

## Clenbuterol Residues in Plasma and Urine Samples of Food-Producing Pigs During and After Subchronic Exposure to a Growth-Promoting Dose

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### Summary

The aim of the study is to evaluate the suitability of plasma and urine as matrices for clenbuterol residue determination during and after its subchronic administration at a growth-promoting dose to male pigs, using previously validated enzyme-linked immunosorbent assay (ELISA) as a screening method and liquid chromatography tandem mass spectrometry (LC-MS/MS) as a confirmation method. A high correlation coefficient between these analytical methods was obtained for both urine ( $R=0.9800$ ) and plasma ( $R=0.9970$ ) concentrations. Study results show the plasma and urine concentration to vary greatly during oral treatment with clenbuterol for 28 days. The peak urine concentration ( $88.54 \pm 50.54$  ng/mL) recorded on day 21 was 40-fold peak plasma concentration ( $2.25 \pm 1.54$  ng/mL). After withdrawal period, the peak urine clenbuterol concentration ( $42.93 \pm 10.52$  ng/mL) recorded on day 0 was 24-fold plasma concentration ( $1.79 \pm 0.97$  ng/mL). The maximum allowed concentration of 0.5 ng/g in the liver as a regulated matrix for control of clenbuterol abuse was achieved in plasma on day 3 ( $0.52 \pm 0.26$  ng/mL) and in urine on day 7 of treatment withdrawal ( $0.45 \pm 0.11$  ng/mL). Study results indicate that urine and plasma may be suitable matrices for the control of clenbuterol abuse during fattening of food-producing pigs but have a limited value because of the rapidly decreasing concentration upon treatment withdrawal, in plasma in particular.

*Key words:* clenbuterol residues, growth promoting dose, subchronic exposure, pig, urine, plasma

### Introduction

Clenbuterol (4-amino- $\alpha$ -[*t*-butylaminomethyl]-3,5-dichlorobenzyl alcohol hydrochloride) is a  $\beta_2$ -adrenergic agonist that, due to its biological activity, has been misused in fattening of food-producing animals to enhance muscle mass and reduce body lipids. In the European Union, clenbuterol is licensed as a bronchospasmolytic

and tocolytic agent in veterinary medicine with therapeutic dose of 0.8  $\mu$ g/kg body mass. In dosages 5–10 times higher than the therapeutic one, clenbuterol provokes anabolic effects (1–5). Clenbuterol is characterized by high bioavailability when administered by oral route (6), persisting in the form of residues in edible and other animal tissues (7,8), with a potential risk of adverse effects with signs of acute intoxication (9,10) or effects on various metabolic processes as the result of its chronic

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action in humans (11). Residues are cleared from plasma, urine and muscle before liver and pigmented retinal epithelium and hair, roughly in that order (7). The maximum residue limit (MRL) for clenbuterol has been set at 0.5 ng/g for liver tissue (12). To detect the illegal use of this compound in anabolic dosages, veterinary and public health control laboratories are required to develop monitoring and screening programs for residues of these drugs.

Unlike most other studies of clenbuterol persistence performed in ruminants as experimental animals, the present study includes pigs as experimental animals, and urine and plasma as study matrices that are readily available from live farm animals in the control of anabolic substance abuse during fattening and at slaughterhouse. Urine has also been used as a monitoring matrix by farmers but little data refer to the study of clenbuterol residue absorption and elimination from blood and urine in pigs. The aim of this study is to obtain information on the level of clenbuterol residues that can be expected in biological fluids of pigs after subchronic exposure to oral anabolic dose of clenbuterol and to establish a generalized relationship between urinary and plasma clenbuterol residue persistence during and after exposure. Clenbuterol concentrations in all samples were determined by the use of previously validated methods of enzyme-linked immunosorbent assay (ELISA) as a screening method and liquid chromatography with mass spectrometry (LC-MS/MS) as a confirmatory method.

## Materials and Methods

### Chemicals and apparatus

A Ridascreen clenbuterol kit for ELISA was provided by R-Biopharm (Darmstadt, Germany). Each kit contains a microtiter plate with 96 wells coated with antibodies to rabbit IgG, clenbuterol standard solutions (0.0, 0.1, 0.3, 0.9, 2.7 and 8.1 ng/mL), peroxidase-conjugated clenbuterol, anti-clenbuterol antibody, substrate (urea peroxide), chromogen (tetramethylbenzidine), stop reagent (0.5 M sulphuric acid), conjugate dilution buffer and washing buffer. Clenbuterol hydrochloride from Sigma-Aldrich Chemie GmbH (Steinheim, Germany) was used for fortification of samples. All other chemicals used in the analysis were of analytical grade. ELISA was performed by using ELx800TM microplate reader and ELx50 washer (BIO-TEK Instruments, Inc., Winooski, VT, USA). The C<sub>18</sub> Bond Elut (100 mg, 3 mL) solid-phase extraction cartridges were purchased from Varian (Harbor City, CA, USA). The LC-MS/MS system consisted of an Agilent Technologies 1100 series HPLC instrument (Waldbronn, Germany) and Applied Biosystems QTrap mass spectrometer (Darmstadt, Germany), the whole system being controlled by the Applied Biosystems Analyst software. An XTerra MS C18 analytical column was used, with 3.5- $\mu$ m particles, 2.1 mm in diameter, 50 mm in length, preceded by a 10-mm guard column of the same type and diameter.

### Animals and sampling procedure

The experiment was carried out in 18 male pigs (15 treated and 3 control) of a known breed (cross-breed

among Swedish Landrace, Large Yorkshire and Pietren) aged 90 days, body mass 50 kg, farm-bred, and kept under the same zoohygienic conditions. The animals were administered clenbuterol at a dose of 20  $\mu$ g/kg of body mass per day *per os* for 28 days. Blood and urine samples were collected on days 1, 7, 14, 21 and 28 during the treatment, and on days 0, 3, 7, 14 and 35 upon treatment discontinuation, on which days the pigs ( $N=3$ ) were randomly sacrificed. The samples were collected and stored at  $-20$  °C until analysis for residual clenbuterol. The experimental protocol was designed according to the Act on Animal Welfare, as stated in the Official Gazette of the Republic of Croatia (13).

### Extraction procedure for ELISA method

Urine samples were first filtered and then diluted with water without the cleaning step. Plasma samples were prepared as described elsewhere (14): 300 mg of plasma samples, 1 mL of water, 15  $\mu$ L of 5 M HCl and 2 mL of ether were shaken for 2 h at room temperature. Ether was separated and discarded. To the aqueous phase, 30  $\mu$ L of 5 M NaOH and 3 mL of ether were added and the samples were shaken overnight. The extraction was repeated, combined ether phases were evaporated at 60 °C, and the residues were dissolved in 100  $\mu$ L of methanol and 300  $\mu$ L of water. Aliquots (20  $\mu$ L) of appropriate dilutions of urine and plasma samples were assayed in duplicate by ELISA.

### Extraction procedure for LC-MS/MS method

Urine (200  $\mu$ L) and plasma (500  $\mu$ L) samples were homogenized in 2 mL of 25 mM sodium citrate buffer (pH=5) and shaken with cooling for 1 h. The resulting solution was purified using solid-phase extraction (SPE). SPE was performed as described elsewhere (15). The cartridges were activated with 2-mL elution solvent (5 % NH<sub>4</sub>OH in methanol), followed by 2 mL of methanol and then equilibrated with 2 mL of 10 mM NH<sub>4</sub>OAc buffer, pH=5. Aliquots corresponding to 200- $\mu$ L equivalents of urine and 500  $\mu$ L of plasma samples were then loaded onto the cartridges and washed with 400  $\mu$ L of 10 mM NH<sub>4</sub>OAc (pH=5), 400  $\mu$ L of 1 M formic acid, dried for 30 s, and finally washed with 400  $\mu$ L of methanol. The analytes were eluted with 1.2 mL of 5 % NH<sub>4</sub>OH in methanol. The eluate was evaporated to dryness using rotavapor and then reconstituted into 200  $\mu$ L of 5 % methanol in 0.1 % formic acid.

### Analysis of clenbuterol by ELISA

Competitive ELISA was performed as described in package inserts provided by the manufacturer. Microtiter strips coated with sheep antibodies directed against anti-clenbuterol rabbit IgG were inserted in the microwell holder for the standards and samples to be analyzed in duplicate. To the microwells, 100  $\mu$ L of diluted antibody solution were added and the plate was incubated at 2–8 °C overnight. The next morning, the wells were emptied completely by inverting them onto absorbent paper and then were washed 3 times with 250  $\mu$ L of washing buffer. Then, 20  $\mu$ L of clenbuterol standards (0, 0.1, 0.3, 0.9, 2.7 and 8.1 ng/L) and the prepared sam-

ples were added to each microwell, followed by 100  $\mu\text{L}$  of diluted enzyme conjugate, mixed gently and incubated for 1 h at room temperature. After washing, 50  $\mu\text{L}$  of the substrate and 50  $\mu\text{L}$  of chromogen were added to each well and incubated in the dark for 30 min at room temperature. The reaction was stopped by adding 100  $\mu\text{L}$  of stop reagent and absorbance was measured on microplate reader at 450 nm.

#### Analysis of clenbuterol by LC-MS/MS

The chromatographic conditions were as follows: the mobile phase consisted of mobile phase constituent A (2 mM ammonium acetate in water) and B (2 mM ammonium acetate in acetonitrile); a gradient elution program was employed, initial 10 % B running for 1 min and then rising to 90 % B in the subsequent 7 min, then staying at that level for the next 2 min before dropping back to the initial conditions and reconditioning the column for the final 5 min of the run. The total runtime was 15 min and the flow rate of the mobile phase 300  $\mu\text{L}/\text{min}$ . The column was held at 35  $^{\circ}\text{C}$ , while the samples in the autosampler were held at 10  $^{\circ}\text{C}$ . The injection volume was 80  $\mu\text{L}$ . Clenbuterol standards were prepared by diluting the substance in a mixture of acetonitrile and water (1:9, by volume) to the concentrations of 0.1, 0.5, 1.0, 2.5, 5.0, 10.0 and 25.0 ng/mL and both the standards and the samples were injected twice. The MS conditions were as follows: Turbo Spray ion source (electrospray ionisation), positive polarity; ion spray voltage 5500 V, source temperature 300  $^{\circ}\text{C}$ , nitrogen level 1 was 30 and level 2 was 60 (these levels are dimensionless because they are defined by the software). The mass spectrometer was equipped with an inject/divert valve, which was programmed to direct the LC flow into the ion source of the mass spectrometer 5 min after the beginning of the run, and to switch the LC flow back to waste 4 min thereafter. The mass spectrometer was operated in multiple reaction monitoring (MRM) mode, two MRM functions were run, one being for quantification and the other as a confirmatory signal. The protonated molecular ion of clenbuterol at  $m/z=277$  was the precursor ion, and the product ions were  $m/z=259$  and  $m/z=203$ .

#### Statistics

Statistical data analysis was performed by the use of STATISTICA v. 6.1 software (StatSoft Inc. 1984–2003, USA). The scatter plot, box plot and surface plot graphs, and

multiple regression and nonparametric statistics (Wilcoxon matched pair test, Friedman ANOVA and Mann-Whitney  $U$  test) were employed. Statistical significance was set at the level of 95 % ( $p=0.05$ ).

## Results and Discussion

#### Validation of ELISA and LC-MS/MS

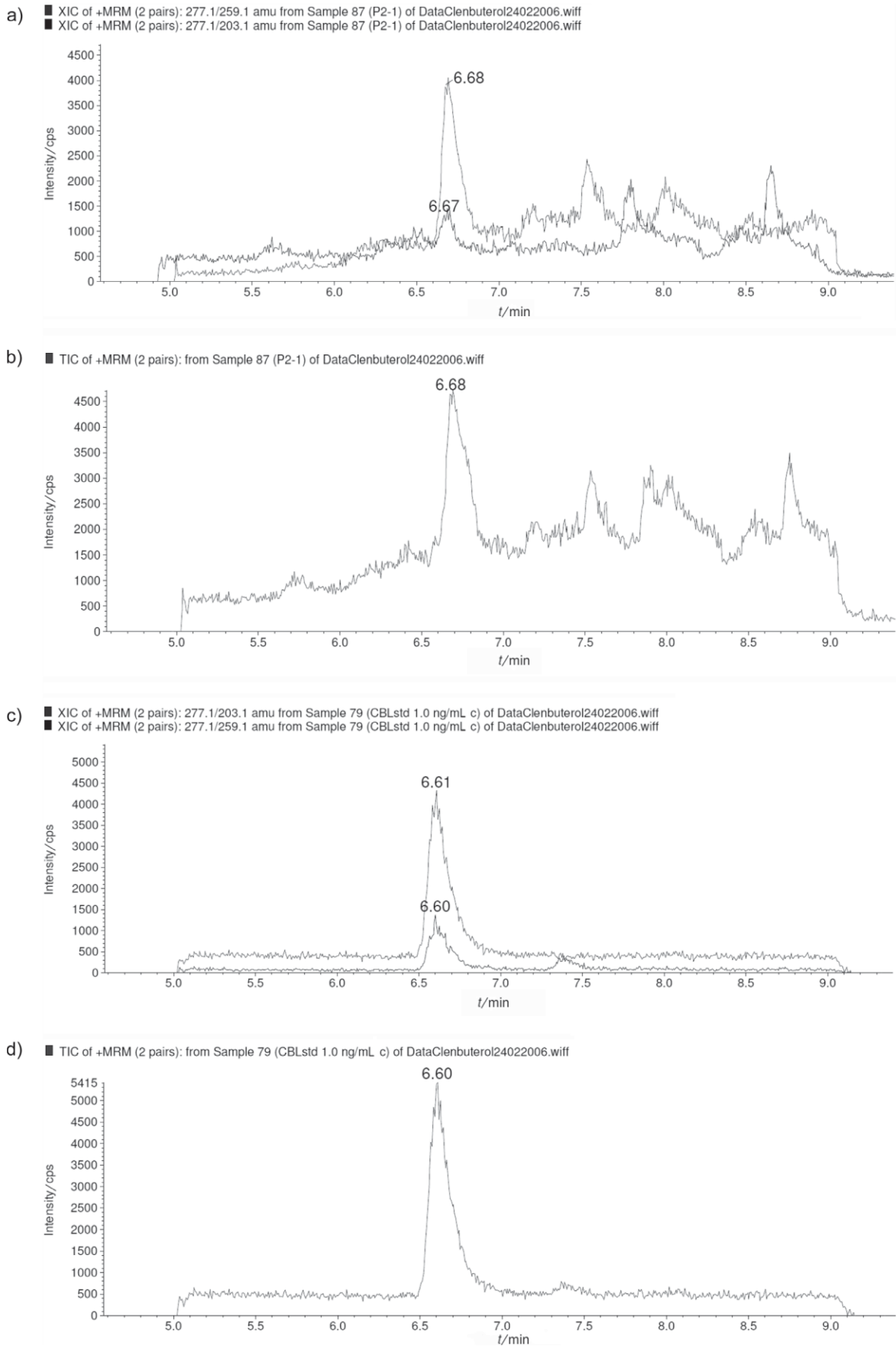
The results of ELISA and LC-MS/MS precision expressed as an intra- and inter-day variation and recovery of urine and plasma samples ( $N=6$ ) are presented in Table 1. The estimated limit of detection (LOD) and limit of quantitation (LOQ), which were calculated from the mean value of ten determinations of a blank urine and plasma material plus three- and tenfold standard deviation were for both matrices and methods 0.1 and 0.3 ng/mL, respectively. Validation of ELISA and LC-MS/MS methods for urine and plasma resulted in mean recoveries ranging from 84.0 to 98.0 % for urine and from 68.0 to 79.0 % for plasma, respectively, with acceptable inter- and intra-day relative standard deviations for both matrices and methods in a range from 6.3 to 10.4 %. The obtained validation results demonstrate the efficiency of the presented sample preparation methods and subsequent ELISA and LC-MS/MS determination of clenbuterol in urine and plasma. Chromatograms for the clenbuterol confirmatory ions ( $m/z$  ions 203 and 259) for real urine and plasma samples (treated pigs) and clenbuterol standard are shown in Figs. 1 and 2. The final results were calculated by taking the average recoveries into account.

#### Correlation between clenbuterol concentrations determined by ELISA and LC-MS/MS

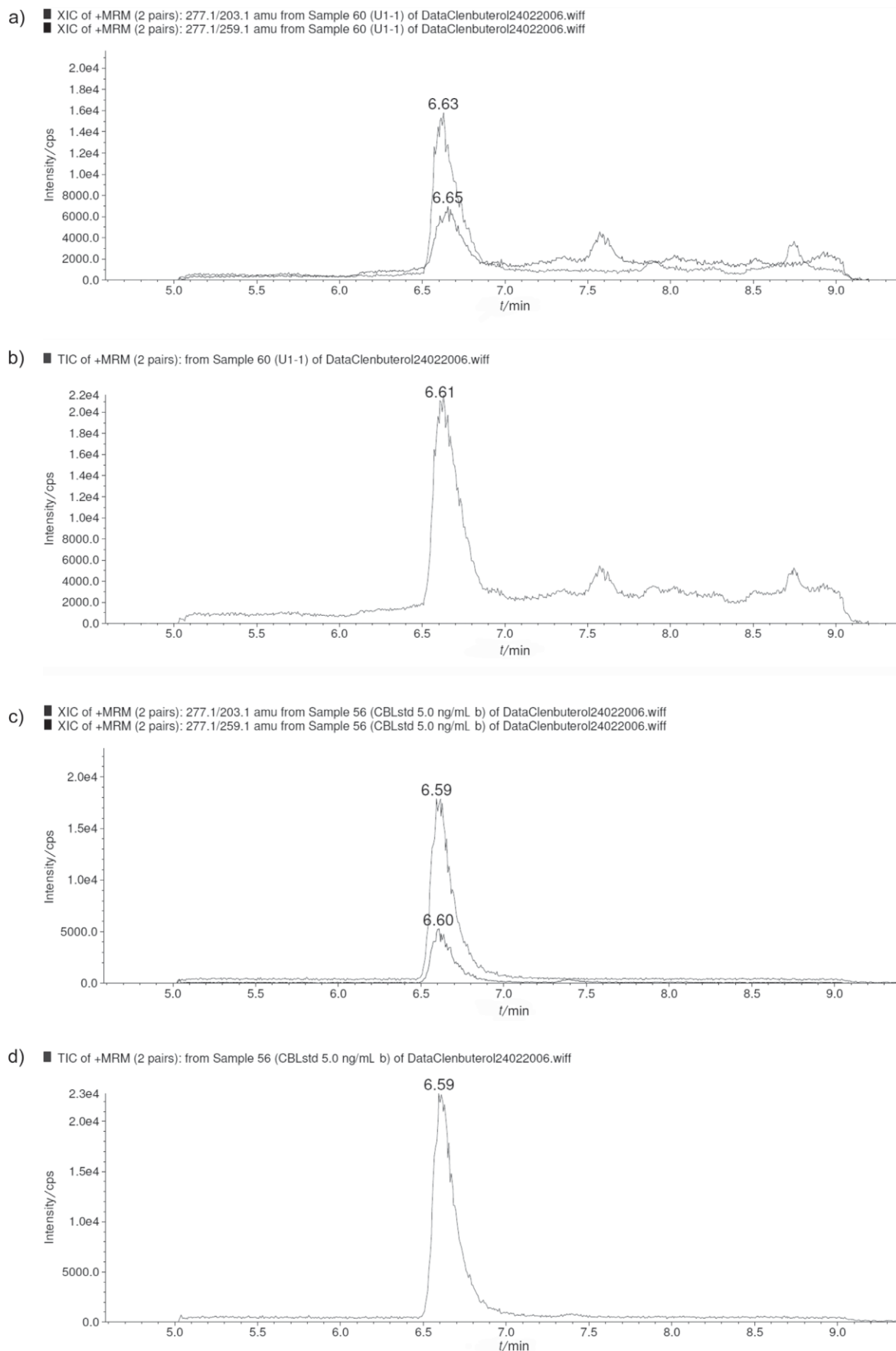
Correlation between urine and plasma clenbuterol concentrations determined by ELISA and LC-MS/MS after cessation of clenbuterol administration to male pigs is shown in Figs. 3 and 4. Correlation coefficients  $R=0.997$  for plasma and  $R=0.98$  for urine were determined by the correlation regression analysis. The correlation of the obtained concentrations of clenbuterol in the plasma samples was possible only in animals sacrificed on day 0 after the treatment (3 points of correlation). On days 3, 7, 14 and 35 after the treatment, clenbuterol was not detected by LC-MS/MS method. Generally, there was a statistically significant correlation between ELISA and LC-MS/MS at the level of  $p<0.05$  for both study matri-

Table 1. Evaluation of validation procedure for urine and plasma samples fortified with clenbuterol (CB)

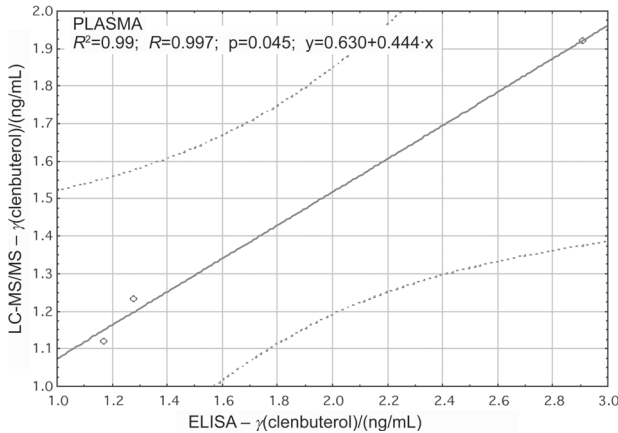
Matrix	Urine						Plasma					
	ELISA		LC-MS/MS				ELISA		LC-MS/MS			
$\gamma(\text{CB}_{\text{added}})/(\text{ng}/\text{mL})$	0.50	0.75	1.00	0.50	0.75	1.00	0.50	0.75	1.00	0.50	0.75	1.00
$\gamma(\text{CB}_{\text{determined}})/(\text{ng}/\text{mL})$	0.44	0.64	0.84	0.46	0.65	0.98	0.35	0.51	0.72	0.38	0.56	0.79
Recovery / %	88.00	85.30	84.00	92.00	86.70	98.00	70.00	68.00	72.00	76.00	74.70	79.00
RSD / %												
Intra-day	7.20	8.60	9.20	7.50	6.30	6.80	9.60	10.40	10.30	8.60	7.80	7.10
Inter-day	9.30	11.20	10.10	9.40	7.80	9.20	11.30	13.50	12.20	11.80	10.90	10.60



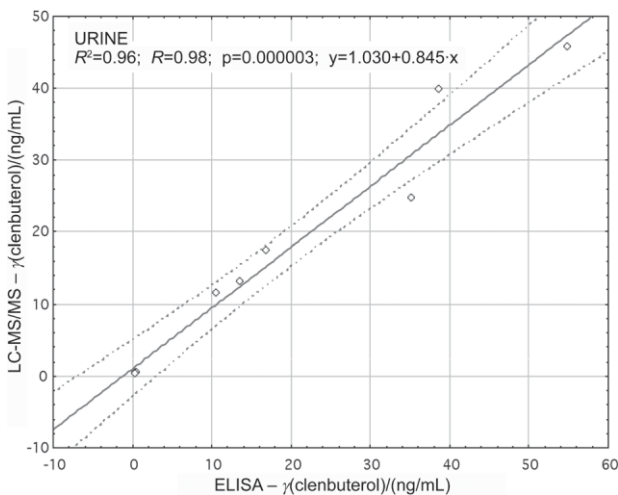
**Fig. 1.** The confirmatory chromatographic signals of (a,b) clenbuterol obtained from LC-MS/MS analysis of the plasma from treated pigs, and (c,d) clenbuterol standard, shown as separate overlaid MRM functions (a,c) and summed signal (b,d)



**Fig. 2.** The confirmatory chromatographic signals of (a,b) clenbuterol obtained from LC-MS/MS analysis of the urine from treated pigs, and (c,d) clenbuterol standard, shown as separate overlaid MRM functions (a,c) and summed signal (b,d)



**Fig. 3.** Correlation between plasma clenbuterol concentration measured by ELISA and LC-MS/MS after cessation of clenbuterol administration in a growth-promoting dose to male pigs

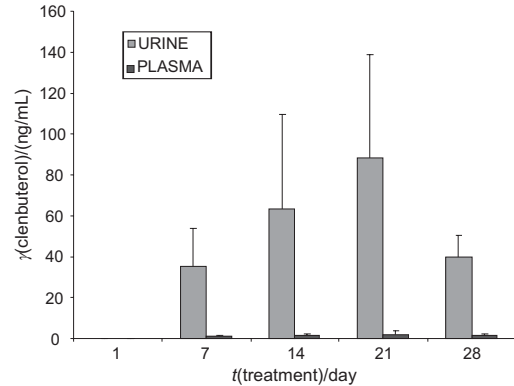


**Fig. 4.** Correlation between urine clenbuterol concentration measured by ELISA and LC-MS/MS after cessation of clenbuterol administration in a growth-promoting dose to male pigs

ces ( $p=0.000003$  for urine and  $p=0.045$  for plasma) indicating high accordance of the two methods. Considering the high values of correlation coefficients, the values obtained by ELISA are presented and discussed below.

#### Plasma and urine clenbuterol concentration during treatment

The mean ( $\pm$ SD) clenbuterol concentrations determined by ELISA in plasma and urine samples collected on days 1, 7, 14, 21 and 28 during treatment period in the experimental group of animals are shown in Fig. 5. The plasma and urine concentrations of clenbuterol increased with the treatment until day 21 and then decreased on day 28, showing great variations in both matrices throughout the treatment period. The peak plasma concentration of clenbuterol was measured on day 21 ( $(2.25 \pm 1.54)$  ng/mL), which declined on day 28 to the concentration recorded on day 14 of the treatment ( $(1.46 \pm 1.00)$  ng/mL). In urine, the peak concentration of clenbuterol was also measured on day 21 ( $(88.54 \pm 50.54)$  ng/mL) and was 40-fold plasma clenbuterol concentration deter-



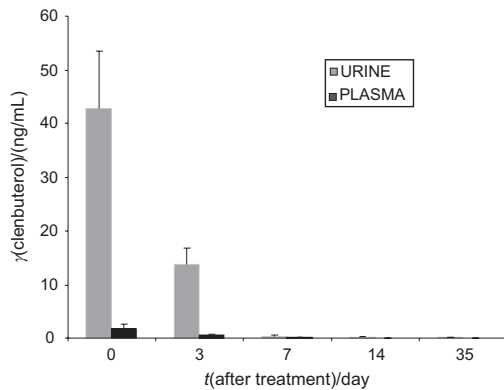
**Fig. 5.** Mean ( $\pm$ SD) clenbuterol concentration in pig\* urine and plasma during treatment\*\*, determined by ELISA  
\*15 male pigs (cross-breed among Swedish Landrace, Large Yorkshire and Pietren) aged 90 days, body mass 50 kg  
\*\*pigs were administered clenbuterol at a dose of 20  $\mu$ g/kg of body mass per day *per os* for 28 days

mined on the same day ( $(2.25 \pm 1.54)$  ng/mL). On day 28, the urine and plasma concentration of clenbuterol ( $(40.08 \pm 10.52)$  ng/mL and  $(1.46 \pm 1.00)$  ng/mL, respectively) was approximately half the value recorded on day 21. Results reported in other studies also show the concentration of clenbuterol to decrease after a certain period of treatment, suggesting that animals adapt to the extraneous compound with time, *i.e.* the affinity of  $\beta$ -adrenergic receptors to bind clenbuterol appears to diminish with the length of treatment (5,16). Our results indicate that the clenbuterol concentration measured in urine samples significantly exceeded the concentration measured in plasma on all days of treatment. Literature data also point to wide variation in plasma and urine clenbuterol concentration during the period of treatment (17), and that it depends on the dose administered, length of treatment, and on the species and mass of experimental animals. Daily plasma and urine sampling, which could not be performed in the present study, would have yielded more substantiated information on the clenbuterol kinetics in body fluids.

#### Plasma and urine clenbuterol concentration after withdrawal

The mean ( $\pm$ SD) clenbuterol concentrations determined by ELISA in plasma and urine samples collected on days 0, 3, 7, 14 and 35 after the treatment discontinuation in the experimental group of animals are shown in Fig. 6. The mean plasma clenbuterol concentration of  $(1.79 \pm 0.97)$  ng/mL was measured on day 0 after treatment withdrawal, and it declined on days 3, 7, 14 and 35 (ranging from 0.52 to 0.05 ng/mL), when it was only detectable by ELISA. These results are consistent with literature data recorded in cows, where plasma concentration of clenbuterol decreased to below 0.5 ng/mL as early as day 4 of treatment discontinuation (5), suggesting that plasma is not reliable enough as a matrix to control clenbuterol abuse. A number of studies point to considerable limitations encountered when quantifying very low concentrations of this substance in plasma (18).

In comparison with plasma samples, significantly higher clenbuterol concentrations were measured in urine



**Fig. 6.** Mean ( $\pm$ SD) clenbuterol concentration in pig<sup>\*</sup> urine and plasma after the last repeated dose<sup>\*\*</sup>, determined by ELISA  
<sup>\*</sup>15 male pigs (cross-breed among Swedish Landrace, Large Yorkshire and Pietren) aged 90 days, body mass 50 kg  
<sup>\*\*</sup>pigs were administered clenbuterol at a dose of 20  $\mu$ g/kg of body mass per day *per os* for 28 days

samples after the treatment discontinuation (24-fold on day 0). The mean urine clenbuterol concentration was (42.93 $\pm$ 10.52) ng/mL on day 0, (13.66 $\pm$ 3.14) ng/mL on day 3, and (0.45 $\pm$ 0.11) ng/mL on day 7 of the treatment discontinuation. Mean values of clenbuterol concentration of (0.27 $\pm$ 0.11) and (0.18 $\pm$ 0.12) ng/mL were determined by ELISA in the urine samples 14 and 35 days after the treatment, respectively, while using LC-MS/MS method the clenbuterol residues on the same days were not determined. The correlation of plasma and urine concentrations of clenbuterol recorded in the present study was comparable to that reported by Meyer and Rinke (7). Upon cessation of clenbuterol administration to veal calves at a dose of 5  $\mu$ g per kg of body mass twice a day for three weeks, in urine samples 40-fold clenbuterol concentration of that determined in plasma samples was measured, while clenbuterol residues could not be detected in urine on day 7 after the treatment discontinuation.

Considering the maximum allowed clenbuterol concentration in the liver as a regulatory matrix for the control of clenbuterol abuse (0.5 ng/mL), respective concentrations were measured in plasma on day 3 ((0.52 $\pm$ 0.26) ng/mL) and in urine on day 7 ((0.45 $\pm$ 0.11) ng/mL) after the treatment discontinuation.

In comparison of plasma and urine as matrices for the control of clenbuterol abuse during animal fattening, urine proved to be more suitable as it enabled determination of clenbuterol residues for a longer period of time (one week) than plasma. Recent studies suggest the use of other matrices for the control of clenbuterol abuse during animal fattening, such as hair, which can also be easily sampled during fattening. In hair, clenbuterol residues persist for a considerably longer time than one week (19–21).

## Conclusion

Results obtained in our study indicate that urine and plasma may be suitable matrices for the control of clenbuterol abuse during fattening in food-producing pigs. Urine was found to be superior to plasma because it en-

abled determination of clenbuterol residues for a longer period of time (one week). In plasma, clenbuterol could not be detected as early as day 3 after the treatment discontinuation. Our future studies in the field of clenbuterol abuse control during animal fattening will address the use of hair as a matrix because the results of the latest studies in the field point to many advantages of hair sampling in the control of  $\beta_2$ -adrenergic agonist residues during fattening of food-producing animals.

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