Tissue depletion of azaperone and its metabolite azaperol after oral administration of azaperone in food-producing pigs

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Abstract: Azaperone is a butyrophenone tranquilizer for swine. Food producing pigs are particularly sensitive to stress during handling and transport to the abattoir. *In vivo*, azaperone is partially metabolised to azaperol, a metabolite with pharmacological activity. The high and persistent concentrations of azaperone and azaperol in the injection site contra-indicates the use of azaperone using the intramuscular route for the transport of the food producing animals, pigs, to the slaughterhouse; the oral use could be an alternative to avoid residues at the injection site. The present study determined the tissue depletion of azaperone and its metabolite azaperol after oral administration of the formulation Stresnil®. Male pigs (30-45 kg of body weight) were treated with Stresnil® (single oral dose of 4 mg azaperone/kg body weight) and were sacrificed 6, 24 and 48 hours after the administration. Muscle, skin + fat, liver and kidney were collected from each animal. Azaperone and azaperol were assayed by HPLC after solid phase extraction. The concentrations of the azaperone plus azaperol in all analysed tissues did not exceed to the Maximum Residue Limit (MRL) established by the European Union (100 µg/kg in muscle, liver, kidney and skin plus fat) at any sampling time. As a consequence, from the results obtained in the present study, edible tissues of pigs treated orally with 4 mg/kg azaperone, 6 hours before to the sacrifice, might be acceptable to guarantee safety for the consumers. Nevertheless a withdrawal time of cero hours was estimated by linear regression analysis.

Keywords: Azaperone, azaperol, pigs, residues, tranquillizers, foods

Resumen: Depleción tisular de azaperona y su metabolito azaperol tras administración oral de azaperona en cerdo. Azaperona es un tranquilizante de tipo butirofenona usado en ganado porcino. Los cerdos son particularmente sensibles al estrés durante el manejo y transporte al matadero. La azaperona es parcialmente metabolizada *in vivo* a azaperol, un metabolito con actividad farmacológica. Las concentraciones altas y persistentes de azaperona y azaperol en el lugar de inyección contraindican el uso de azaperona por vía intramuscular para el transporte de cerdos de producción de carne al matadero; el uso oral podría ser una alternativa para evitar residuos en el lugar de inyección. El presente estudio determinó la depleción en los tejidos de azaperona y su metabolito azaperol después de la administración oral de la formulación Stresnil®. Cerdos machos (30-45 kg de peso corporal) fueron tratados con Stresnil® (dosis oral única de 4 mg azaperona/kg de peso corporal) y se sacrificaron 6, 24 y 48 horas después de la administración. De cada animal se obtuvo músculo, piel + grasa, hígado y riñón. Azaperona y azaperol se analizaron por HPLC tras la extracción en fase sólida. Las concentraciones de azaperona más azaperol en todos los tejidos analizados no superaron el Límite Máximo de Residuos (LMR) establecidos por la Unión Europea (100 mg / kg en el músculo, el hígado, los riñones y la piel + grasa) en ningún momento del muestreo. Como consecuencia, según los resultados obtenidos en el presente estudio, los tejidos comestibles de los cerdos tratados por vía oral con 4 mg/kg de azaperona, 6 horas antes al sacrificio, podrían ser aceptables para garantizar la seguridad de los consumidores. Sin embargo, se estimó un tiempo de espera de cero horas por análisis de regresión lineal.

Palabras clave: azaperona, azaperol, cerdo, residuos, tranquilizantes, alimentos

Introduction

Intensive livestock production increased meat production and reduced costs in industrialised countries. Swine represents nearly 50% of the meat consumed in some European countries. Pigs are particularly sensitive to stress during handling and transport to the abattoir. This phenomenon leads to high mortality rates and poor-quality meat qualified as "Pale Soft Exudative" (PSE) [1]. For this reason, the use of tranquillizers has become generalised since 1970s. Phenothiazine derivatives (acepromazine, chlorpromazine and propionylpromazine) were the first to appear on the market. Currently their use is banned in food production animals as veterinary medicinal products.

Azaperone, the active principle of Stresnil®, a butyrophenone neuroleptic, developed for swine and widely used in pig production [2]. In veterinary medicine, azaperone is used in pigs for a wide variety of indications (anti-aggressiveness, obstetrics, stress, sedation and anaesthesia). Azaperone is partially metabolised to azaperol, a metabolite with some pharmacological activity [3,4]. Azaperone has been also used to reduce aggressive behavior in young pigs and has been claimed to increase food conversion efficiency in groups where previously unacquainted pigs were mixed together [5]. Azaperone acts by blocking dopamine receptors in the brain, and unlike the most antipsychotic neuroleptics, azaperone is a potent α-receptor blocker at low dose, while dopamine receptor is blocked only at high doses. Azaperone is thought to depress the activity in the reticular activating system. This fact decreases the ability of the animal to respond to external signals making the animal relatively indifferent to its surroundings [6]. The clinical effects remain during 4 to 8 hours [7]. Many boars are transported together to slaughterhouse and they often fight under these circumstances. This may result in unnecessary suffering and economic loss due to damaged carcasses. For this reason azaperone is widely used.

Current industry standards and codes of practice have attempted to alleviate the problems associated with shipping cull boars for

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slaughter by requiring individual segregation, but welfare concerns remain. It has been well documented that the mixing of unfamiliar groups of pigs results in increased aggressive behavior and fighting among the animals in order to establish a new dominance hierarchy [8]. The most severe aggression normally occurs during the first 30 to 90 min after the groups have been mixed [9], and although the overt aggressive acts are not seen commonly after the first 24 hours of mixing and only low levels of fighting can be observed up to 8 days after regrouping, sufficient stresses is presented causing a reduction in performance [10]. A study in Australia showed that mixing a relatively small number of boars during lairage induced fighting among the pigs, resulting in a high incidence of skin lesions and inferior pork quality [8].

During the pre-slaughter handling of pigs, unfamiliar groups of pigs will often be mixed together. Currently, in countries as Canada, the Health of Animals Regulations prohibits the transportation of groups of animals that are "incompatible by nature". These Regulations state that groups of bulls, de-tusked boars, rams and goat bucks, if mature, shall be segregated from all other animals during transport [11]. Although not specifically, this suggests that cull boars being transported in groups are to be detusked prior to mixing and if not, must be transported in individual compartments.

Azaperone is thought to act on CNS to make animals indifferent to their surrounding environment [6-12]. Studies have shown that azaperone causes quantitative changes in pig behavior that could be interpreted as indicating an anxiolytic effect [13,14]. Azaperone have been also described as an effective sedative in small and medium-sized antelope and deer species at dose range of 0.05–2.83 mg/kg body weight [15]. Azaperone used as a sedative at low doses has been found to reduce emotionality in sheep tested in an open field test [16] and to increase inter-individual distance and lower shade preference when given to sheep before testing in a novel environment [17].

Biotransformation of azaperone leads primarily to the production of its reduced metabolite azaperol (Fig. 1). Azaperone has 4–30 times the biological potency of azaperol [3]. Azaperol is the only metabolite with pharmacological activity. Residues of azaperone are evaluated as the sum of azaperone and azaperol because the activity of azaperol may be associated with its ready intercom version with azaperone. Although some evidence exists that azaperol is pharmacologically less effective than azaperone, for an adequate safety evaluation for the consumer, residues of azaperol are considered to be as harmful as those of azaperone [18]. The Committee for Veterinary Medicinal Products (CVMP) of the European Agency for the Evaluation of Medicinal Products (EMEA) recommends the marker residue as sum of azaperone and azaperol for establish the MRL. European Union Commission Regulation (EC) No 1958/98 [19] as an amendment to Council Regulation (EEC) No 2377/90 [20] classifies azaperone as a substance with MRL in the animal species porcine of 100 µg/kg in muscle, skin + fat, liver and kidney being the marker residue the sum of azaperone and azaperol [21].

The pharmacologically active compounds azaperone and azaperol deplete to undetectable levels within a few days after intramuscular administration in all edible tissues except injection site. The high and more persistent concentrations of azaperone and azaperol in the injection site might contra-indicate the use of azaperone for transport of pigs to the slaughterhouse, however due to the stress and aggressivity triggered by this situation it might be necessary its use. Taking into account the more persistence concentrations of azaperone and azaperol in the injection-site, the oral use could be a solution to avoid residues at the injection site of animals being transported.

The objectives of this paper were to determine the residue depletion of azaperone and its metabolite, azaperol in edible tissues of pigs after single oral administration

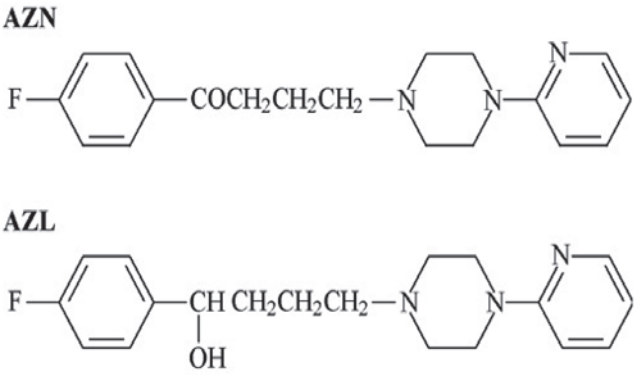

Figure 1. Chemical structure of azaperone (AZA) and azaperol (AZOL)

Materials and Methods

Animals and Experimental Design

Thirteen young, 3 months old Duroc Jersey pigs with a body weight between 30-45 kg, were treated with the formulation Stresnil® (Janssen Animal Health) at the recommended single oral dose of 4 mg azaperone/kg body weight (i.e., equivalent to 2 mL per 20 kg body weight). The Stresnil formulation was diluted in water and administered orally directly into the mouth by a syringe attached to a cannula. All the animals were housed in the experimental farm of the La Plata University and allowed to acclimatize for 7 days prior the study start. The animals were kept in individual pens with free access to water and food (a proprietary pig feed) in amounts adjusted to their body weight. The protocol followed the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (Federation of Animal Science societies -FASS-). Five pigs were slaughtered by insensitization and exsanguinations at 6 hours after oral drug administration. Four pigs were sacrificed at 24 hours and another 4 pigs at 48 hours after the oral drug administration. Samples of 100 g of liver, kidney, muscle and skin + fat were obtained from each treated animal and stored in individual bags at -40°C until assay. Tissue samples from two untreated animals (control tissues) were also obtained to be used in the validation analytical method.

Chemicals and Reagents

The reagents used were HPLC-grade acetonitrile (Sigma Aldrich®), sodium chloride (J.T. Baker®), sulfuric acid (J.T. Baker®), acetic acid (J.T. Baker®), and anhydrous sodium acetate (J.T. Baker®). Azaperone and azaperole reference standards were supplied by Janssen Animal Health BVBA (Beerse, Belgium). Stock standard solutions (1 mg/mL) were prepared in acetonitrile.

Analytical Method and Validation

Azaperone and azaperol concentrations in tissues were measured using a HPLC technique.

Extraction and clean-up procedure. The extraction was performed in an automatic solid-phase extraction system (Gilson Aspec XL, France) using Strata (C18, 100 mg, 1 mL, Phenomenex Torrance, CA,

USA) cartridges. Tissues were homogenized with a grinder. One g of sample was weighed and 4 mL acetonitrile added under continuous mixing. The mixture was vortexed for 15 min, sonicated 2 min, centrifuged at 4000 G for 20 min. The supernatant was mixed with 8 mL 10% NaCl and added to the cartridge, the cartridge was washed with 1 mL 10 mM sulphuric acid, then 2 mL air and eluted with 2 mL acidic acetonitrile (1 mL 50 mM sulphuric acid plus 100 mL acetonitrile). The eluate was placed in a tube and evaporated to dryness in a thermostatic bath at 70°C and under nitrogen flow. The dried extract was dissolved in 300 µL acetonitrile and 10 mM sulphuric acid (1:1). A 100 µL aliquot of this solution was injected into the HPLC system. The retention times of azaperone and azaperol, under the above-described conditions, were about 4.95 and 7.5 min., respectively (Figure 2).

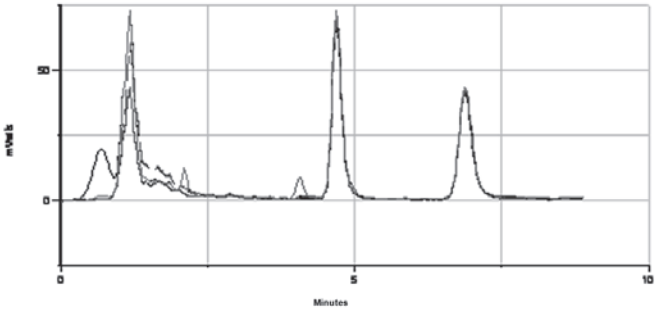

Figure 2. HPLC Chromatogram corresponding to an injection of muscle sample spiked with 200 ng/g of azaperone (AZA) and azaperol (AZOL)

HPLC analysis. The chromatographic system consisted of an isocratic pump (Gilson Inc. 307), an automatic injector (Gilson Inc. 234), a UV-VIS detector (Gilson Inc. 155) set at a wavelength of 240 nm and Eppendorf CH-30 ColumnHeater (set at a 50ºC). The system is controlled through the Unipoint® Gilson system. An C18 column (Gemini 5 µm, 4.6 mm x 150 mm, 5 µm; Phenomenex, Torrance,CA, USA) was eluted with a mixture of acetonitrile : water 55:45 (with 2,46 g/L anhydrous sodium acetate, pH adjusted to 6.5 with acetic acid) at a flow rate of 1.2 mL/min. Identification of the metabolites in pig tissues were made by comparison with the retention times of the reference standards. The analytical method was fully validated for the compounds azaperone and azaperol (linearity, recovery rate, accuracy, precision, trueness, quantification limit (LOQ), detection limit (LOD) and specificity). The precision of the extraction procedure and chromatography was evaluated by processing as replicates in six different occasions, aliquots of pooled different tissue samples containing known amounts of azaperone and azaperol. Drug concentrations were determined from peak areas and the use of calibration curves obtained by running tissue samples from pigs not treated with Stresnil® (i.e. pigs control group) that were spiked with known concentrations of azaperone and azaperol. For tissue specimens as determined using the linear least squares regression procedure, a linear relationship existed in the calibration curve of azaperone and azaperol over the range of 0.03 to 0.5 µg/g for muscle, liver and kidney, which always yielded a correlation coefficient exceeding 0.9889 (range 0.9889 to 0.9999) (Figure 3). Overall mean recovery of azaperone and azaperol from was greater than 99% and 89%, respectively. Within-day and day-to-day precision were < 2.365% for azaperone and <10.18% for azaperol. The LOD was 0.002 µg/g for azaperone and 0.003 µg/g for azaperol in muscle, liver, skin + fat and kidney. The LOQ was 0.010 µg/g for azaperone and azaperol with an accuracy of 89.54% with a CV of 12.72% in all tissues. The method was selective for those substances analyzed; no endogenous interference was observed on the HPLC chromatograms.

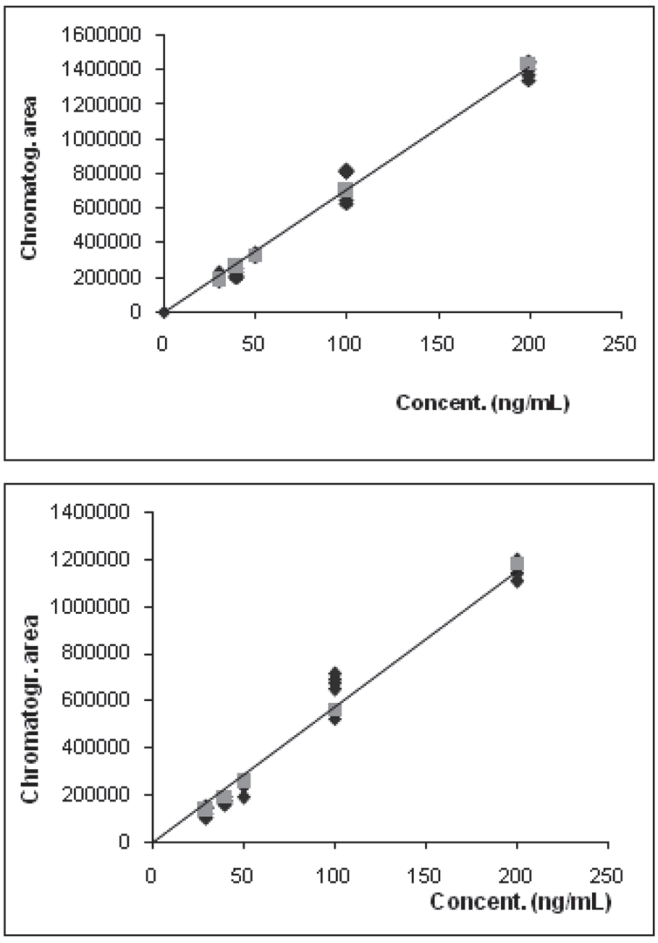

Figure 3. Representative calibration curves (A) for azaperone and (B) azaperol. The calibration curves were obtained by running muscle samples from control pigs that were spiked with known concentrations of azaperone and azaperol.

Results and Discussion

Many techniques are currently available for detecting the presence of azaperone residues. Immunological methods [23] allow efficient screening, but also positive results have been confirmed by physic-chemical methods. Among the techniques that have been reported are thin-layer chromatography [24] and liquid chromatography extraction coupled with electrochemical detection [25] or fluorescence detection [26]. These methods require extensive clean-up and do not allow sufficiently sensitive detection. In recent years several studies were performed with techniques of liquid chromatography with UV detection and liquid chromatography-tandem mass spectrometry [27,28]. These techniques have the advantage of being very sensitive. For this reason we have validated a technique by HPLC with UV detection. The method described has good sensitivity and specificity, the limits of detection were low and the quantification was well below the MRL established by the EMEA. Validation parameters of the procedure for determining azaperone + azaperol residues in pig tissues were in accordance with

internationally required criteria [29].

In the present study, the concentrations of the residues in all tissues did not exceed 0.1 µg/g or 100 µg/kg at any sampling time. Six hours post-administration, levels of azaperone + azaperol were below their respective MRL in all tissues assayed. Our results are consistent with those of Delahaut et al. [28], although these authors administered a dose of 0.025 mg azaperone/kg body weight by the intramuscular route. In the study of Delahaut et al., the animals were slaughtered 2, 6, or 24 hours after azaperone intramuscular injection. This timing was chosen to mimic field conditions as closely as possible (2 hours for a farm located near the abattoir, 6 hours for transport over a medium distance, and 24 hours for slaughter postponed to the next day). On the other hand, it was reported the plasma levels of azaperone after single intramuscular administration to pigs at a dose of 1 mg/kg body weight; plasma levels of azaperone reached a maximum concentration within 30 minutes and showed a biphasical depletion with half-lives between 60 minutes and 2.5 hours thereafter. Azaperone was rapidly distributed to tissues (with highest levels in kidney, liver and lung and lower levels in fat, brain and muscle) followed by a high rate of metabolism and elimination [21]. Excretion was with the urine (62 to 89% for single intramuscular doses of 4 and 1 mg/kg bodyweight, respectively), mainly between 8 and 24 hours [21].

The results found in the present study on the tissue concentrations (µg/g) of azaperone + azaperol for pigs treated orally with Stresnil® (azaperone at a single oral dose of 4 mg/kg body weight) are shown in Table 1 and Figures 4-7. The mean ± SD azaperone + azaperol concentrations after oral administration of Stresnil® (single oral dose of 4 mg azaperone/kg body weight) to pigs in liver, muscle, kidney and skin+fat pigs tissues are presented in Figures 4 to 7. The sum of the concentrations of azaperone and azaperol is considered the most appropriate marker residue, due to both azaperone and its metabolite azaperol have pharmacological activity; on the basis of pharmacological activity, a pharmacological ADI of 0.8 µg/kg body weight (equivalent to 48 µg for a 60 kg person) has been set by the EMEA, formally European Medicines Agency (EMA). Besides, azaperol can be reconverted to azaperone. Although azaperol is less pharmacologically active than azaperone, as a worst case scenario in order to adequately protect the consumer, azaperol is considered as potent as azaperone. In our study, 6 hours after Stresnil® oral administration (4 mg azaperone/kg body weight), levels of azaperone plus azaperol were below the MRL (0.1 µg/g or 100 µg/kg) in all target tissues assayed.

Table 1. *Residues (µg/g) mean ± SD of azaperone (AZA) and azaperol (AZOL) in tissues of pigs slaughtered 6, 24 and 48 hours after PO administration of stresnil® (oral single dose of 4 mg azaperone/kg body weight)*

Skin + Fat	AZA	SD	AZOL	SD	AZA + AZOL	SD
6 h	0.025	0.010	0.006	0.002	0.031	0.011
24 h	0.030	0.02	0.006	0.002	0.036	0.016
48 h	0.021	0.01	0.012	0.005	0.033	0.007
Muscle	**AZA**	**SD**	**AZOL**	**SD**	**AZA + AZOL**	**SD**
6 h	0.016	0.009	0.012	0.004	0.028	0.010
24 h	0.016	0.010	0.009	0.005	0.025	0.012
48 h	0.011	0.005	0.009	0.004	0.020	0.009
Kidney	**AZA**	**SD**	**AZOL**	**SD**	**AZA + AZOL**	**SD**
6 h	0.040	0.023	0.011	0.007	0.051	0.024
24 h	0.032	0.010	0.014	0.009	0.047	0.021
48 h	0.013	0.003	0.020	0.012	0.033	0.022
Liver	**AZA**	**SD**	**AZOL**	**SD**	**AZA + AZOL**	**SD**
6 h	0.026	0.011	0.006	0.001	0.032	0.011
24 h	0.013	0.009	0.009	0.002	0.022	0.009
48 h	0.009	0.005	0.007	0.002	0.016	0.006

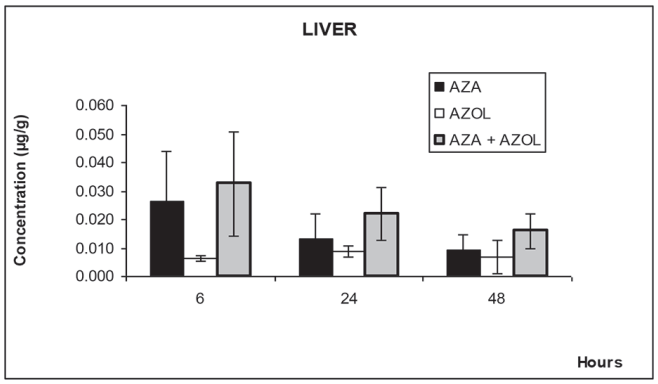

Figure 4. Mean liver concentrations of azaperone (AZA), azaperol (AZOL) and azaperone + azaperol (AZA + AZOL) in pigs slaughtered 6, 24 and 48 h after oral administration of Strenil® (single dose of 4 mg azaperone/kg body weight).

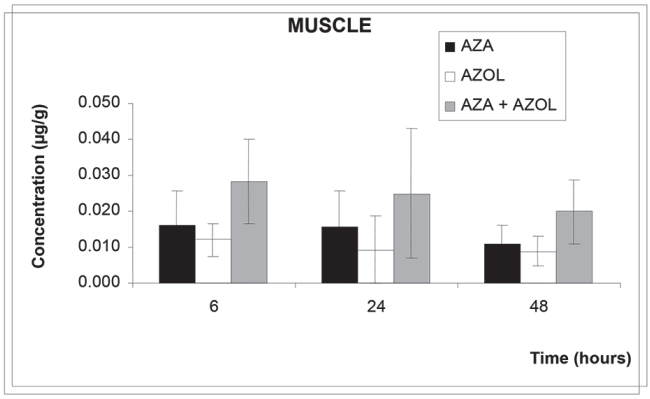

Figure 5. Mean muscle concentrations of azaperone (AZA), azaperol (AZOL) and azaperone + azaperol (AZA + AZOL) in pigs slaughtered 6, 24 and 48 h after oral administration of Strenil® (single dose of 4 mg azaperone/kg body weight).

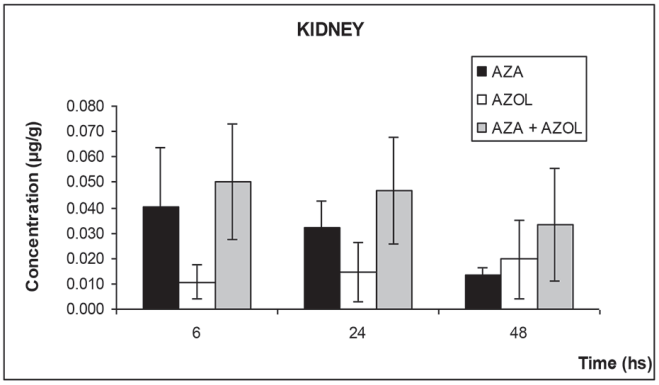

Figure 6. Mean kidney concentrations of azaperone (AZA), azaperol (AZOL) and azaperone + azaperol (AZA + AZOL) in pigs slaughtered 6, 24 and 48 h after oral administration of Strenil® (single dose of 4 mg azaperone/kg body weight).

One of the major disadvantages that entail the administration of tranquilizers, in pigs for slaughter, by the intramuscular route is the high persistence of residual levels at the injection site. This problem could be absolutely solved by the oral administration. In this line, the present investigation is the first study that describes the residue tissue depletion of azaperone after oral administration to pigs. The sampling time chosen for this study is consistent with the peak concentrations observed for azaperone during this residue depletion study.

Numerous experimental designs and statistical approach are used to establish the withdrawal time. The EMEA recommends the use of a linear regression technique as the choice method [30]. In our study, taking into account the MRLs in pigs and considering that the marker residue is the sum of azaperone and azaperol, the calculated withdrawal time was cero hours (Figure 8). Our results demonstrate that oral azaperone administration at the recommended dose not require withdrawal time respecting the MRL fixed for azaperone [31] and thus 0-day withdrawal time is proposed. Nevertheless, as precaution measure, we can conclude that, from the results obtained in the present experiment, edible tissues of pigs treated orally with azaperone at 4 mg/kg body weight, 6 hours previously to the sacrifice, should be a withdrawal time acceptable to guarantee safety of foods for the consumers.

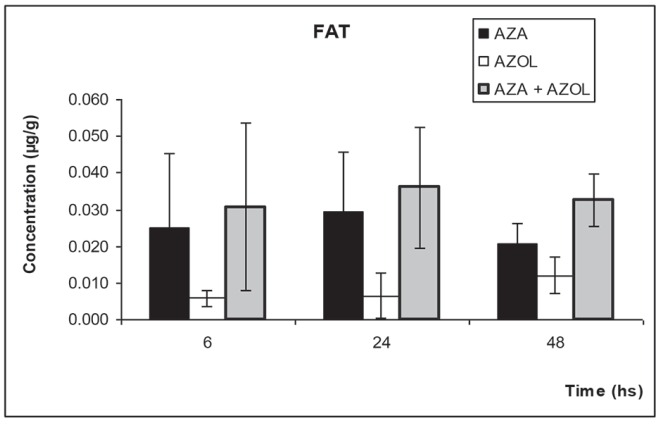

Figure 7. Mean skin + fat concentrations of azaperone (AZA), azaperol (AZOL) and azaperone + azaperol (AZA + AZOL) in pigs slaughtered 6, 24 and 48 h after oral administration of Strenil® (single dose of 4 mg azaperone/kg body weight).

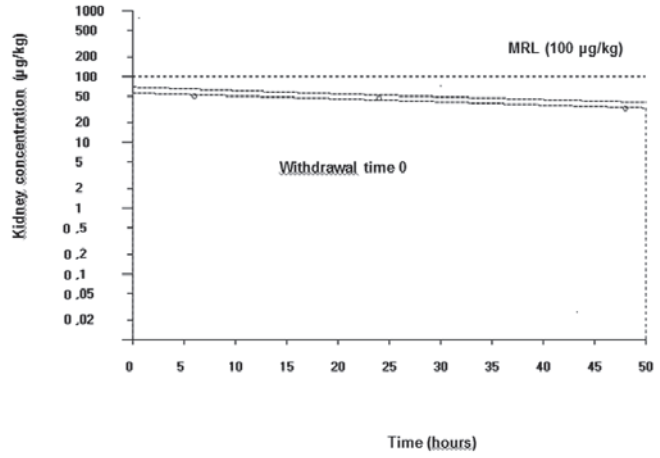

Figure 8. Plot of the withdrawal time calculation for azaperone in pig kidney at the time when the one-sided 95% upper tolerance limit is below the EU MRL for azaperone (100 µg/kg) after oral administration of Strenil® (single dose of 4 mg azaperone/kg body weight). [Residue marker: azaperone + azaperol].

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