Rapid Methods for detection of Veterinary Drug residues in Meat

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Abstract

The use of substances having hormonal or thyreostatic action as well as b-agonists is banned in many countries. However, sometimes forbidden drugs may be added to feeds for illegal administration to farm animals for promoting increased muscle development or increased water retention and thus obtain an economical benefit. The result is a fraudulent overweight of meat but, what is worse, residues of these substances may remain in meat and may pose a real threat to the consumer either through exposure to the residues, transfer of antibiotic resistance or allergy risk. This has exerted a great concern among the meat consumers. The control of the absence of these forbidden substances in animal foods and feeds is regulated in the European Union by Directive 96/23/EC on measures to monitor certain substances and residues in live animals and animal products. Analytical methodology, including criteria for identification and confirmation, for the monitoring of compliance was also given in Decisions 93/256/EEC and 93/257/EEC. More recently, Decision 2002/657/EC provided rules for the analytical methods to be used in testing of official samples. New substances with anabolic properties are being detected year by year increasing the list of forbidden compounds to be tested. Furthermore, the extended practice consisting in the use of "cocktails" (mixtures of low amounts of several substances that exert a synergistic effect) to have a similar growth promotion, reduces the margin for an effective analytical detection. Thus, the evolution of the "black market" is making really difficult to have an effective analytical control of the residues of these substances in foods of animal origin. Control laboratories must face an increasing demand of analysis like the growing number of residues to be analysed in different types of samples, the strict guidelines for analytical methodologies according to the latest Directives, the increased costs of such new methodologies, the variety of residues to search per sample and the need to invest on powerful new instruments for identification and confirmatory purposes. Rapid and versatile screening methodologies make its control easier and reduce the number of non-compliant samples to be confirmed through tedious and costly confirmatory analytical methodologies. For instance, the multiresidue analysis can be performed better by using fast LC methods. Thus, the availability of new screening methodologies and the improvement of the existing ones will contribute to a better safety assurance of meat and other foods of animal origin.

Keywords: Drug Residue, Meat, Residues in food, Hormone.

Introduction

Veterinary drugs are generally used in farm animals for therapeutic and prophylactic purposes and they include a large number of different types of compounds which can be administered in the feed or in the drinking water. In some cases, the residues may proceed from contaminated animal feedstuffs (McEvoy, 2002). But many of these substances may exert other effects when administered to animals for other purposes like growth promotion. A primary effect is the increase in the protein deposition, usually linked to fat utilisation that decreases the fat content in the carcass and increases meat leanness (Lone, 1997).

This allows a better efficiency in the feed conversion rate and a leaner meat. In addition, some practices consists in the use of "cocktails" (mixtures of low amounts of several substances that exert a synergistic effect) to have a similar growth promotion and reduce the margin for an effective analytical detection. The residues of these substances or its metabolites in meat and other foods of animal origin may cause adverse effects on consumers' health as described below. The presence of residues and its associated harmful health effects on humans makes the control of veterinary drug residue an important measure in ensuring consumer protection. The many countries were strictly regulated the use of veterinary

drugs in food animal species. Some of these drugs can be permitted only in specific circumstances (therapeutic purposes) but under strict control and administration by a veterinarian (Van Peteguem & Daeselaire, 2004). This review is summarising the main effects of veterinary drugs on human health as well as its effects on meat quality and revising the current methods for rapid detection.

Concerns of the presence of veterinary drugs residues in foods of animal origin.

The residues of veterinary drugs or its metabolites in meat and other foods of animal origin may cause adverse toxic effects on consumers' health. In fact, the European Food Safety Authority has recently issued an opinion on the effect of hormones residues in meat and reflected that epidemiological data provided evidence for an association between some forms of hormone-dependent cancers and red meat consumption (EFSA, 2007). Furthermore, recent intoxications by consumption of lamb and bovine meat containing residues of clenbuterol resulted in 50 intoxicated people with symptoms described as gross tremors of the extremities, tachycardia, nausea, headaches and dizziness (Barbosa *et al.,* 2005). Other important effects mainly due to the presence of residual antibiotics consist in allergic reactions or the selection of a resistant bacteria that could be transferred to humans through the food chain (Butaye *et al.,* 2001). In addition, the consumption of trace levels of antimicrobial residues in foods from animal origin may have consequences on the indigenous human intestinal microflora which constitutes an essential component of human physiology. This flora acts as a barrier against colonization of the gastrointestinal tract by pathogenic bacteria and has an important role for food digestion. So, the ingestion of trace levels of antimicrobials in foods must take into account potentially harmful effects on the human gut flora (Cerniglia & Kotarski, 1999). In view of all these circumstances, foods of animal origin must be monitored for the presence of veterinary drug residues.

Effects of veterinary drugs on meat quality

Most of the drugs used as growth promoters may exert more or less important effects on meat quality, usually towards poorer eating quality. The meat tends to be tougher because there is an increase in connective tissue production and also a higher rate of collagen cross-linking as well as an increase in the insoluble fraction of the intramuscular collagen (Miller *et al.,* 1990). Another factor, which is important from the point of view of meat tenderness, consists in the inhibitory action that these substances may exert against muscle proteases, enzymes responsible for protein breakdown in postmortem meat. For instance, myofibrillar protein fragmentation has been reported to be decreased in agonists-treated animals probably due to calpains inhibition by b-agonists (Fiems *et al.,* 1990). On the contrary, other endogenous enzymes like the hormone-sensitive lipase appear to be activated, increasing the lipolysis rate and the breakdown of triacylglycerols (Brockman & Laarveld, 1986). The final result of the altered lipid metabolism is a sensible reduction in the amount of fat although this reduction in fat is associated to a lower sensory quality (poor juiciness and flavour). As mentioned above, some toxic effects in humans have been reported after consumption of lamb and bovine meat containing residues of clenbuterol (Barbosa *et al.,* 2005). In other cases, like thiouracyls, the result is a substantial retention of water which is rapidly lost during cooking, giving a meat with lower juiciness.

Control of veterinary drugs residues in meat

The use of substances having hormonal or thyreostatic action as well as b-agonists is controlled by official inspection and analytical services following Commission Directive 96/23/EC on measures to monitor certain substances and residues in live animals and animal products. This Directive contributed to a sensible reduction in the number of growth promoting reported cases. However, laboratories in charge of residues control usually face a large number of samples with great varieties of residues to search in short periods of time making it rather difficult. The availability of simple and useful screening techniques is really necessary for an effective control.

Main veterinary drugs and substances with anabolic effect are listed in Tables 1 and 2.

Table-1. Lists of substances having anabolic effect belonging to group A according to Council Directive 96/23/EC (Group A: substances having anabolic effect)

Substance	**Main representative**
1.Stilbenes	(Diethylestilbestrol)
2. Antithyroid agents	(Thiouracyls)
3. Steroids	
Androgens	(Trenboloneacetate)
Gestagens	(Melengestrolacetate)
Estrogens	(17-b estradiol)
4. Resorcycilic acid lactones	(Zeranol)
5. b-agonists	(Clenbuterol)
6. Other compounds	(Nitrofurans)

Groups of substances may be differentiated: those unauthorized substances having anabolic effect belonging to group A and those veterinary drugs of group B, some of them having established maximum residue limits (MRL). Analytical methodology, including criteria for identification and confirmation, for the monitoring of compliance was also given in the Commission Decisions 93/256/EEC and 93/257/EEC. The Commission Decision 2002/657/EC, which is in force since 1 September 2002, implemented Council

Directive 96/23/EC by providing rules for the analytical methods to be used in testing of official samples and specific common criteria for the interpretation of analytical results of official control laboratories for such samples. For instance, substances in group A (Table 1) would require 4 identification points while those in group B (Table 2) only require a minimum of 3. The guidelines given in the new Directive imply new concepts like the decision limit (CCa) which means the limit at and above which it can be concluded with an error probability of that sample is non-compliant, and the detection capability (CCb) that means the smallest content of the substance that may be detected, identified and/or quantified in a sample with an error probability of b. Recently, the EC Quality of Life Programme supported European collaborative projects in the area of antimicrobials and hormone residues analysis (Boenke, 2002). These projects consisted in the development and validation of screening and confirmatory analytical methods and sensors for a cost-effective and time efficient control of synthetic glucocorticoids, nitrofurans, coccidiostatics, b-lactam residues and androgen residues in live and postmortem animals.

Analytical methods for rapid screening

These controls are based on the screening of a large number of samples. Compliant samples are accepted while those suspected non-compliant samples have to be confirmed through other confirmatory methods that are described later. High throughput methods with low cost and able to detect an analyte or class of analytes at the level of interest are thus required (Van Peteguem *et al.,* 2001). Main requirements for a screening method are summarised in Table 3. In case the residue has a MRL, the screening method must be capable to detect the residue below this limit. The screening methods must also avoid or reduce to a minimum the number of false negative results because they will be considered as compliant samples and will not be further analysed. According to the Commission Decision 2002/657/EC, the methods must be validated and have a detection capability (CCb) with an error probability (b) lower than 5%. On the other hand, it must not give an excessive number of false non-compliant samples, which will be later confirmed as compliant, due to the excessive cost and time involved.

Table-2. Lists of veterinary drugs belonging to group B according to Council Directive 96/23/EC

Group B: veterinary drug
1. Antibacterial substances
Sulphonamides and quinolone
2. Other veterinary drugs
(a) Antihelmintics
(b) Anticoccidials, including nitroimidazoles
(c) Carbamates and pyrethroids
(d) Sedatives
(e) Non-steroideal anti-inflammatory drugs
(f) Other pharmacologically active substances (dexamethasone)

Table 3. Main requirements for a screening method

Easy to use and handle
Low set-up and running costs
High throughput
Possibility of automatisation
Reduced time to obtain the result
Good sensitivity and specificity
Detection capability (CCb) with an error probability (b)<5%

Preparation procedures and handling of samples, especially solid and heterogeneous foods like meat, kidney or liver, are very important in order to ensure better sensitivity of the screening tests (McCracken *et al.,* 2000). Samples are usually cut, blended, homogenised and liquid extracted. Next step is usually based on solid-phase extraction for sample clean-up and concentration. The type of cartridge is chosen depending on the analyte for the appropriate elimination of potential interferents. In other cases, the residues may be bound or conjugated and need further cleavage before the analysis is performed. The main types of screening techniques are summarised below.

1. Immunological techniques

The immunological methods are based on the interaction antigen–antibody which is very specific for a particular residue. The most usual technique consists in the ELISA and the detection system is usually based on enzyme-labeled reagents. There are different formats for antigen quantification like the double antibody or sandwich ELISA tests and direct competitive ELISA tests. Radioimmunoassay (RIA) is based on the measurement of the radioactivity of the immunological complex (Samarajeewa *et al.,* 1991). Other assays have enhanced detectability by using of a luminescence detector if using a chemiluminescent compound or a fluorimeter in the case of using a fluorescent compound (Roda *et al.,* 2003). Today, there are many different types of ELISA kits commercially available for a large number of substances within each group listed in Table 1 like B-agonists, corticoids, steroids, stilbenes, resorcylic acid lactones and several antibiotics. ELISA kits are available for a specific residue (i.e., sulphametazine) or a group of related compounds (i.e., sulphonamides). In some cases, the possibility of cross reactions must be taken into account. These kits allow the analysis of a large number of samples per kit, do not require sophisticated instrumentation, the results are available in a few hours and are quite specific and sensitive. ELISA kits have shown good performance for the analysis of antibiotic residues in meat like tylosin and tetracyclin,

chloramphenicol (Gaudin *et al.,* 2003), nitroimidazoles (Huet *et al.,* 2005) and sulphonamides and also for sedatives. In general, ELISA tests require some manual operation (pipetting and discarding of liquids) that is progressively replaced by automated systems. Dipstick constitutes another system which basically consists of a membrane strip with the receptor ligands. The sample with the antibiotics is applied and left to interact and, after dipping into two different solutions, the developed colour can be quantified either by comparison to a standardised colour scale or by measuring spectrophotometrically (Link *et al.,* 2007).

2. Biosensors

Different types of biosensors have been developed in recent years as an alternative approach to screen veterinary drugs in meat. In general, these sensors usually contain an antibody as a recognition element that interacts with the analyte. The resulting biochemical signal is measured optically or converted into an electronic signal that is further processed in appropriate equipments (White, 2004). Biosensors can be able to detect simultaneously multiple veterinary drugs residues in a sample at a time and some authors have reported no need for sample clean-up (Elliott *et al.,* 1998). In general, these sensors are valid for control laboratories because they can detect multiple residues in one sample and can thus allow the analysis of a large number of residues and samples (Franek & Hruska, 2005). There are differences in the design of the biosensors depending on how the interaction between the recognition molecule and the analyte is performed and the type of detection. In some sensors, the biomolecular interaction analysis is based on surface plasmon resonance (SPR). This type of optical biosensors measures variations in the refractive index of the solution close to the sensor when there are changes in the mass concentration of molecules in that solution (Gillis *et al.,* 2002). The biosensor allows real time monitoring on the interaction analyte- receptor at the sensor chip surface. Some recent applications of this SPR sensor for the detection of antibiotics, b-agonists and antiparasitic drugs in foodstuffs have been recently reviewed (Haughey & Baxter, 2006). Other biosensors are based on the use of biochip arrays, specific for a certain number of residues, which also allow a real time monitoring of the interaction of the analyte with the recognition molecule. These sensors are affected by several factors like the surface ligand density in the array, the concentration of active antibody and the flow rate.

Some residues like chloramphenicol, clenbuterol and tylosin have been reported to be detected with a small molecule microarray. The drug molecules were immobilised on glass slides and after incubation with corresponding antibodies and samples, the binding was detected using cy5 labeled secondary antibody (Zuo & Ye, 2006). Other types of biosensors are designed against specific classes of antibiotics and, in fact, have shown good detection of tetracycline, streptogramin and macrolide antibiotics in milk and serum. The design of these sensors is compatible with the ELISA-type format and the loss of colour gives a readout that is proportional to the antibiotic concentration (Weber *et al*., 2004).

3. High performance thin-layer chromatography (HPTLC)

HPTLC allows the qualitative and quantitative detection of multi-residues in meat but its use has rapidly decreased due to the expansion of other techniques like HPLC. Reported uses of HPTLC applied to meat include the detection of residues like clenbuterol and other agonists (Degroodt *et al.,* 1991), nitroimidazol (Gaugain & Abjean, 1996) and sulphonamides and thyreostatic drugs. The plates are sprayed with an appropriate chromogenic reagent or viewed under UV light for visualisation of compounds. Detection by fluorescence is also applied. Quantitation is achieved by measuring the relative intensity of the spot vs that of the internal standard by scanning densitometry. Modern HPTLC has been automatised at a high level.

4. High performance liquid chromatography (HPLC)

HPLC expanded its use in the 1990s due to the availability of columns, good performance, variety of available detectors and possibility of automation. Recent developments like the ultraperformance liquid chromatography systems or types of columns with improved packagings in terms of smaller size, geometry and inertness are also very valuable. HPLC is a separative technique where the choice of the detection system is very important for selectivity and sensitivity. Some analytes require chemical modifications to render chromophore, fluorescent or UV-absorbing compounds (Bergweff & Schloesser, 2003). Typical detections of multi-residues in meat samples are relatively simple and rapid, requiring a preliminary clean-up through solid-phase extraction followed by filtration before injection into a reverse-phase HPLC with diode array detection. This procedure has been applied to meat for detection of antibiotics like quinolones (Kirbi *et al.,* 2005), sulphonamides, b-lactams and macrolides and tetracyclines, veterinary drugs (Reig *et al.,* 2005a), anabolic steroids and corticosteroids like dexamethasone (Reig *et al.,* 2006). In some cases, the compounds can be further identified through diode array or fluorescence detection. Ten quinolone residues (ciprofloxacin, danofloxacin, difloxacin, enrofloxacin, flumequine, marbofloxacin, nalidixic acid, norfloxacin, oxolinic acid, sarafloxacin) in meat have been screened and confirmed with HPLC and fluorescence detection.

Latest developments in liquid chromatography include the development of new smaller columns with packagings of reduced size as well as the use of higher pressures (i.e.,UPLC systems).

This allows considerable reductions in elution times increasing substantially the number of samples per day. Liquid chromatography techniques are getting expanded use in control laboratories due to the possibility of automation (injection, elution, washing of column, detection), computer-controlled use and data manipulation and the relatively short time needed per sample. Recent developments in new systems and columns that allow high speed and reduced analysis time are being already commercialized and will contribute its expanded use. It must be taken into account that sample extraction and clean-up are the rate-determining steps in drug analyses. The use of on-line solid-phase extraction (SPE) with chromatography coupled to mass spectrometry or other spectroscopic techniques are getting widely used in recent years. They allow for screening with simultaneous confirmation for those suspicious samples. Even though the cost of the instrument is high, when a large number of samples are analysed the costs are reduced and are more competitive. For instance, nineteen veterinary drugs have been reported to be screened in meat by using an extraction cartridge packed with hydrophilic–hydrophobic polymer sorbent followed by fast LC using a short C18 column and direct analysis by LC/MS/MS (Tang, Ho, & Lai, 2006). Other analytical strategies consist in the use of liquid chromatography–tandem mass spectrometry (LC–MS–MS) for the analysis of different groups of substances in meat like corticosteroids (Antignac, *et al.,* 2004), b-agonists, chloramphenicol and penicillins, sulphonamides or ionophore coccidiostats in broiler meat (Rokka & Peltonen, 2006).

Confirmatory analytical methodologies

The next step after initial screening consists in the unambiguous identification and confirmation of the veterinary drug residues in foods of animal origin. The full procedure and the methodologies for confirmatory analysis are costly in time, equipments and chemicals. In addition, they require trained personnel with high expertise. Different analytical techniques are available for such purpose. When the target analyte is clearly identified and quantified above the decision limit for a forbidden substance (i.e., substances of group A) or exceeding the maximum residue limit (MRL) in the case of substances having a MRL, the sample is considered as non-compliant (unfit for human consumption). Identification is easier for a limited number of target analytes and matrices of constant composition. Some examples of the available confirmatory methodologies are as follows: The use of HPLC-electrospray ionisation (ESI) tandem mass spectrometry (Thevis *et al.,* 2003) or liquid chromatography- mass spectrometry with atmospheric pressure chemical ionisation (APCI). ESI ionisation technique facilitates the analysis of small to relatively large and hydrophobic to hydrophilic molecules and is thus very adequate for the analysis of veterinary drug residues even though it is more sensible to matrix effects than APCI ionisation (Dams *et al.,* 2003). ESI and APCI interfaces are the sources of choice to promote the ionization of antibiotics and both complement each other well with regards to polarity and molecular mass of analytes (Gentili *et al.,* 2005). The assay of chloramphenicol in meat has been successfully identified and quantitated by liquid chromatography/electrospray ionisation tandem mass spectrometry (ESI- LC/MS/MS) in the negative ion mode coupled to ion trap analyzer. The same technique with positive ESI has been successfully applied to the analysis of four nitrofuran compounds (furazolidone, furaltadone, nitrofurantoin, and nitrofurazone) in meat.

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