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Drug Residue Analysis in Food and Feed : State-of-the-Art for Growth Promoters

C. Van Peteghem[1] and E. Daeseleire[2]

[1] Ghent University, Department of Bioanalysis, Laboratory of Food Analysis, Harelbekestraat 72, B-9000 Gent, Belgium.
[2] Ministry of Small Enterprises, Traders and Agriculture, Agricultural Research Centre – Ghent, Department of animal product quality and transformation technology, Brusselsesteenweg 370, B-9090 Melle, Belgium.

INTRODUCTION

Veterinary drugs in general are used for both therapeutic and prophylactic purposes. A third application is the use of certain compounds or mixtures of compounds with a view to better breeding efficiency. To these belong, among others, the steroids and other substances that have similar pharmacological activity and are used to improve the efficiency of protein conversion. The better this conversion, the faster the animal grows and the earlier it can be slaughtered. These are obvious economic reasons that do not take into account possible harmful effects for the consumer of the products derived from the carcass of the slaughtered animal.
Another group of compounds are the thyrostats, which are sometimes called antihormones because they inhibit the activity of the thyroid gland.
A new class of compounds found its way into animal breeding about two decades ago. The beta-agonists, sometimes referred to as repartitioners, are licensed only for respiratory diseases in cattle and horses. They are being used, however, because they increase the ratio of lean tissue to fat.
Finally, the corticosteroids, of which dexamethasone is probably the most frequently used representative, are also being used, apparently with varying success. There is still no unanimity as to their mode of action.
An important feature of thyrostats, beta-agonists, and corticosteroids is that hey are in general orally active. This means that they can be given via the fodder or drinking water,leaving no trace of percutaneous administration, which is one of the annoying indications when orally inactive steroids are injected.
The European Union (EU) prohibits by Council Directive 96/22/EC the use in stockfarming of certain substances having a hormonal or thyrostatic action and of beta-agonists (1). In particular, the member states must prohibit.

(a) the administering to a farm or aquaculture animal, by any means whatsoever, of substances having a thyrostatic, oestrogenic, androgenic, or gestagenic action and of beta-agonists ;
(b) the holding, except under official control, of animals referred to in (a) on a farm, the placing on the market or slaughter for human consumption of farm animals that contain the substances has been established, unless proof can be given that the animals in question have been treated for the exceptional cases mentioned later ;
(c) the placing on the market for human consumption of aquaculture animals to which substances referred to in (a) have been administered and of processed products derived from such animals ;
(d) the placing on the market of meat of the animals referred to in (b) ;
(e) the processing of meat referred to in (d).

For therapeutic purposes and under the control of a responsible veterinarian, the administration to farm animals of 17ß-estradiol, testosterone, and progesterone and derivatives that readily yield the parent compound on hydrolysis after absorption at the site of application may be authorized veterinary medicinal products containing (i) allyl trenbolone, administered orally, or

beta-agonists to equidae and pets, provided they are used in accordance with the manufacturer's instructions, (ii) beta-agonists, in the form of an injection to induce tocolysis in cows when calving may be authorized . Again, these veterinary medicinal products must be administered by a veterinarian under his direct responsibility.
Certain zootechnical treatments constitute the second exception to the general prohibition of medicinal products having an estrogenic, androgenic, or gestagenic action. In particular, EU member states may allow the synchronization of oestrus and the preparation of donors and recipients for the implantation of embryos. With regard to aquaculture animals, young fish may be treated for the first three months, for the purpose of sex inversion, with veterinary medicinal products that have an androgenic action.
Till August 30, 2002 the organization of the monitoring was maid down by Council Directive 96/23/EC on measures to monitor certain substances and residues thereof in live animals and animal products (2).
The list of substances and groups of residues to be monitored is show in Table 1.

Table 1 – List of substances and residues listed in Annex I of the Council Directive 96/23/EC

GROUP A – Substances having anabolic effect and unauthorized substances

(1) Stilbenes, stilbene derivatives, and their salts and esters
(2) Anti thyroid agents
(3) Steroids
(4) Resorcylic acid lactones including zeranol
(5) Beta-agonists
(6) COMPOUNDS INLUDED IN Annex IV to Council Regulation (EEC) N° 2377/90 of 26 June 1990.

GROUP B – Veterinary drugs and contaminants

(1) Antibacterial substances, including sulphonalides, quinolones
(2) Other veterinary drugs
    (a) Anthelmintics
    (b) Anticoccidials, including nitroimidazoles
    (c) Carbamates and pyrethroids
    (d) Sedatives
    (e) Non-steroid anti-inflammatory drugs (NSAIDs)
    (f) Other pharmacologically active substances
(3) Other substances and environmental contaminants
    (a) Organochlorine compounds including PCBs
    (b) Organophosphorus compounds
    (c) Chemical elements
    (d) Mycotoxins
    (e) Dyes
    (f) Others

PERFORMANCE CRITERIA

It is quite obvious that a sound analytical methodology is required for the monitoring of the compliance with the directive 96/22/EC.
Criteria for identification and confirmation, both for qualitative and quantitative methods by means of various analytical principles were set out in Decision 93/256/EEC (3) and 93/257/EEC (4). These criteria have now been re-examined in other to take account of developments in

scientific and technical knowledge. A provisional document has been circulating for a couple of years and has been used semi-officially as the basis of future criteria in many publications. It was referred to as the SANCO document 1085/2000. By Commission Decision 2002/657/EC of 12 August 2002, this document, after long political discussions, has finally been adopted. The Decision shall apply from 1 September 2002 (5). Nevertheless transitional provisions have been included. Methods for the analysis of official satisfy the criteria set out in Decisions 93/256/EEC and 93/257/EEC may be used for up to two years after this Decision enters into force. Methods currently applied for substances listed in group B of that Annex shall comply with this Decision at the latest five years after the date of application of this Decision.
The Council Regulation 2377/90 fixes maximum residue limits or MRL'S (6). These are the maximum concentrations of residue resulting form the use of a veterinary medicinal product (expressed in mg/kg or µg/kg on a fresh weight basis which may be accepted by the Community to be legally permitted or reorganized as acceptable in or on a food. It is based on the tyre and amount of residue considered to be without any toxicological hazard, for human health as expressed by the acceptable daily intake (ADI), or on the basis of a temporary ADI that utilizes and additional safety factor.
The Regulation has for annexes, each containing a list of substances. Annex I is the list of pharmacologically active substances for which maximum residue levels have been fixed. It contains anti-infections agents, anti-parasitic agents, agents acting on the central nervous system, anti-inflammatory agents, corticoids, agents acting on the reproductive system. Annex II is the list of substances not subject to maximum residue levels consequently, these are of no importance for this publication. They comprise inorganic chemical, organic compounds, substances generally recognised as safe, substances used in homeopathie veterinary medicinal products, substances used as food additives in foodstuffs for human consumption (substances with an E number) and substances of vegetable origin.
Annex III is the list of pharmacologically active substances used in veterinary medicinal products for which provisional maximum residue limits have been fixed. They include anti-infections agents, antiparasitic agents, anti-inflammatory agents and agent acting on the reproductive system. Finally the annex IV contains the list of pharmacologically active substances for which no maximum levels can be fixed. This means in other terms that no residues of these substances may be detectable. The forbidden substances are updated to 25.11.2002. Aristolochia spp. and preparations thereof, Chloramphenicol, Chloroform, Chlorpromazine, Colchicine, Dapsone, Dimetridazole, Metronidazole, Nitrofurans (including furazolidone), Ronidazole.

GENERAL ANALYTICAL APPROACH

In the preambles of the Commission Decision 2002/657/EC it is stated that it is necessary to ensure the quality and comparability of the analytical results generated by laboratories approved for official residue controls. This should be achieved by using quality assurance systems and specifically by applying of methods validated according to common procedures and performance criteria and by ensuring tracebility to common standards or standards commonly agreed upon.
Official control laboratories must be accredited according to ISO 17025 from January 2002 onwards. Participation in an internationally recognised external quality control assessment and accreditation scheme is required for approved laboratories. Moreover, approved laboratories must prove their competence by regular and successful participation in adequate proficiency testing schemes, recognised or organised by the national or Community reference laboratories.
As a result of advances in analytical chemistry since the adoption of Directive 96/23/EC the concepts of routine methods and reference methods have been superseded by criteria approach, in which performance criteria and procedures for the validation of screening and confirmatory methods are established.

In order to ensure harmonised implementation of Directive 96/23/EC it is necessary to provide for the progressive establishment of minimum required performance limits (MRPL) of analytical methods for substances for which no permitted limit has been established and in particular for those substances whose use it not authorised, or is specifically prohibited in the Community. Therefore the Commission Decisions 93/256/EEC and 93/257/EEC have been repealed with the Decision 2002/657/EC. The latter introduces new approaches with regard to the interpretation of results. Terms like positive or negative are being replaced by non-compliant and compliant. The result of an analysis shall be considered non-compliant if the decision limit of the confirmatory method for the analyte is exceeded. If a permitted limit has been established for a substance, the decision limit is the concentration above which it can be decided with a statistical certainty of 1-á that the permitted limit has been duly exceeded. If no permitted limit has been established for a substance, the decision limit is the lowest concentration at which a method can discriminate with a statistical certainty of 1-á that the particular analyte is present. For substances listed in Group A (Table 1) of Annex I to Directive 96/23/EC, the á error shall be 1% or lower. For all other substances, the á error shall be 5% or lower.

The annex of Commission Decision contains a list of 46 definitions, all related to performance criteria, other requirements and procedures for analytical methods. The most relevant ones with regard to the theme of this lecture are the following :

Alpha (á) error means the probability that the tested sample is compliant, even though a non-compliant measurement has been obtained (false non-compliant decision).

Beta (ß) error means the probability that the tested sample is truly non-compliant, even though a compliant measurement has been obtained (false compliant decision).

Confirmatory method means methods that provide full or complementary information enabling the substance to be unequivocally identified and if necessary quantified at the level of interest.

Decision limit (CCá) means the limit at and above which it can be concluded with an error probability of á that a sample is non-compliant.

Detection capability (CCß) means the smallest content of the substance that may be detected, identified and/or quantified in a sample with an error probability of ß. In the case of substances for which no permitted limit has been established, the detection capability is the lowest concentration at which a method is able to detect truly contaminated samples with a statistical certainty of 1 – ß. In the case of substances with an established permitted limit, this means that the detection capability is the concentration at which the method is able to detect permitted limit concentrations with a statistical certainty of 1 – ß.

Minimum required performance limit (MRPL) means minimum content of an analyte in a sample, which at least has to be detected and confirmed. It is intended to harmonise the analytical performance of methods for substances for which no permitted limit has been established.

Screening method means methods that are used to detect the presence of a substance or class of substances at the level of interest. These methods have the capability for a high sample throughput and are used to sift large numbers of samples for potential non-compliant results. They are specifically designed to avoid false compliant results.

Only those analytical techniques, for which it can be demonstrated in a documented traceable manner that they are validated and have a false compliant rate < 5% (ß-error) at the level of interest shall be used for screening purposes. In the case of a suspected non-compliant result, this result shall be confirmed by a confirmatory method.

Confirmatory methods for organic residues or contaminants shall provide information on the chemical structure of the analyte. Consequently methods based only on chromatographic analysis without the use of spectrometric techniques are not suitable on their own for use as confirmatory methods. However if a single technique lacks sufficient specificity, the desired specificity shall be achieved by analytical procedures consisting of suitable combinations of clean-up, chromatographic separation(s) and spectrometric detection.

An extensive discussion on the performance of analytical methods and the interpretation of the results, by the same others of this paper will be published in the near future (7).

SAMPLE PREPARATION

Basically sample preparation includes, except for liquid samples, an homogenization with water or an appropriate aqueous solution, followed by a centrifugation step. For concentrations below the µg/kg or ppb range, concentration and thus removal of potentially interfering substances is required (clean up).
An alternative to homogenization is the enzymatic digestion of the tissue by means of proteolytic enzymes such as subtilisin.
Homogenates as well as digestates subsequently need to extracted with an organic solvent.
Deconjugation of sulphates and glucuronides is easily performed by means of Helix pomatia juice or similar preparation. Solid phase extraction (SPE) on commercially available disposable column has become the most common technique for selective sample clean up. As some cases semi-preparative HPLC automated fraction collection is performed. Matrix solid-phase dispersion (MSPD), as proposed initially by Barker in 1989 (8) has lost some of its importance in the last decade, mainly because of the lost of the required adsorptive material.
On the other hand there is an increasing popusanty of Immunoaffinity chromatography.
A more recent technique to gain selectivity, and thus a more efficient clean-up, is to use molecularly imprinted polymers (MIPs) as sorbent material in solid phase extraction. MIPs are artificial recognition systems normally based on non-covalent forces, thus trying to mimic nature (9). A template, the analyte itself or a structurally related analogue is first allowed to form bonds with functional monomers in a solvent. Subsequently the monomers are crosslinked and the template extracted from the polymer, leaving specific cavities in regard to shape and functionality. These cavities can rebind the analyte of structurally related compounds and thus a selective clean-up can be obtained. When compared to antibodies, MIPs offer several advantages in that they are stable at relatively high temperatures, in organic solvents and over a wide pH range. Moreover MIPs often can be developed within a couple of weeks, in contrast to the long development times normally required for antibody production in animals.

SCREENING METHODS

The development of radioimmunoassay (RIA) by Yalow and Berson in 1960 (10) as an extremely sensitive technique for the detection and quantification of very small quantities of endogenous compounds in biological matrices caused a real revolution in clinical chemistry. Since then, the analytical principle made its way into other disciplines, including veterinary drug residue analysis, where it is still of crucial importance for screening large sample numbers.
The term enzyme immunoassay (EIA) encompasses all immunoassays in which either the antigen or the antibody is enzyme-labeled. In a more restricted definition it is analogous to RIA. Like RIA it requires the separation of free and bound phases, and it is therefore referred to as a heterogenous immunoassay. The homogenous EIA or enzyme-mediated immunoassay technique (EMIT) does not depend on free and bound label separation to enable the degree of competition. It relies on the use of enzyme-hapten conjugates in which enzyme activity is altered when it binds with the antibody. These tests can provide very rapid results (a few minutes) but something suffer from a lack of sensitivity. Heterogenous immunoassays are 100-1000 times more sensitive.
Enzyme-linked immunosorbent assays (ELISA) encompass all solid-phase immunoassays using enzyme-labeled reagents. Three versions are of common use in food analysis : direct competitive ELISA, indirect ELISA, and double-antibody or sandwich ELISA.
Several fluorescent molecules have been used for labeling both antigens and antibodies. The fluorescent signal of the assay endpoint can be measured quickly and simply by means of readily available and relatively inexpensive equipment. Fluorescein has been the most commonly used label. Others are rhodamines, umbelliferones, and rare earth metal chelates. The main disadvantages that have limited the development of fluorescence immunoassay (FIA) are

light-scattering effects, inner filter effects, quenching, and, especially endogenous fluorophores such as bilirubin in the samples.
Luminescent labels were first introduced in clinical chemistry. Luminescence is divided into bioluminescence and chemiluminescence. Bioluminescence occurs in many living organisms, the oxidation step leading to high emission being catalyzed by a group of enzymes, the luciferases. The substrates for luciferase are kwown as luciferin. Both firefly and bacterial luciferase have been used to label antigens. Also the cofactors such as ATP and NAD that are necessary for the luciferase reaction can be used for labeling purposes.
Chemiluminescence is produced in certain chemical reactions in which the energy released during the reaction is sufficient to produce light quanta. Most chemiluminescent reactions are oxidation reactions. Luminescent compounds that have been used as labels include luminol, isoluminol, ABEI [N-(4-aminobutyl)-N-ethyl isoluminol], AHEI [N-(6-aminohexyl)-N-ethyl isoluminol] and ABEI-H [N-(4-aminobutyl)-N-ethyl isoluminol hemisuccinamide]. The light is produced by oxidation by hydrogen peroxides or other peroxides. In most cases the reaction is catalyzed by metal-containing complexes or enzymes containing heme such as microperoxidase. To a lesser extent, aryl acridinium esters have also been used for labeling purposes.
Luminescent labels can be easily incorporated into established RIA techniques, yielding fluorescence immunoassays (FIAs) with similar or improved sensitivity and short measurement times. The feasability of homogenous or nonseparation assays has been demonstrated by Kohen et al. (11,12).
A rather novel technique in food analysis is the use of biosensors. The optical surface plasmon resonance (SPR) biosensor has in recent years to be proven to be a useful technique for simple, rapid and nonlabeled determination of various biochemical analytes. Proteins, complex conjugates, toxins, allergens, drugs and pesticides can be determined using natural antibodies or synthetic receptors. When small molecules are to be measured an inhibition assay format is used, since low molecular weight substances are difficult to measure directly due to mass sensitivity limitations of current instruments. In the inhibition assay format a fixed concentration of antibody is added to the sample and if analyte is present binding will occur and some of the antibody sites will be blocked. An interchangeable and reusable chip (gold coated) with the analyte covalently immobilised on the sensor surface is used to determine free antibody concentration in solutions versus a calibration curve. Analyte concentration is consequently measured in terms of inhibition of antibody binding to the surface.

CONFIRMATORY METHODS

Whereas immunochemical methods play a predominant role in screening for the presence of forbidden growth promoters in food products, the various existing chromatographic techniques are the most intensively used methods for confirmatory purposes. The use of thin-layer chromatography is very limited nowadays due to the availability of mass spectrometry techniques in the routine laboratories. The last few years, liquid chromatography coupled to mass spectrometry has become the method of choice for the confirmation and quantification of residues of growth promoters, especially for corticosteroids. Non-volatile and thermolabile compounds can be analysed directly without derivatization.
Specificities in the analysis of veterinary drug residues, usually occurring at sub-µg/kg levels, can be achieved by either a lengthy sample preparation and cleanup to separate the analyte from potential interferences or by using a less selective workup but a more specific detector. As a direct consequence of the latter approach, increased MS specificity is pursued by the use of tandem mass spectrometry, which becomes more and more available to routine laboratories. Because there are no maximum residue limits for most growth promoters, emphasis is laid on specificity rather then on accuracy and precision. The use of isotope-labeled internal standards is limited because of the unavailability of most deuterated homologs of the analyte.
The target matrices for the detection of residues of growth promoters in edible products of animal origin are mainly muscle tissue, liver, kidney and fat. Non-edible matrices (urine, faeces,

retina, hair, …) are also frequently used to perform the control on the abuse of growth promoters.
All these materials have a highly complex composition that is subject to variations, depending on animal species, tissue type, and the nutritional regime of the animal. In most cases the parent compound – the molecule that has been administered – is considered the target molecule and therefore traced. Little is known about the chemical form in which residues are deposited in the animal tissues. Well-known exceptions are 19-nortestosterone, stanozolol and chlorotestosterone acetate.

SUMMARY

A review of the most recent methodology is about to be published by the same authors of this contribution (7).

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