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Prevalence of steroid hormone residues by GC-MS/MS screening in animal matrices in Romania

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Abstract

The aim of this one year survey study was to assess, the prevalence of steroid hormones residues in theaim of National Residue Control Plan. GC-MS/MS in urine, serum, liver and muscle tissue in conformity withSOP ARO/492-2006, respectively SOP ARO/534-2008 was performed on 273 samples. Samples were purified using a one-step cleaning, detection being performed to a Varian 320-MS GC-MS/MS spectrophotometer (able to identify and confirm the matrix presence at 0.5 $\mu g.kg^{-1}$ or 0.5 $\mu g.L^{-1}$), the CCα values for most compounds being between 0.1-0.3 $\mu g.kg^{-1}$ or $\mu g.L^{-1}$. The percentage of analyzed samples/species was: 40.29% poultry; 34.43%, cattle; 22.71% swine; 1.43% sheep and goats and respectively 1.09% equines. From total number of samples analyzed for detection of natural or synthetic steroid hormones / category, considered in this study, regardless of the matrix (urine, serum, and muscle tissue), 0.36% of samples were for medroxyprogesterone, 12.09% for estradiol, 31.5% for nortestosterone, respectively 56.05% for trenbolone. Samples analyzed / animal specieswere compliant for: medroxyprogesterone, estradiol, testosterone and trenbolone-cloud. In the case of estradiol, values identified were between 0.01-0.03 ppb for β fraction and of 0.01-0.06 ppb, for α fraction, but below detection limit of the method used.

Keywords: steroid hormones, residues, GC-MS/MS screening, animal matrices

1. Introduction

Steroid hormones stimulate livestock due to increased feed efficiency, and its conversion into muscle tissue. The use of hormones such as: 17β-estradiol, progesterone, trenbolone acetate, melengestrol, nandrolone, in order to accelerate muscle growth, causes major risks to human consumers, deleterious activity of these disruptors being demonstrated in some experiments (A. ANDERSSON & N. SKAKKEBAEK [1]; R.T. CRISTINA & al. [2]; R.T. CRISTINA & al. [3]); SCHWARZENBERGER & al. [4]).

The undesirable effects on human health could be:cyto-morphopathologic, genetic, neurobiological, genotoxic, carcinogenic etc, andthese may occur due to the substance itself or the presence of its metabolites in products or animal by-products(T. COLBORN & al. [5]; A. DAXENBERGER & al. [6]; S. FRITSCHE & H. STEINHART [7]; P.M. MATIAS & al. [8]).

Quality control, assurance and food safety are required to be a priority for numerous and diversespecialized bodies and/or supervisory authorities in all European Union countries and USA, the main objective of food safety policy in all these countries being to achieve the highest possible degree of protection, human health and consumer interests related to food (S.L. LEVY [9]; EDSTAC [10]; FDA [11]; OECD [12]; WTO [13]).

In European Union the control of prohibited substance residues and various contaminants that may be used in the veterinary field, or administered to farm animals (such as live animals and products of animal origin) is decided by the European Commission According to Council

Directive 96/23/EC[14] two main groups of substances should be monitored in order to ensure a high level of human health protection in relation to products of animal origin intended for consumption, and certain substances in Groups A and B. Annex IV, Directive 96/23/EC stipulates, the sampling rules and frequency of sampling for cattle, swine, sheep, goat, equine, poultry and aquaculture products andfor milk, eggs, honey, rabbit meat and game, Commission Directive 97/747/EC [15]establishes the sampling levels and frequency control. Group A is comprised byprohibited substances and growth stimulating agents,which could not establish a Maximum Residue Limit (MRL) and group B includes veterinary medicines and contaminants that have established MRL. Group A containsthe substances with anabolic effect and prohibited substances like: stilbene and derivatives; ant thyroid agents; steroids; resorcylic acid lactones; β-agonists, all substances included in Annex IV of the Council Regulation, being now findable in Regulation 470/2009[16].

In this respect, European Union Member states must submit their residue monitoring results at the latest by 31 March of each year. In Romania, the National Sanitary Veterinary and Food Safety Authority (ANSVSA)together with the Institute of Hygiene and Veterinary Public Health (IHVPH)prepared the National Residue Control Planhaving in responsibility the residues monitoring. In consequence, ANSVSA emitted the Order 2/2010 of 20 January 2010[17], for approval of the food safety program for surveillance and control for 2010, where for the first time, in Section 11, it was introduced the chapter "*Expertise of residues in live animals and their products*". In this respect, for the first time in our country, research was focused on the residues of steroid hormone prevalence in accordance with, the National Residue Control Plan for food safety and consumer health protection monitoring for year 2010, the main objective being the creation of a residue survey image from different matrices of animals over a one year period. The general examination scheme of residues in live animals and their products is presented in Table 1.

Table1. Examination scheme for several animal species of the substances from A3 group[16-20].

A3 – group substances							
Species	**Substance**	**Matrix**	**Screening method**	**Confirmation method**	**Sampling place**	**Animal's category**	**Results interpretation referential**
Cattle	17β-estradiol	Serum	GC-MS/MS	GC-MS/MS	Exploitation	young cattle, cows	ANSVSA Order 199/2006, as amended and supplemented. Commission Decision 2005/34/EC, Directive 2008/97/EC
	Nortestosterone	Urine	GC-MS/MS	GC-MS/MS	Exploitation or abattoir	calves, young cattle, cows	
	Trenbolone	Urine	LC-MS/MS	LC-MS/MS	Exploitation or abattoir	young cattle, cows	
	17β-testosterone	Serum	GC-MS/MS	GC-MS/MS	Exploitation	young cattle, cows	
	Medroxyprogesterone	Muscles	GC-MS/MS	GC-MS/MS	Abattoir	calves, young cattle, cows	
	Melengestrol						
Swine	Nortestosterone	Urine	GC-MS/MS	GC-MS/MS	Exploitation or abattoir	young and adult	ANSVSA Order 199/2006, as amended

	Trenbolone	Urine, Liver	LC-MS/MS	LC-MS/MS	Abattoir		and supplemented.Commission Decision 2005/34/EC
	Medroxyprogesterone	Muscles, Liver	GC-MS/MS	GC-MS/MS			RegulationEC /470/2009
	Melengestrol		LC-MS/MS	LC-MS/MS			
	Altrenogest						
Sheep / Goats	Nortestosterone	Urine	GC-MS/MS	GC-MS/MS	Abattoir	young and adults	ANSVSA Order 199/2006, as amended and supplemented.Commission Decision 2005/34/EC, Directive 2008/97/EC

2. Materials and methods

In Europe, Directorate-General for Health and Food Safety (DG SANCO) is responsible for coordinating the collection of data on residues from all Member States. In Romania, according to ANSVSA Order 205/2007[21], the National Reference Laboratory (NRL) for steroids confirmation appointed the DSVSA Constanța.

Sampling

Samples from farms and/or slaughterhouses like serum, urine, liver or muscle tissue were collected by authorized veterinarians as follows:

- *blood* was collected from the ventral coccygeal sinus artery or jugular vein, using sterile vacuum tubes without anticoagulant after a standard sterilization. After blood sampling, the expressed serum was frozen.
- *urine* was collected in special containers by stimulating urination, then frozen and samples sent for analysis.
- *tissues and organs* were collected at the slaughterhouse. Isolates were individually wrapped and marked with identification numbers and then samples sent to the laboratory.

Methodology

GC-MS/MS in urine, serum, liver and muscle tissue in conformity with reference documents SOP ARO/492-2006 [22], respectively SOP ARO/534-2008 [23]it was performed. The method was validated by the Romanian Accreditation Association (RENAR), dated 26 April 2010. The method could be used for detecting a large number of anabolic compounds in the tissues and urine, being able to identify and confirm the presence of the matrix at a proposed control 0.5 $\mu g.kg^{-1}$ or 0.5 $\mu g.L^{-1}$.

Samples were purified using a one-step cleaning, and detection was performed to a Varian 320-MS GC-MS/MS spectrophotometer,the CCα values for most compounds being between 0.1-0.3 $\mu g.kg^{-1}$or $\mu g.L^{-1}$. The working principle consisted of the following steps:

- muscle tissue sample preparation and methyl tert-butyl ether (MTBE) extraction,
- the urine sample enzymatic hydrolysation, and extraction with MTBE,
- purification, both the matrix and cartridges C18,
- derivatization,
- detection by GC-MS/MS.

The ionization mode was accomplished by electronic impact (EI), in the case of the appearance of suspect samples,confirmation can be achieved on the same extracts.

In the case of the *serum*, method consisted of: solid phase extraction (SPE) derivatization of heptafluorobutyric acid (HFBA) (Sigma-Aldrich) and GC-MS/MS detection. In this case, the mode used was also EI.

Detailed operations like: preparation of primary tissue and urine extracts;purification on C18 cartridges;derivatisation; GC-MS / MS detection and expression of resultsare presentedin Table 2.

Table 2. Detailed operations steps per each phase

Phase	Detailed operations
Preparation of *primary tissue extract*	Weighing of one gram of the tissue, adequately mixed in a 50 mL tube; introduction of internal standards and stirring the mixture in the vortex for a few seconds;addition of 10 mL of water and stirring the mixture 30 sec; putting the tube in an ultrasonic bath for 15 min;addition of 10 mL of TBME and rotating for 15 min., thencentrifugation for 10 min at 4000 rpm, then putting the tube for 20 min at -80°C and decanting the TBME layer in a 10 ml tube and finally, evaporation at 55°C under nitrogen;dissolving the extract with 4 mL of methanol / water (80/20); adding 4 mL of n-heptane and stirring for 30 sec; centrifugation for 10 min at 3000 rpm and the removal of the n-heptane layer; dissolving the extract with 4 mL of methanol / water (80/20); evaporation to a volume <0.5 mL by addition of water at 4 mL; followed by the purification step on C18 cartridges.
Preparation of *primary urine extract*	urine 5 mL pipetting into a 50 mL tube and adding the internal standards; addition of a 2 mol/L acetate solution buffer, the pH (5.2 ±0.2) check and adding a 1 mol/L solution of NaOH or a 1 mol/L HCl solution if necessary; addition of 0.05 mL of β-glucuronidase / sulfatase and incubating overnight at 37°C; addition of 10 ml of TBME and stir for 30 sec; centrifugation for 10 min at 4000 rpm, putting the tubes for 20 min at -80°C and decanting the TBME layer in a 10 ml tube evaporation at 55°C under nitrogen;addition of 10 mL of TBME and stirring for 30 sec;Centrifugation for 10 min at 4000 rpm, putting the tubes for 20 min at -80°C and decanting the TBME layer in a 10 ml tube evaporation at 55°C under nitrogen;dissolving the extract in 0.2 mL of methanol followed by 4 mL of water; followed by the purification step on C18 cartridges.
Purification on C18 cartridges	Pre-washing the cartridge with 5 mL of methanol followed by 5 mL of water; passing the extract through the column;washing the column with 5 mL of water;washing the column with 5 mL of methanol / water 40/60 (v/v);column eluting with 5 mL of methanol / water 80/20 (v/v); evaporation at 55°C under nitrogen; dissolving the extract in 3 mL of water and extraction with 5 mL of n-pentane; centrifugation for 10 min at 3000 rpm and n-pentane layer transfer to clean tubes; repeating the extraction with n-pentane, n-pentane layers combining and evaporating them; dissolving dry extract with 0.4 ml of ethanol.
Derivatisation	Residue transfer into derivatization bottles; pipetting of standards used for calibration curve; samples in ethanol and for calibration evaporated, then 25 µL of MSTFA ++added; the vials are shaken and the mixture incubated for one hour at 60°C; after incubation the tubes are evaporated under a nitrogen stream at 55°C; dissolving the residue in 50 µL of isooctane and transferring in glass inserts for injection, placing them in the GC-MS / MS automatic injector.
GC-MS/MS detection	Injection of 2 µL in the pulsed splitless mode at 260°C; (initial temperature of 110°C (1 minute)then temperature increase 20°C / min up to 340 °C, left for 5 minutes at 340°C); transfer line of temperature 330°C; constant flow mode, 1.1 mL/min. Measurements are for detection (quantification) on MRM I (Multiple Reaction Monitoring). To confirm the measurements are made both on MRM I and II.Both methods can be combined. If a result is inconsistent, MRM II is used for confirmation; the ratio of transitions I/II must meet the criteria Decision 2002/657/EC [182].
Expression of results	Ion selected areas for standard and internal standard are calculated, the report being response variable. The quantitative results are obtained by constructing a linear graph by plotting the points corresponding to linear regression, calculation of concentration vs. response variable. The unknown concentrations are calculated by interpolation, quantification being valid only if: the analyte signal has a >3 signal/noise ratio (S/N > 3); the blank test samples are present in all internal standards (S/N>3, for all internal standards); the enriched sample materials are present in all of the compounds (S/N>3, internal standards for the undeuterated compounds).

3. Results and discussions

Prevalence and residue levels of steroids

Minimum number of animals to be checked each year for different types of waste is specified in Annex III of Directive 96/23/EC [24].

For *cattle* it must be at least equal to 0.4% of the total number of animals slaughtered the previous year; 0.05% for*swine*, and for *sheep* and *goats* of 0.05%.

For *horses,* the annual number of samples for control is determined by each Member State, partly depending on existing diseases in these species.

For *poultry*, the minimum number of samples per category (broilers, turkeys, etc.) must be one per 200 tons of annual output with a minimum of 100 samples for each group of substances, when annual production for each category is of 5000 tons. The monitoring of natural and synthetic steroid hormone concentrations in living animals or food, such as meat,

by-products or organs, contributes to the knowledge of the animals hormonal status, the environmental impact of excretion, and most importantly, the impact on human health.

In the period studied, for steroids determination, a total of 273 samples were analyzed. During the monitoring the percentage of analyzed samples/species was of: 40.29% - poultry; 34.43% -cattle; 22.71% - swine;1.43% -sheep and goatsand respectively, 1.09% - equines, as presented in figure 1.

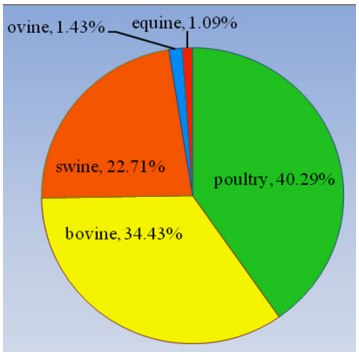

Figure 1. Percentage of analyzed samples in animal groups

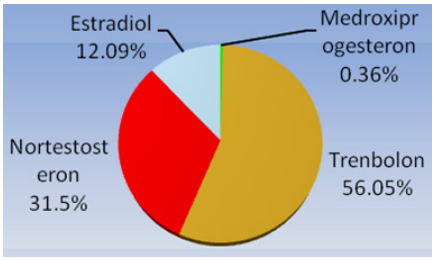

Figure2. Results distribution (%) in different substance groups

Fromtotal number of samples analyzed for detection of natural or synthetic steroid hormones / category, considered in this study, regardless of the matrix (urine, serum, and muscle tissue),0.36% of sampleswere for medroxyprogesterone, 12.09% for estradiol, 31.5% for nortestosterone,respectively 56.05% for trenbolone, as presented in figure 2.

Medroxyprogesterone, a synthetic progestagen, had the smallest share in our study, led by swine muscle tissue. The result was in line, below the detection capability (CCβ = 0.26 $\mu g.kg^{-1}$) for this compound,the minimum required performance limit was of 1 ppb ($\mu g.kg^{-1}$).

Estradiol was analyzed both for 17α and 17 β-estradiol isomers. Measurements were performed in the serum. CC for the*alpha*fraction was of 0.07 ng/mL and 0.05 ng/mL for the *beta* fraction. Only when the values obtained for the analyte werebetween: 0.01-0.06 ppb (ng/mL) to 30.3% of samples, for 17α-estradiol, respectively, ranged from 0.01-0.03 ppb, for 17β-estradiol, in 15.15% of the total samples, but all results being under the CCβ. Following the interpretation of the chromatograms, all the results were declared compliant.

19-nortestosterone (nandrolone or 17β-19-nortestosterone) and *trenbolone* are thelast compounds that were supervised in our study, being in our opinion with a great importance as disruptors. These growth promoters from the anabolic androgen group can be identified in meat, when they are used in a fraudulent manner, before their removal from the body of the animal as some research ascertained(C.J.M. ARTS & al.[25]; J.P. SCARTH & al. [26, 27]).

Inthe 17 β-19-nortestosterone case, the metabolite marker used for its screening was 17α-19-nortestosterone, which can occur in different matrices, if the animals were slaughtered in the last period of gestation or following an exogenous administration (B. LE BIZEC [28]; N.A. SHAHIDI [29]).

The observations from our tests wereof 86% in urine and 14% in the muscle tissue. The number of nortestosterone samples analyzed per animal species is shown in figure 3.

Detection capability for the 17α -19-nortestosterone was of 0.22 ppb and respectively of 0.26 ppb for 17β-19-nortestosterone. The results obtained were found to be compliant, with values below the detection limit, which means that the samples tested originated either from young male or non-pregnant females.

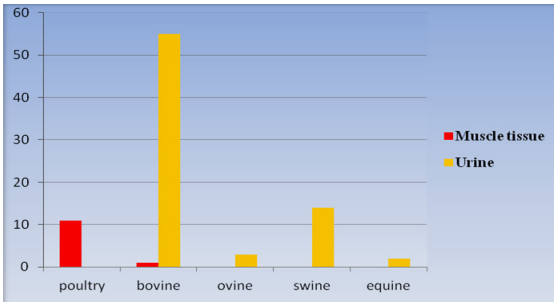

Figure3. Global analysis for nortestosterone by species

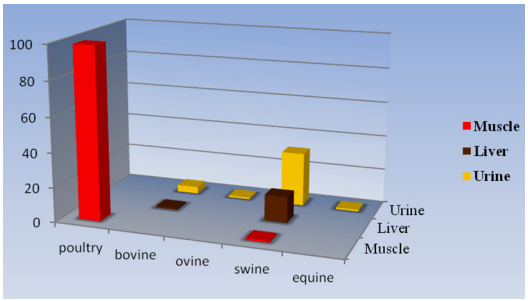

Figure 4. Global distribution for trenbolone on animal species

Trenbolone, an anabolic steroid with a testosterone-like chemical structure, when administered to animals is rapidly hydrolyzed into the body, in 17α-trenbolone and 17β-trenbolone major metabolites, which then, will be eliminated from the body through urine, faeces and bile (W. SCHÄNZER [30]). The method used for screening has a CCβ value for the *alpha* fraction of 0.33 ppb, and the *beta*fraction of 0.27 ppb. Most of the analysis for the trenbolone matrix was made using muscle tissue, followed by urine and liver. The results were declared compliant, and in figure 4,are presented the species and samples used for the determination of this compound.

According to the last Regulation 37/2010/EC[31], steroid hormones are part of the banned substances category, which is why, MRL cannot be fixed in case of these substances, except for medroxyprogesterone acetate where MRL value were established to 1 $\mu g.kg^{-1}$ in the fat around pig's kidney, in the aim that residues at whatever limit could constitute a potential risk, especially for different categories of consumers as many authors proved(F. COURANT [32]; F. COURANT & al. [33]; G.C. GATTI &al.[34]; D.B. GOWER & al. [35]; M.L. SCIPPO &al. [36]; F., SCHWARZENBERGER & al.[37]).

The great steps that were accomplished in this field are due on the one hand on the extraordinary development of analytical criteria, intensely debated in the last decade, in developing more and more sensitive methods, the number of researches on this topic being significant (S. BIDDLE, & al. [38];B. LE BIZEC & al. [39]; E.A.I. DAESELEIRE & al. [40]; P M. DONIKE & al. [41]. SCHMIDLEY [42]; L. VANHAECKE & al. [43]and, on the other hand, due to rapid developing of related legislation into verifyand to take rapid and the most appropriateCommunitymeasures(S.L. LEVY [9]; EDSTAC [10]; WTO [13]; Commission Decision 2005/34/EC [19]; Regulation 37/2010/CE [24]).

The results obtained, though at a glance, seems to be disproportioned in what it concerns the proportion of the studied four matrices, want to show how important did nortestosterone (nandrolone) and trenbolone for our study. Though the values were below the detection limit, the obtained values showed us that at least for poultry and swine species showing the appearance tendency of these disruptors in animal tissues. In this aim we agree with other research groups who put these two disruptors in the top of the threats that can be expected in this decade (A. ANDERSSON & N. SKAKKEBAEK [1]; P. VAN DER WAL & P.L.M. BERENE [2]; T. COLBORN & al. [5]; S. FRITSCHE & H. STEINHART [7];C.J.M. ARTS & al. [25]).

Among the multifarious detection methods proposed by the topic's researchers we consideras the most influential for the steroid residues screening are the serum and urine ones, being more rapid and "real time" relevant (B. LE BIZEC [28]; F. COURANT & al. [33]; G.C. GATTI & al. [34]; M.L. SCIPPO & al. [36]; F., SCHWARZENBERGER & al. [37]; S. BIDDLE & al. [38]; B. LE BIZEC & al. [39]; M. DONIKE &al. [41]; L. VANHAECKE & al. [43]).

4. Conclusion

The samples analyzed in this study / animal species, for the period of one year were declared as compliant for: medroxyprogesterone, oestradiol, testosterone and trenbolone-cloud. If the case of oestradiol, values identified were between 0.01-0.03 ppb for *beta* fraction and of 0.01-0.06 ppb, for *alpha* fraction, but below the detection limit of the method used.

5. Acknowledgements

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