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Ractopamine and Clenbuterol Urinary Residues in Pigs as Food-Producing Animals

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Summary

The aim of the study is to determine residual ractopamine (RCT) and clenbuterol (CLB) concentrations in urine during and after their administration in anabolic dose to male pigs. RCT and CLB residues were determined using previously validated enzyme-linked immunosorbent assay (ELISA) as a quantitative screening method. Hydrolysis of urine samples with β-glucuronidase showed significantly higher ($p<0.05$) RCT residues. Study results showed RCT and CLB urine concentrations to vary greatly during oral treatment for 28 days, with maximal RCT and CLB concentration recorded on day 25 ((327.4±161.0) ng/mL) and day 20 ((68.4±32.2) ng/mL), respectively. RCT concentration of (57.1±10.6) ng/mL and CLB concentration of (38.8±20.1) ng/mL were measured on day 0 of treatment withdrawal; on day 7 of treatment withdrawal, the measured concentration of RCT ((5.0±0.9) ng/mL) was 20-fold of CLB concentration ((0.3±0.2) ng/mL). Study results indicate that the excretion of RCT and CLB in pig urine could clearly point to their abuse in pigs as food-producing animals, in particular when using sample hydrolysis with β-glucuronidase on RCT determination.

Key words: ractopamine, clenbuterol, ELISA, pig, urine

Introduction

Numerous studies have reported that fat and lean deposition in animals for food production can be influenced by the administration of β_2-adrenergic agonists such as clenbuterol (*1,2*) and ractopamine (*3–5*). They promote muscle growth by increasing nitrogen retention (*6*) and protein synthesis (*7*) while reducing fat growth by increasing lipolysis and suppressing lipogenesis (*7–9*), which results in favourable shift in the lean/fat ratio of growing animals (*10,11*). The physiological activity of a β-agonist depends on its inherent activity at the receptor, on its absorption, metabolism and elimination rates, and distribution to target tissues (*12*). Some of the diversity may be explained by species and/or tissue differences in the distribution of β-adrenergic receptor subtypes (*13*). Clenbuterol (CLB) is used in medicine for its bronchiolytic and tocolytic action but if used in dosages 5–10 times higher than the therapeutic one, it provokes anabolic effects (*14,15*). It is the most effective β-agonist as growth-promoting agent (*12*); because of that, it has been widely misused in meat producing animals since the 1980s (*16*). In recent years, other β-agonists with lower efficiency than CLB such as ractopamine (RCT) have also been used for the same purpose. RCT is approved as a feed additive for swine and cattle in the United States and some other countries but remains strictly forbidden in the EU (*17*). Performance improvements associated with RCT feeding in pigs are affected by several factors, including but not limited to nutrient concentrations of the diet, dietary RCT concentration and duration of RCT feeding (*18*). Literature data show that dietary RCT concen-

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trations of 10 to 20 ppm and feeding duration of 28 to 35 days result in significant improvements in carcass characteristics (*11*,*19*,*20*). The oral administration of RCT in humans is also likely to be by three orders of magnitude less potent than that of CLB (*12*).

However, illegal use of β-adrenergic agonists implies risk to consumers of products prepared from illegally treated animals, as the usage of these pharmacologically active substances can result in severe cases of food poisoning (*14*,*21*,*22*). That is why a ban has been placed on the use of these substances in the European Union countries (*23*), and their detection is of interest to governmental regulatory officials, importers, exporters and consumers. In order to provide quality assurance for the consumer and to satisfy legal testing obligations, the ability to detect residues of the drug at low concentrations has become a very important issue. Urine has proved to be a suitable matrix to control the abuse of these substances in meat industry.

In the present study, residual concentrations of RCT and CLB will be assessed in pig urine as a matrix that is readily available from live farm animals in the control of anabolic substance abuse during fattening and at slaughterhouse. The aim of the study is to determine the levels and compare the excretion of the RCT and CLB β-adrenergic agonists in the urine of pigs during treatment and after withdrawal using the enzyme-linked immunosorbent assay (ELISA) as a quantitative screening method.

Materials and Methods

Chemicals and apparatus

A Ridascreen RCT and CLB kits for ELISA were provided by R-Biopharm (Darmstadt, Germany). Each kit contains a microtiter plate with 96 wells coated with antibodies to rabbit IgG, RCT/CLB standard solutions (0, 100, 300, 900, 2700 and 8100 ng/mL), peroxidase-conjugated RCT/CLB, anti-RCT/CLB antibody, substrate/chromogen (tetramethylbenzidine); stop reagent (0.5 M sulphuric acid), sample dilution buffer and washing buffer (10 mM phosphate buffer, pH=7.4). Standards of ractopamine and clenbuterol hydrochloride (for animal treatment and method validation) and β-glucuronidase from *Escherichia coli* (Art. no. G7646) were provided by Sigma-Aldrich Chemie (Steinheim, Germany). Standard solutions were prepared as aqueous stock and working solution in concentrations of 10 000 and 20 ng/mL, stored at 4 °C until analysis and used for sample fortification, respectively. All other chemicals used in the analysis were of analytical grade. ELISA was performed by use of EL×800TM microplate reader and EL×50 washer (BioTek Instruments, Inc., Winooski, VT, USA).

Animals and sampling procedure

The experiment was carried out in 24 male pigs, Zegers hybrid type, aged 90 days, body mass 60 kg, farm-bred, and kept under the same zoohygienic conditions. Animals were divided into three groups: 9 pigs treated with RCT, 9 pigs treated with CLB, and 6 control animals. The first two groups were administered 0.1 mg per kg of body mass per day RCT and 10 μg per kg of body mass per day CLB, respectively. The pigs were administered RCT and CLB once daily *per os* for 28 days, in the form of a pure chemical capsule admixed to feed. Six animals served as a control group and were not treated with either RCT or CLB. Urine samples were collected on treatment days 1, 5, 10, 15, 20, 25 and 28 in the morning at the time of giving the daily dose of RCT and CLB, and also after slaughtering. On days 1, 3 and 7 of treatment withdrawal, 6 pigs (3 treated with RCT and 3 treated with CLB) were randomly sacrificed. Control animals (N=6) were sacrificed on day 1 of the experimental group treatment withdrawal. The samples were collected and stored at –20 °C until analysis for residual RCT and CLB. The experimental protocol was designed according to the Act on Animal Welfare, as stated in the Official Gazette of the Republic of Croatia (*24*).

Analysis of ractopamine and clenbuterol

On determination of both analytes, urine samples were filtered and aliquots were assayed in duplicate by ELISA. Urine RCT was first determined without hydrolysis, and then with sample hydrolysis with β-glucuronidase, whereas urine CLB was determined without sample hydrolysis. All steps in sample preparation for both analytes and competitive ELISA for quantitative determination of RCT and CLB were performed according to the kit manufacturer's instructions. For determination of RCT with hydrolysis, step method was performed with β-glucuronidase from *Escherichia coli*, 2-hour incubation at 37 °C and using 25 mM borate buffer (pH=9.0), also according to the same instructions. All samples were analyzed in duplicate. After absorbance measurement, the analyte concentrations were calculated using the RidaSoft Win programme (R-Biopharm, v. 1.67, Darmstadt, Germany). Statistical data analysis was performed by use of STATISTICA v. 6.1 software (StatSoft Inc., Tulsa, OK, USA) with statistical significance set at the level of 95 % (p= 0.05).

Method validation

For both analytical methods, the limit of detection (LOD) and limit of quantitation (LOQ) were obtained by adding 3 and 10 times, respectively, standard deviation of 10 blank samples analyzed to the mean blank value. The method recovery was determined at three different levels by spiking urine samples with working solution of the RCT or CLB standard to yield concentrations equivalent to 1, 5 and 10 ng/mL (six replicates per concentration level) and calculated from six-point calibration curves. For determination of repeatability, the same steps were repeated on two other occasions under the same analysis conditions. Final results of RCT and CLB concentrations in the urine of treated animals were calculated by taking the average recoveries into account.

Results

The estimated LOD and LOQ values for RCT were 0.3 and 0.5 ng/mL without hydrolysis and 0.4 and 0.6 ng/mL with hydrolysis, respectively. For CLB, the estimated LOD was 0.1 ng/mL and LOQ was 0.2 ng/mL. The results of method recovery (N=18) and repeatability (N=54) are presented in Table 1.

Table 1. Determination of recovery and repeatability in blank pig urine samples spiked with RCT and CLB using ELISA method

Validation parameter	Compound	Number of replicates	γ(spiked)	γ(determined)	Mean recovery	Relative standard deviation
			ng/mL	ng/mL	%	%
Recovery	RCT[a]	6	1	0.98	98.0	3.2
		6	5	4.68	93.6	4.3
		6	10	9.24	92.4	2.7
	RCT[b]	6	1	0.96	95.8	4.1
		6	5	4.91	98.2	2.7
		6	10	9.85	98.5	3.9
	CLB	6	1	0.93	93.0	2.9
		6	5	4.44	88.8	3.7
		6	10	8.23	82.3	4.6
Repeatability	RCT[a]	18	1	0.95	95.0	7.1
		18	5	4.64	92.8	6.3
		18	10	9.01	90.1	5.8
	RCT[b]	18	1	0.91	91.0	6.1
		18	5	4.99	99.8	5.8
		18	10	10.25	102.5	7.2
	CLB	18	1	0.91	91.0	5.9
		18	5	4.27	85.4	5.1
		18	10	8.03	80.3	6.4

[a]without hydrolysis, [b]with hydrolysis

Comparison of RCT concentrations in urine with and without hydrolysis with β-glucuronidase is shown in Table 2. Figs. 1 and 2 show RCT (with hydrolysis) and CLB (without hydrolysis) concentrations determined in the urine of pigs during treatment and after treatment withdrawal, respectively.

Table 2. Concentration of RCT (mean values±S.D.) measured in urine of pigs during the treatment and after the withdrawal, with and without hydrolysis

	Days	Number of animals	γ(RCT)/(ng/mL)	
			without hydrolysis	with hydrolysis
During treatment	1	9	0.5±0.2	0.6±0.1
	5	9	1.6±0.8	13.4±3.2
	10	9	4.6±2.1	23.1±4.0
	15	9	3.0±1.1	11.3±1.1
	20	9	3.6±0.6	23.1±11.2
	25	9	9.1±5.5	327.4±161.0
	28	9	5.8±1.9	57.1±10.6
After withdrawal	1	9	1.4±0.5	7.0±6.3
	3	6	0.9±0.4	8.3±3.1
	7	3	2.1±0.2	5.0±0.9

Discussion

Urine is a common matrix for the analysis of β-adrenergic agonists, although residual concentrations of some may be higher in liver and hair (*25*). Literature data show that swine urine has not been sufficiently studied for RCT depletion compared to bovine urine (*17*) and also that urine samples provide an excellent matrix (*e.g.*

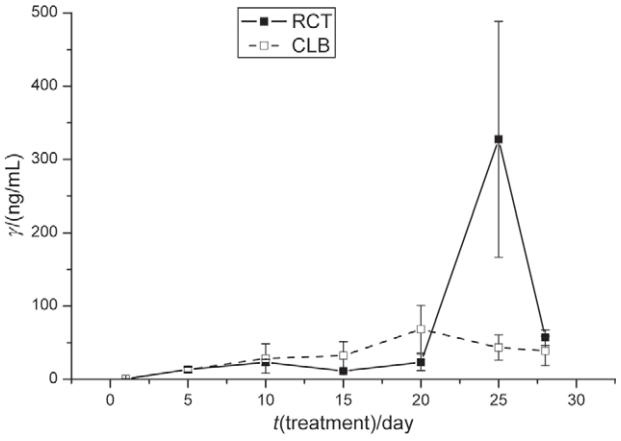

Fig. 1. Concentration (mean values±S.D.) of RCT and CLB in the urine of pigs during treatment

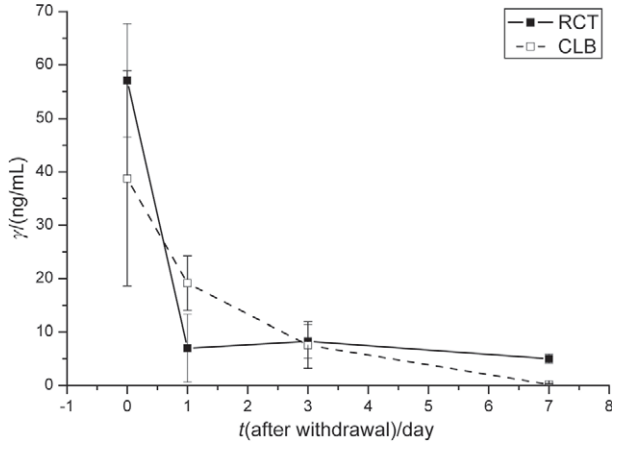

Fig. 2. Concentration (mean values±S.D.) of RCT and CLB in the urine of pigs upon treatment withdrawal

relative to liver) for the detection of RCT residues for an extended period post withdrawal (*26*). Studies suggest that the use of liquid chromatography/mass spectrometry (LC/MS/MS) systems or an immunoassay could probably be the best methods to improve sensitivity in determination of β-adrenergic agonists, while retaining excellent selectivity (*27*). The enzyme-linked immunosorbent assay (ELISA) enables rapid screening and quantification of very low analyte concentrations in large number of samples, easy performance, accuracy and availability of kits of different manufacturers. Several authors have proposed screening methods for RCT in calf urine using ELISA (*28–30*).

In the present study, RCT and CLB urine concentrations were assessed on particular days during and after animal treatment with anabolic dose of 0.1 mg per kg of body mass per day and 10 μg per kg of body mass per day, respectively, during the same treatment period (28 days) and in the same animal species. Literature data show the concentration of RCT, if given to experimental animals at a dose of 0.1 mg per kg of body mass per day, to be in the range required to cause growth promotion (*28,29*). Our previous studies including CLB alone, administered at a higher anabolic dose (20 μg per kg of body mass per day), had indicated that CLB residues could be detected in urine for one week after treatment discontinuation (*31*). In the present study, urinary concentrations of RCT and CLB were determined in animals administered a lower anabolic dose, in order to establish whether urinary RCT residues might serve as a useful screening tool to determine animal exposure to anabolic treatment, and to compare RCT and CLB excretion in urine. ELISA with and without β-glucuronidase hydrolysis was used to determine RCT urine residues, whereas ELISA without hydrolysis was employed to determine CLB urine residues.

The estimated LOD and LOQ were 0.3 and 0.5 ng/mL without hydrolysis and 0.4 and 0.6 ng/mL with hydrolysis for RCT, and 0.1 and 0.2 ng/mL for CLB, respectively. The results of method validation are presented in Table 1. Validation resulted in good mean recoveries (R >80 %) with acceptable inter- and intra-day relative standard deviations (RSD<8 %). Validation results demonstrated the method efficiency in determination of urinary RCT (with and without hydrolysis) and CLB concentrations. Considering the results of ELISA validation and the ability of RCT and CLB residue determination at the suggested minimum required performance levels (MRPL) of 1 and 0.2 ng/mL (*32*), respectively, the method can be used for monitoring RCT and CLB abuse as anabolics in meat production, with a confirmation method required in case of noncompliant samples.

The mean (±S.D.) RCT and CLB concentrations determined in urine samples collected on days 1, 5, 15, 20, 25 and 28 during the treatment in the experimental group of animals are shown in Fig. 1. Urine concentrations of RCT and CLB increased with the length of treatment and then decreased, showing great variations of both analytes throughout the treatment period. Determined RCT concentrations were much greater after β-glucuronidase hydrolysis than those determined without this analytical step (Table 2). Deconjugation step confirmed RCT to be excreted mainly in the form of glucuronide metabolites, as reported previously (*14,29*). Urine concentrations measured upon sample hydrolysis were as follows: on day 28 of the treatment, urine concentrations of RCT and CLB were (57.1±10.6) and (38.8±20.1) ng/mL, respectively. The highest RCT and CLB concentrations were measured on treatment day 25 ((327.4±161.0) ng/mL) and day 20 ((68.4±32.2) ng/mL), respectively. In comparison with some literature data (*27,33*), low concentrations of RCT urinary residues could be explained by low exsposure of animals to RCT (0.1 mg per kg of body mass per day). Other authors also report large variations and significant differences in RCT concentrations during treatment with several-fold concentrations determined in hydrolysed compared to unhydrolysed urine samples (*26,27,33*). The significantly higher RCT concentrations recorded on treatment day 25 were most likely due to inconsistent time of sampling relative to dosing; namely, because of the complex sampling procedure, it was impossible to collect all urine samples over a short period of time. In our previous study, urinary CLB concentrations had also been found to vary greatly with time and decrease after a certain period of treatment (*31*). Literature data indicate that swine eliminate nearly 85 % of the administered RCT during the first day, resulting in relatively low tissue residues (*34*). CLB is almost exclusively excreted *via* urine, mostly as unchanged parent compound, whereas RCT metabolites are almost exclusively glucuronic acid conjugates.

The mean (±S.D.) RCT and CLB concentrations measured in study animals on days 1, 3 and 7 after treatment discontinuation are shown in Fig. 2. On day 1 of treatment withdrawal, RCT and CLB residue levels were (7.0±6.3) and (19.1±5.1) ng/mL, respectively. On day 7 of treatment withdrawal, the measured RCT concentration ((5.0±0.9) ng/mL) was 20-fold of CLB ((0.3±0.2) ng/mL) concentration. Other studies carried out on cattle and sheep report on detectable RCT residues in urine 5 to 7 days after the last exposure to dietary RCT, stating that hydrolysis of RCT metabolites may extend the period in which it is detected in cattle (*27*). In their study, Thompson *et al.* (*26*) found RCT residues in pig urine to be detectable by both screening and confirmatory methods until day 21 after treatment withdrawal. In calf, the RCT residue concentrations found after drug withdrawal were also substantially lower than during the medication period, and were only detectable in one animal 2 weeks after the removal of medication from the diet (*29*). Some of the reported CLB concentrations were statistically significantly lower ($p>0.05$) than the concentrations recorded in our previous study with the use of twofold CLB dosage (*31*).

Conclusion

Urine as a matrix for the control of β-adrenergic agonist abuse during animal fattening proved to be suitable as it enabled determination of CLB and RCT residues during and after the treatment. The concentrations of RCT and CLB residues increased with the length of treatment and then decreased, showing great variations of

both analytes throughout the treatment period. Under our study conditions, RCT was excreted in urine for a longer period after withdrawal as compared to CLB. The period of detection of the hydrolysis of RCT metabolites was extended. Our data indicate that the excretion and determination of RCT and CLB in pig urine enable simple control of their abuse in food-producing animals.

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