oral administration. In recent years, the pharmacokinetics of ractopamine via oral administration were studied. The residual time and pharmacokinetics of six beta-adrenoceptor agonists in dogs, cattle, pigs, rats, and turkeys by oral administration or intravenous injection. The beta-adrenoceptor agonists such as terbutaline, salbutamol, clenbuterol, and other beta-adrenoceptor agonists have the shorter C_{max} and $t_{1/2}$ after intravenous administration than oral administration (Smith, 1998). The metabolism of ractopamine in pigs found that ractopamine extended to other isomers through enzymes during the metabolism (Smith, 1998), however, there is no research on this aspect in fish. In this experiment, the pharmacokinetics of ractopamine hydrochloride in tilapia can be further extended to fish species. In addition, the different administration routes such as intramuscular injection, intravascular injection, or intraperitoneal injection can be further tested to explore the effect of different administration routes on the pharmacokinetic analysis.

The research on the toxicity of ractopamine has gradually attracted attention in recent years with the use of clenbuterol (Adeola and Young, 1992; Amin and Singh, 2011; Brooks and Conrad, 2009). Although the toxicity of ractopamine is very low compared with other beta-adrenoceptor agonists such as clenbuterol and salbutamol (Burniston et al., 2005), it is used for feed addition. However, there is a hidden concern of being ingested by the human body, so the toxicity of ractopamine is still in progress (Chesley et al., 2000; Burniston et al., 2005). Ractopamine enhances muscle performance through its beta-adrenergic receptor agonist properties or its assimilation. Toxicity of ractopamine was even studied in dogs (Greyhound). According to the results, the authors speculated that ractopamine (1 ppm) might induce segmental arterial mediolysis in dogs (Greyhound). The ractopamine might induce splanchnic varicose veins by stimulating peripheral sympathetic nerves to release norepinephrine and stimulate α -1 receptors and induce damage and apoptosis at the junction of the inner and outer muscle fibers of the arterial vessel wall. It can be speculated that dogs were highly sensitive to ractopamine (Yaeger et al. 2012). In this experiment, the ractopamine hydrochloride concentrations of the high dose and low dose group were respectively set at 30 and 60 ppm according to the previous study (Mustin and Lovell, 1993). After 3 months of continuous feeding, pathological dissection was performed and examined on the heart and no lesions were found.

Most of the studies on the use of ractopamine in aquatic animals focus on the growth and body components (Mustin and Lovell, 1993; Mustin and Lovell, 1995; Vandenberg et al., 1998a; Vandenberg and Moccia, 1998b; Atapattu et al., 2005; Jalali et al., 2010; Bicudo et al., 2012). There are no studies related to pathology. Therefore, two speculations were made regarding the phenomenon that ractopamine did not cause damage to the heart of tilapia. One reason is the structure of the heart of fish is very different from that of mammals, so the physiological mechanisms of ractopamine are not the same. The pathological damage caused by ractopamine in mammals may have a harmless impact on fish. The second reason is the heart of fish has the ability to self-repair, so the damage caused by ractopamine may disappear due to the self-repair effect. Future studies for heart toxicity can take heart pathological sections at shorter time points in order to rule out the possibility of fishes' cardiac damage being repaired.

Most of the current researches on ractopamine for fish focuses on increasing fishes' BW and improving body composition (Mustin and Lovell, 1993; Vandenberg et al., 1998; Bicudo et al., 2012). Based these studies, it can be seen that the effects of ractopamine hydrochloride used in tilapia are effective and the obvious effects of BW gain and the improvement of body composition. The results of this experiment showed that the residue problems of ractopamine hydrochloride in the use of aquatic animals were not serious and did not cause major side effects or harm to the fish. However, the effects of ractopamine varies greatly among various fish species. If the ractopamine is used in other fish species, additional experiments must be carried out to confirm the effects and residual problems. Although the legal application of ractopamine to aquaculture has not been carried out in various countries in the world. The withdrawal period of each beta-adrenoceptor agonist for each fish species is clearly established. It is believed that ractopamine can increase the yield of fish and help the problem of food shortage.

Abbreviations

- ADI: Acceptable daily intake;
- $AUC_{0-\infty}$: Area under the concentration-time curve from 0 h to infinity;
- BW: Body weight;
- C_{max} : Maximum concentration;
- CL_{tot} : total body clearance;
- CV: Coefficient of variation;
- FAO: Food and Agriculture Organization of the United Nations;
- HPLC: High-performance liquid chromatography;
- IACUC: Institutional animal care and utilization committee;
- JECFA: Joint FAO/WHO Expert Committee on Food Additives;

- LC-MS/MS: Liquid chromatography with tandem mass spectrometry;
- LOD: Limit of detection;
- LOQ: Limit of quantification;
- MRL: Maximum residue limits;
- *MRT*: Mean residence time;
- p.o.: Oral administration;
- RAC: Ractopamine hydrochloride;
- SD: Standard deviation;
- SPE: solid-phase extraction;
- T_{max} : Time to maximum concentration;
- $t_{1/2}$: Half-lives;
- UPLC-MS/MS: Ultrapformance liquid chromatography-tandem mass spectrometry;
- US: United States;
- UV: Ultraviolet

5. Conclusion

An accurate, precise, and quite sensitive HPLC-UV method for ractopamine hydrochloride determination in tilapia (*Oreochromis mossambicus*) was developed. This is the first report on pharmacokinetic study of ractopamine hydrochloride in tilapia (*Oreochromis mossambicus*). The results of this experiment showed that the residue problems of ractopamine hydrochloride in the use of aquatic animals were not serious and did not cause major side effects or harm to the fish. However, the effects of ractopamine hydrochloride varies greatly among various fish species. If the ractopamine is used in other fish species, additional experiments must be carried out to confirm the effects and residual problems. Although the legal application of ractopamine to aquaculture has not been carried out in various countries in the world. The withdrawal period of each beta-adrenoceptor agonist for each fish species is clearly established. It is believed that ractopamine can increase the yield of fish and help the problem of food shortage.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declared no conflict of interest.

Statement of ethical approval

Animal care in this study were carried out according to previously described the guideline of the Institutional Animal Care and Utilization Committee (IACUC), National Chung Hsing University.

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