

Monitoring of residues of hormonally active and prohibited substances in food materials of animal origin

Martina Rejtharová¹, Alfred Hera²

¹Institute for the State Control of Veterinary Biologicals and Medicines in Brno

²Department of Pharmacology and Pharmacy

Faculty of Veterinary Medicine

University of Veterinary and Pharmaceutical Sciences Brno

Brno, Czech Republic

Abstract

Hormonally active substances (e.g. anabolic steroids) and residues of pharmaceutical drugs prohibited from use in food-producing animals (e.g. chloramphenicol or metronidazole) are monitored in biological matrices (tissues, urine, blood plasma) as part of regular official tests for the presence of banned substances in animals intended for food production. Any such residues found in farm animals are considered a proof of illegal administration of a banned substance, and in such cases, an official investigation of the livestock's owner is conducted, a fine is imposed and the contaminated food materials are prevented from entering the food chain and are destroyed at the owner's expense. The finding of residues of substances that are banned for use in food-producing animals but could, at the same time, be endogenous in animals, poses a problem in practice. This article describes several procedures that could be used to distinguish between illegal administration of a banned substance and endogenous residues in farm animals.

Biological matrices, residues of hormonally active and prohibited substances

Introduction

The European Commission through the Directorate General for Health and Food Safety (DG SANTE) coordinates the control of food safety in each EU member state so as to ensure the compliance with the common rules for the production of foodstuffs of animal origin. Each member state guarantees that uniform requirements for its domestic production are fulfilled, and based on that, the smooth cross-border movement of animals and trade in food of animal origin is possible across the EU. The same rules also apply to the inspection of foodstuffs of animal origin imported from the so-called third countries.

The European legislation lists substances which are strictly prohibited in food-producing animals. Council Directive No. 96/22/EC bans the use of growth promoters in the fattening of food-producing animals, which includes hormonally active substances having an anabolic effect, thyrostatics or beta-agonists. It has also prohibited the use of certain substances formerly used as pharmaceutical drugs (these are listed in Table 2 of the Regulation (EC) No. 37/2010), which were later demonstrated to have adverse effects that could affect a consumer of food coming from treated animals. The harmful effects on the consumer are not dependent on the concentration of residues present, so any (even a minimum) amount of these substances can be dangerous for the consumer.

The rules for checking the compliance with the ban on using these substances are described in Council Directive No. 96/23/EC. In each EU member state, an official authority is established which carries out regular checks that should lead to the detection of a possible breach of this ban. In the Czech Republic, the authority responsible for the enforcement is the State Veterinary Administration (SVA CR). In addition to inspections on farms and at slaughterhouses of food animals, the SVA in cooperation with official laboratories prepares an annual National Residue Monitoring Plan, which gives details about the numbers and types of samples to be taken and analysed for the presence of residues of prohibited substances. The number of samples from each species of animals is based on

Address for correspondence:

Mgr. Martina Rejtharová
Institute for the State Control of Veterinary Biologicals and Medicines in Brno
Hudecova 56a, 621 00 Brno, Czech Republic

Phone: +420 541 594 501
E-mail: rejtharova@uskvbl.cz
www.maso-international.cz

the production of each commodity in the preceding year. Not only the animal species are determined from which the samples should be taken, but also matrices of samples (types of biological material, e.g. urine or muscle tissue) and the regional distribution of sampling sites. The substances in the samples to be determined and the analytical methods to be used are also exactly defined. The samples are collected by inspectors of the respective Regional Veterinary Administrations of the SVA and handed over to officially appointed accredited laboratories. Residues of hormonally active and prohibited substances are determined in the laboratories of the Institute for the State Control of Veterinary Biologicals and Medicines in Brno (ÚSKVBL), which acts as the National Reference Laboratory for Residues of Prohibited Substances. Table 1 lists the groups of prohibited substances routinely analysed at the ÚSKVBL in official samples and some examples of characteristic compounds.

Table 1. Prohibited substances determined in official samples in the National Reference Laboratory ÚSKVBL in Brno, Czech Republic

Group of analysed substances	Examples of compounds
Stilbenes	diethylstilbestrol, hexestrol, benzeestrol
Thyreostatics	thiouracil, tapazol, methylthiouracil
Steroids with androgenic action	testosterone, methyltestosterone, boldenon
Steroids with estrogenic action	estradiol, ethinylestradiol
Steroids with gestagenicaction	medroxyprogesterone, chlormadinon, megestrol
Corticosteroids	dexamethazone, triamcinolone, prednisolone
Resorcylic acid lactones	zeranol, taleranol, zearalanon
Beta-agonists	clenbuterol, zilpaterol, ractopamine
Chloramphenicol	chloramphenicol
Nitroimidazoles	metronidazole, dimetridazole, ronidazole
Nitrofurans (metabolites)	semicarbazide, AMOZ - furaltadon metabolite
Dapsone	dapsone
Sedatives	chlorpromazine, propionylpromazine, carazolol

Residues of hormonally active and prohibited substances are determined in biological materials by using gas or liquid chromatography coupled with mass detection. The selection of analytical methods and procedures for processing these samples is defined by the rigorous requirements for selectivity and sensitivity of the determination. Biological materials are extremely complex matrices, and analytes in them occur in a very low physiologically active concentrations (i.e. micrograms per kilogram or litre of the sample). At the same time, the results of the determination must be correct and indisputable. These strict requirements for the quality of the results are necessary in order to avoid putting consumers at risk with hazardous residues in foodstuffs on one hand, and to avoid false accusations of producers and their subsequent economic losses on the other.

If residues of prohibited substance are detected in any sample, the farm in question is subject to an official investigation, which is described in detail in Council Directive No. 96/23/EC. The aim of the investigation is to identify the reason for non-compliant results, and first and foremost, to prevent the entry of contaminated food materials into the food chain. The movement of animals and food products from the farm is suspended, and then targeted control samples are collected. Any circumstances that could lead to the presence of residues of prohibited substances in the samples are investigated on the farm of origin (feedstuffs, watering, handling and treatment of animals, drug administration and mandatory records). Based on analyses of the control samples, measures are taken to

prevent contaminated food materials from reaching the consumer. If prohibited substances are proven in the control samples, it means that the illegally treated animals and their products (e.g. milk) must be destroyed with no possibility of compensation from the state. The producer may be requested to pay the costs of the analyses performed, and an administrative fine may be imposed.

Interpretation of the results

The Commission Decision No. 2002/657/EC provides – inter alia – a detailed description of the rules for interpreting the results of determining the residues of prohibited substances in official samples. In order to be used for the official analyses of residues of prohibited substances, each analytical method must have the so-called decision limit ($CC\alpha$) determined during the validation process in the laboratory. This limit is defined as the lowest concentration level at which the method used can confirm the presence of the analysed substance with 99% probability. If the measured concentration of residues is below the decision limit (the residues were not detected), such a sample is evaluated as compliant. If the concentration of the analysed substance in the sample is found to be higher than the decision limit, such a sample is evaluated as non-compliant, and the above-described official investigation is conducted in the place of the sample origin.

The interpretation of a non-compliant result is quite clear when residues of some synthetic substances that could not be naturally produced in a living organism are confirmed in the sample. An example of such substances are synthetic steroids ethinylestradiol or methyltestosterone, or the prohibited pharmaceutical drugs metronidazole and dimetridazole from the group of nitroimidazoles. The problem with interpretation occurs if a non-compliant sample contains substances whose administration to food animals is prohibited but which may occur naturally in animal tissue or body fluids. Typical examples of such compounds are natural hormones such as estradiol or testosterone, or thiouracil from the group of thyreostatics, or semicarbazide from the group of metabolites of prohibited nitrofurans. Experts from the EU Reference Laboratories for residues in cooperation with the National Reference Laboratories in each member state strive to find the decision-making procedures which would make it possible to objectively assess whether prohibited substance residues found are naturally present or whether they are due to the illegal treatment of the animal. The following text provides a more detailed description of the procedures for assessing some of these problematic substances.

Thiouracil

Thiouracil (Fig. 1) is a drug from the group of thyreostatics. By influencing the activity of the thyroid gland, it causes higher water retention in the tissues, which could be abused to increase the body weight of fattened animals before slaughtering. In addition, these thyreostatics are probable human carcinogens, and therefore their use has been prohibited in Europe since 1981. If thiouracil is administered to fattened cattle in doses that would ensure weight gain, thiouracil residues would appear in animal urine in concentrations higher than $100 \mu\text{g}\cdot\text{l}^{-1}$. With the development of analytical techniques,

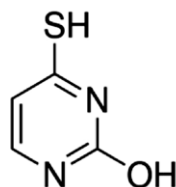


Fig. 1. Thiouracil

the sensitivity of the methods used gradually increased and official laboratories reached the decision limit in units of micrograms per litre. When these more sensitive methods began to be used in routine checks, non-compliant results of cattle urine tests for thiouracil were reported. Subsequent official investigations of these cases, however, failed to prove any unauthorized administration of this substance to animals, and neither were any other indications found of thyreostatics having been administered to the cattle (no morphological changes in the thyroid were observed, and muscle structure was not affected by water absorption).

Several animal studies have shown a possible link between these non-compliant results of thiouracil in urine and feeding cattle with plants of the brassica (cruciferous) family (e.g. Pinel et al. 2006). It has been also proven that microflora present in the digestive tract of ruminants actively participate in the formation of thiouracil molecules from compounds contained in brassica plants (Kiebooms et al. 2012). However, concentrations of the thiouracil residues formed by this mechanism never exceeded $30 \mu\text{g}\cdot\text{l}^{-1}$, and only rarely exceed $10 \mu\text{g}\cdot\text{l}^{-1}$. In contrast, administration of thiouracil to animals in order to affect weight gain left thiouracil urine residue concentrations even several orders of magnitude higher ($\text{mg}\cdot\text{l}^{-1}$). The European Reference Laboratory therefore did not recommended applying the so-called zero tolerance of residues - as is done for other prohibited substances – in the evaluation of thiouracil residues in urine, and to consider samples with thiouracil levels lower than $30 \mu\text{g}\cdot\text{l}^{-1}$ as compliant.

Semicarbazide

Nitrofurans are antimicrobial synthetic substances with proven mutagenic and carcinogenic effects on humans and whose use in food animals in the EU is prohibited. Because they rapidly metabolize in the body, major metabolites of nitrofurans are determined when samples of biological materials from food animals are analysed. Semicarbazide (Fig. 2) is the main metabolite of nitrofurazone. Although semicarbazide toxicity is significantly lower than that of nitrofurazone, semicarbazide residues in the samples inspected are determined as the main indicator of the use of prohibited nitrofurazone. The decision limits of analytical methods used for semicarbazide detection are below $1 \mu\text{g}\cdot\text{l}^{-1}$. In several cases in the past, semicarbazide residues were found in samples of shellfish, pork and poultry meat, fish, eggs and honey. However, during the investigation into the place of the sample origin, the application of nitrofurazone to animals was not proven, and in some cases the administration of this substance to the animals was highly unlikely, if not impossible.

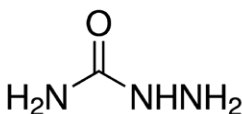


Fig. 2. Semicarbazide

Investigation into other potential semicarbazide sources in animal products showed that semicarbazide in living organisms or in the processing of food materials may be formed from natural compounds, such as arginine or creatine (Hoenicke et al. 2004; De la Calle and Anklam 2005). Semicarbazide concentrations measured in biological materials varied greatly, and it was not possible to determine the decision limit for the evaluation of results which would differentiate between semicarbazide formed naturally and its residues as the metabolite of the banned nitrofurazone. Strategies for deciding about the food material with the detected content of semicarbazide continue to be based on official investigations

in the place of sample origin, identification of any other sources of the substance and analyses of other related control samples.

Steroids

In terms of official control, the evaluation of what constitutes natural (endogenous) levels of, e.g., testosterone or estradiol poses a problem since it is extremely difficult to decide whether the presence of the steroid compound in the sample is the result of metabolic processes in the animal (in dependence on the sex and age) or of an illegal use of the same substance intended to stimulate the animal's growth. So far, this problem has been dealt by administrative determination of average natural concentration levels of hormones for each animal species in dependence of the sex and age of the animal. An example of testosterone concentration in blood plasma of young cattle considered as natural is presented in Table 2.

The detection of a higher concentration would be a reason for an official investigation on the farm, and the non-compliant result would have to be explained. These "above-the-safety limits" results are in practice usually explained by the biological diversity of animals, hormonal disorders, poorly performed castration, or stress or injury to the animals, which can also influence hormonal balance in the body.

Table 2. Maximum concentrations of testosterone in the blood plasma of young cattle, which are considered as natural

Sex	Age	Maximum concentration
Young bull	Less than 6 months	10 ng·ml ⁻¹
Young bull	6 – 18 months	30 ng·ml ⁻¹
Heifer	Less than 18 months	0.5 ng·ml ⁻¹

One way for a laboratory to distinguish endogenous hormones from synthetically prepared "natural" hormones is to measure the ratio of the carbon isotopes ¹²C and ¹³C in the residues of the hormones in samples of animal origin. The ratio of isotopes ¹²C / ¹³C in synthetically created molecules is different from that in organic molecules commonly found in nature. The natural ratio of carbon isotopes ¹²C / ¹³C in the body of the animal that has been administered synthetic molecules is changed. Measuring isotope ratios, however, is extremely difficult and requires special expensive equipment; in practice, unfortunately, these measurements are often not sufficiently conclusive.

Hormonal products illegally used to promote the growth of food-producing animals usually contain an active substance in the form of a fatty acid ester (because of the stability or solubility of the active substance). Another option for the detection of the illegal use of "natural" hormones is the direct monitoring of these original forms of active substances.

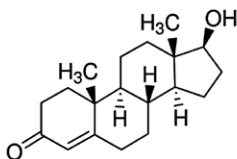


Fig. 3. Testosterone

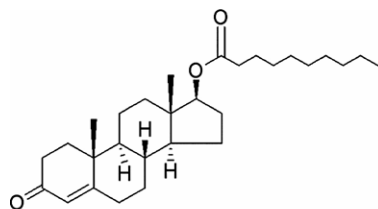


Fig. 4. Testosterone decanoate

An example of that is a product containing a “natural” hormone, testosterone, in the form of a decanoic acid ester (testosterone decanoate in Fig. 4). When administered to an animal, the ester is rapidly hydrolysed in the body to free hormone (testosterone in Fig. 3), which affects the metabolism of the animal (greater growth of the muscle mass).

In residual studies of products containing steroid esters in cattle and pigs, it was found that for a short period after the injection application it is possible to determine residues of the originally applied substance, namely ester, in the blood plasma of the animal, but in very low concentrations (tens of $\text{pg}\cdot\text{ml}^{-1}$). With the gradual development of analytical techniques and laboratory methods, however, it is possible to achieve lower and lower limits for the determination of residues in the biological material, so even this extremely difficult analysis is reliably performed.

Besides, residues of substances administered are stored in skin derivatives, i.e. in animal hair and/or bristles, also in the original form of esters. Here they persist longer and are more easily detectable. Samples of hair and/or bristles can also be easily collected from live animals, and therefore constitute a suitable matrix for monitoring the possible illegal use of anabolic growth stimulators. If an indisputable method of confirmation detects anabolic steroid ester residues in the biological material collected from a food animal, that finding is evidence of the illegal treatment of the food-producing animal, even if it was a hormone which could, at the same time, be present in the animal naturally.

Conclusions

Official inspections of hormonally active and prohibited substances in food materials of animal origin use “zero tolerance approach” for the presence of residues of these banned substances. For substances of a synthetic origin, the laboratory finding of residues can be unequivocally interpreted as proof of non-compliance, and appropriate measures can be taken to prevent the entry of contaminated materials into the food chain. In the case of substances that may be of both synthetic and natural origin, it is necessary to use other procedures to decide whether they are endogenous substances formed by natural processes (such as thiouracil in low concentrations in the urine of cattle), or whether they are residues of prohibited substances illegally administered to food animals. The compounds whose interpretation is difficult are investigated in residue studies by experts from the European and National Reference Laboratories. An example of a successful solution to the hitherto most problematic area, i.e. the interpretation of the detection of steroid hormones, is the introduction of the determination of steroid esters in animal blood plasma, hair and bristles. Any detection of steroid esters residues is clear evidence of the illegal use of a prohibited substance in food-producing animals.

References

- Commission Decision No. 2002/657/EC of 12th August 2002 implementing Council Directive No. 96/23/EC concerning the performance of analytical methods and the interpretation of results. Official Journal of the European Communities, L 221: 8-36
- Commission Regulation (EU) No. 37/2010 of 22nd December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin. Official Journal of the European Union, L 15: 1-72
- Council Directive No. 96/22/EC of 29th April 1996 concerning the prohibition on the use in stock farming of certain substances having a hormonal or thyreostatic action and of beta-agonists. Official Journal of the European Communities, L 125: 3-8
- Council Directive No. 96/23/EC of 29th April 1996 on measures to monitor certain substances and residues thereof in live animals and animal products. Official Journal of the European Communities. L 125: 10-32
- De la Calle MB, Anklam E 2005: Semicarbazide: occurrence in food products and state-of-the-art in analytical methods used for its determination. *Anal Bioanal Chem* **382**(4): 968-77

- Duffy E, Rambaud L, Le Bizec B, O'keeffe M 2009: Determination of hormonal growth promoters in bovine hair: comparison of liquid chromatography-mass spectrometry and gas chromatography-mass spectrometry methods for estradiol benzoate and nortestosterone decanoate. *Anal Chimic Acta* **637(1-2)**: 165-172
- Hoenicke K, Gatermann R, Hartig L, Mandix M, Otte S 2004: Formation of semicarbazide (SEM) in food by hypochlorite treatment: is SEM a specific marker for nitrofurazone abuse? *Food Addit Contam* **21(6)**: 526-37
- Kiebooms JA, Vanden Bussche J, Hemeryck LY, Fievez V, Vanhaecke L 2012: Intestinal microbiota contribute to the endogenous formation of thiouracil in livestock. *J Agric Food Chem* **60(32)**: 7769-76
- Pinel G, Mathieu S, Cesbron N, Maume D, De Brabander HF, Andre F, Le Bizec B 2006: Evidence that urinary excretion of thiouracil in adult bovine submitted to a cruciferous diet can give erroneous indications of the possible illegal use of thyrostats in meat production. *Food Addit Contam* **23(10)**: 974-980
- You Y, Uboh CE, Soma LR, Guan F, Li X, Liu Y, Rudy JA, Chen J, Tsang D 2011: Simultaneous separation and determination of 16 testosterone and nandrolone esters in equine plasma using ultra high performance liquid chromatography-tandem mass spectrometry for doping control. *J Chromat A* **1218**: 3982-3993