Methods for Screening Veterinary Drug Residues in Animal Products: A review

Ishraga G. Ibrahim1,*, Amna E. Khalafalla2, Yarsan3, E., Altintas3, L., Tumer4, I.

1Veterinary Research Institute, Soba P.O. Box 8067 Alamarat, Khartoum, Sudan
2Faculty of Veterinary Medicine, University of Khartoum, Khartoum North, Sudan
3Faculty of Veterinary Medicine, University of Ankara, Ankara, Turkey
4Pendik Veterinary Control and Research Institute, Istanbul, Turkey

*Corresponding author: ishragaiibrahim0@gmail.com

Summary

With strict regulations and government controls worldwide, the monitoring of animal drug residues is becoming increasingly complex and demanding. The risk of failing to comply with legally permitted maximum limits can affect consumer health. For this reason, accurate results of residue levels in animal products are needed. International food safety authorities outline the concept of reference method criteria in which minimum requirements of analytical methods used in the analysis of veterinary drug residues, being screening or confirmatory, were described. In this document, the most important analytical methods used in the screening of antimicrobial and some other veterinary drug residues are described.

Introduction

Antimicrobials, anti-inflammatory drugs and hormones have been commonly used in farming practices to treat diseases and to promote growth in animals. The majority of animal products remain safe but the illegal use of these drugs can leave harmful residues in animal products that enter the food chain (McEvoy, 2002). The main veterinary drugs and substances with anabolic effect stated by European Commission are listed in Table1. Only a few substances are authorized for therapeutic purposes and under the control of a responsible veterinarian. In all countries, the presence of these substances in food is controlled by official inspection and analytical services following national and international regulations measure to monitor certain substances and residues in live animal and animal products (Toldra and Reig, 2006).

The Codex Alimentarius and Joint FAO/WHO programme have developed the standards concerning the residues in foods since 1985. These standards are based upon scientific assessments performed by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) which determines the acceptable daily intakes (ADIs) and gives recommendations for maximum residue limits (MRLs) (Ellis, 2008; Navartilova, 2008). The guidelines for reference methods were established indicating which analysis techniques and methods are considered suitable for the detection of specific residues. The minimum requirements of analytical methods used in the analysis of veterinary drug residues, being screening or confirmatory methods, were described.
Table 1: Lists of veterinary drugs and substances with anabolic effect, with some examples (Council Directive 96/23/EC)

<table>
<thead>
<tr>
<th>Group B: veterinary drugs</th>
<th>Group A: substances having anabolic effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibacterial substances Sulfonamides and quinolones</td>
<td>Stilbenes, stilbene derivatives, and their salts and esters (diethylstilbestrol)</td>
</tr>
<tr>
<td>Other veterinary drugs</td>
<td>Antithyroid agents (thiouracils)</td>
</tr>
<tr>
<td>Anthelmintics</td>
<td>Steroids</td>
</tr>
<tr>
<td>Anticoccidials, including nitroimidazoles</td>
<td>Androgen</td>
</tr>
<tr>
<td>Carbamates and pyrethroids</td>
<td>Gestagens (melengestrolacetate)</td>
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<tr>
<td>Sedatives</td>
<td>Estrogens (17-b estradiol)</td>
</tr>
<tr>
<td>Non-steroidal anti-inflammatory drugs</td>
<td>Resorcycilic acid lactones (zeranol)</td>
</tr>
<tr>
<td>Other pharmacologically active substances (dexamethasone)</td>
<td>Beta-agonists (clenbuterol)</td>
</tr>
<tr>
<td>Other substances and environmental contaminants</td>
<td>Other compounds (nitrofurans)</td>
</tr>
<tr>
<td>Organochlorine compounds including PCBs</td>
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<tr>
<td>A. Organophosphorus compounds</td>
<td></td>
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<tr>
<td>B. Chemical elements</td>
<td></td>
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<tr>
<td>C. Mycotoxins</td>
<td></td>
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<tr>
<td>D. Dyes</td>
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<tr>
<td>E. Others</td>
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</table>

This includes criteria for, amongst others, trueness, repeatability and within-laboratory reproducibility. General requirement for analytical methods, the concepts of the minimum required performance limit (MRPL), the concept of the decision limit ($CC_\alpha$) and detection capability ($CC_\beta$), and regulations on the confirmation of an analytical result were introduced (Boenke, 2002; Zeleny et al., 2006; Ellis, 2008).

To protect consumer health and meet worldwide regulations, accurate results of the residue levels are required for all animal products. Harmful animal drug residues in the food chain pose a threat to consumers. Whether residues arrive in the food chain by illegal use of prohibited drugs, or inappropriately administered legal drugs, the fact remains that human ingestion of animal drug residues has been linked to increased drug-resistance of bacteria that cause human diseases; also consist in allergic reactions and interference with indigenous human intestinal microflora (Butaye et al., 2001).

In addition, data had provided evidences for an association of some forms of hormone-dependent cancer and red meat consumption (Shankar et al., 2010). In view of all these circumstances, foods of animal origin must be monitored for the presence of veterinary drug residues. Therefore, this review is summarizing the most common screening methods used for antimicrobial and a few other therapeutic drugs detection.

**Methods of analysis**

Methods are generally divided into screening and confirmatory methods. For antimicrobial residue control, some laboratories add a third intermediary level based on post-screening test which gives structural or biological activity information about the residue (Stolker and Brinkman, 2005).

Screening method can be defined as 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
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a high sample throughput and are used for large numbers of samples for potential non-compliant results. They are specifically designed to avoid false compliant results (Sanders, 2007). Screening methods are usually inexpensive, easily to use and handle, rapid, suitable for high-throughput analysis, and have good sensitivity, specificity and detection capability (ccβ) with a probability of error of p < 5%. They usually do not provide unequivocal identification and usually do not result in exact quantitative results (Berendsen, 2013).

Confirmatory methods are methods that provide full or complementary information enabling the substance to be unequivocally identified and if necessary quantified at the level of interest. They must be instrumental spectrometric techniques and therefore are more expensive and time-consuming, but are supposed to be highly selective in order to provide unequivocal identification (Berendsen, 2013).

Screening methods
Historically, screening of antimicrobial activity in animal production started in the 60’s with problem of inhibitory activity detected by dairy industry during milk processing (yoghurt or cheese) (Mitchell et al., 1998).

Several bio-based tests have been reported for the screening of antibiotic substances in different matrices. Bio-based screening methods used for the detection of antibiotics in products of animal origin have been reviewed (Popelka et al., 2004; Pikkemaat, 2009; Cháfer-Pericás et al., 2010; Meng and Xi, 2011). The most commonly applied bio-based screening techniques for antibiotics are microbiological inhibition assays, immunoassays and reporter gene assays (Bovee and Pikkemaat, 2009).

Microbiological inhibition assays
Microbiological inhibition assays are based on a reaction between a bacteria and the antibiotic present in the sample. Different inhibitory tests were developed to screen different animal products (Popelka et al., 2004).

The tube and plate tests are the most common formats for this type of screening assays (Berendsen, 2013).

The tube test consists of a growth medium inoculated with a bacterium, supplemented with a pH or redox indicator. If no specific antibiotics are present, the bacteria start to grow and produce acid, which will cause a detectable colour change. If antibiotics are present that inhibit bacterial growth, no colour change will occur (Hoff et al., 2011).

The plate test consists of a layer of Nutrient Agar inoculated with bacteria and the samples are brought onto the surface. If no specific antibiotics are present, the bacteria start to grow throughout the plate. If a specific antibiotic is present, no bacterial growth will occur around the sample, which can be observed from the bacteria free inhibition zone (Berendsen, 2013).

Some of these tests are available as kits with a high sample throughput. They need limited laboratory capacity to ensure reproducible conditions of application. Few of them need a more experienced laboratory that is able to produce a medium and a bacterial suspension. They are widely used to perform residue control and self-control by industry (Pikkemaat, 2009).

An important advantage, compared to immunoassays and instrumental methods, is that microbiological tests can detect any antibiotic compound that shows antibacterial activity (Picó and Barceló, 2008). Moreover, they have the potential to cover the entire antibiotic spectrum within one test (Pikkemaat, 2009). The most important drawbacks of the microbiological tests are their lack of selectivity, especially the tube test, relatively high detection limits and the long incubation time. As a result, microbiological inhibition assays are not suitable for detection of banned antibiotic compounds like chloramphenicol (Berendsen, 2013).
Immuno- and Receptor-assay
Some screening tests are also developed using antibodies; they are more or less selective of a compound or the structure of an antimicrobial family (Gustavsson and Sternesjo, 2004). Different types of signals are used to report the binding competition between free drugs and control. In regards to the maximum residue limit (MRL), these tests are valid for a list of compounds (Sanders, 2007).

Immunoassay
Immunoassays are based upon a binding reaction between a compound and an antibody. The most commonly applied immunoassay in antibiotic analysis is the enzyme-linked immuno sorbent assay (ELISA) (Meng and Xi, 2011). There are different test formats for antigen quantification like the double antibody or sandwich ELISA tests and direct competitive ELISA tests (Shankar et al., 2010), but all tests are based upon the same principle. The sample that is screened for antibiotic content is incubated with antibodies, under the production of an analyte antibody binding complex. The degree of binding, which is related to the level of antibiotics present in the sample, is determined (Cháfer-Pericás et al., 2010; Davis and Higson, 2010; Meng and Xi, 2011). The assays based on immune or receptor affinity are designed to give a positive response in line with the level of interest in the matrix tested. They are used daily for self-control in milk industry to screen residue of several antimicrobial residues such as ß-lactams, sulphonamides and tetracyclines (Navratilova, 2008). Effective kits are developed for some aminoglycosides and are used to screen these compounds in kidney or urine by some countries. Some laboratories use them also as post screening test after a positive inhibitory test to give information about the chemical family of the residue. ELISA tests for analysis of ß-lactams, chloramphenicol, tylosin, tetracyclines, nitromidazoles, sulphonamides and also sedatives were reported (Sai et al., 2010; Fernández et al., 2010 Babington et al., 2012).

An important advantage of immunoassays is that they are able to detect the presence of antibiotics at very low levels, which makes them even useful for screening of banned substances. The kits allow the analysis of a large number of samples per kit, don’t require sophisticated instrumentation, the results are available in a few hours and are quite specific and sensitive. However, the main challenge of immunoassays is the production and supply of antibodies that should be selective about the targetted antibiotic compound or group (Shankar et al., 2010).

Charm test
The Charm I and Charm II tests are microbial receptors or antibody assays for the detection of antimicrobial drugs (Toldra and Reig, 2006). Separate tests for the antibiotic drug families’ ß-lactams, macrolides, sulphonamides, tetracyclines and chloramphenicol are available. The tetracyclines and chloramphenicol assays are antibody assays and the remaining are microbial receptor assays. A binding reagent (a microbial cell with specific receptors for the antibiotic in question or, for tetracyclines and chloramphenicol, an antibody specific to the antibiotic) is added to the sample (McGrane, 2000). The test employs C14 or H3 radio labelled antibacterial to compete for the binding sites. If antibiotic is present it will bind to the receptors on the microbial cell (or antibody) and prevent the binding of radio labelled antibiotic, which is added subsequently. Therefore, the more radio labelled antibiotic detected the lower the concentration of antibiotic in the sample (Navratilova, 2008).

Detection of sulphonamides, streptomycin and erythromycin antimicrobial residues in suspected meat samples using the Charm II receptor assay, was compared with the results obtained using thin layer chromatography or liquid chromatography; the comparison showed that, Charm II test is an acceptable alternative, with a lower limit of detection. Also results showed the incidence of false positives to be higher using the Charm II test. This may be due to the lower sensitivity of
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the Charm II assay. The estimated detection sensitivities of the Charm II test assay, were 10 ppb for penicillin G (which has an MRL of 4 ppb in milk), 200 ppb for gentamicin (which has an MRL of 100 ppb in milk), and 300 ppb for tetracycline (which has an MRL of 100 ppb in milk) (McGrane, 2000). The most disadvantage of charm tests are the cost and the need for waste disposal (Toldra and Reig, 2006).

Biosensors

Different types of biosensors have been developed to screen veterinary drugs (Berendsen, 2013). They utilize biological molecules, such as enzymes, or antibodies, capable of recognizing specific targeted analytes. The molecules are coupled to a transducer that responds to the reaction between the analyte and the bound biological molecule. The resulting biochemical signal is measured optically or converted into electronic signal that is further processed in appropriate equipment. Biosensors are able to detect simultaneously multiple veterinary drugs residues in samples at a time. Some authors have reported that there is no need for sample clean up (Shankar et al., 2010).

There are differences in the design of the biosensors depending on how the interaction between the recognition molecule and the analysis performed and the type of detection. In some sensors, the biomolecular interaction analysis based on the surface plasmon resonance (SPR). This type of optical biosensor 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 enzymic biosensors use specific enzyme for the capture and catalytic generation of the product. ß-lactam antibiotics were detected using a biosensor containing penicillinase immobilized in the surface, either membrane or porous glass, that produces pencilloic acid and thus reduction in pH and either decrease in the fluorescence intensity of the dye or an increase in the electrical conductivity (Patel, 2002).

The reporter gene assays biosensors consist of a genetically modified bacterium, containing an inducible promoter, responsive to a particular antibiotic, coupled to a reporter gene or operon (Bovee and Pikkemaat, 2009). Based on the presence or absence of responsive antibiotics, the reporter gene induces a fluorescent signal or the operon affects the transcription to produce or inhibit a signaling process (Berendsen, 2013). An example is that, which is based on specific, tetracycline-controlled expression of bacterial luciferase genes that code for enzymes responsible for light emission. When tetracycline enters the genetically engineered cell, it releases a repressor protein from the luciferase operon allowing synthesis of the luciferase reporter genes, resulting in a luminescence signal (Bovee and Pikkemaat, 2009; Virolainen et al., 2008). A comparable assay has been reported for the screening of macrolide antibiotics (Möhrle et al., 2007).

The tetracycline cell-biosensor was found to be more sensitive and faster compared with the microbial inhibition test (Pikkemaat et al., 2010).

The important advantages of biosensor are easy to use, availability of results in short time, analysis of multiples residues in one shot, full automatisation, and has a high productivity (high throughput technique of up to 120 samples per hour). Disadvantages of the technique include high operative costs and the analysis is restricted to available chips (Toldra and Reig, 2006).

Thin layer chromatography

In the early 70s, thin layer chromatography (TLC) was the method of choice for the qualitative detection of banned substances (thyreostats and certain anabolics at that time). The reasons therefore were the specificity, the simplicity of development in two dimensions and the possibility
of reaching low limits of detection for an acceptable budget (McGrane 2000). TLC methods are also employed for antibiotic screening, as they can provide high sample throughput and relatively high detection sensitivities. Silica and cellulose acetate plates are commonly employed and a variety of solvents and mixtures of solvents used for analyte separation (Shankar et al., 2010). Various detection techniques have been employed. Detection may require the addition of a visualization agent, such as Fast Violet. Direct UV scanning of the plate is frequently employed. Derivatisation of the analytes on the plate by fluorescent dyes provides a rapid, inexpensive method of detection (Toldra and Reig, 2006).

TLC-bioautography is a popular method for the analysis of antibiotic residues. Following separation by TLC, the analytes are detected by a microbial inhibition assay. The chromatography plate may be incubated against an agar plate seeded with sensitive micro-organisms (Choma et al., 2002).

TLC has been applied to a large range of antibiotic residues including sulphonamides, aminoglycosides, macrolides, ß-lactams (McGrane, 2000), clenbutrol, nitroimidazole, other agonist and thyrostatic drugs (Shankar et al., 2010).

TLC-mass spectrometry has been reported as an antibiotic residue screening method (McGrane, 2000). The method can be performed manually or with on line detection. Manual detection requires removal of the spot from the TLC plate followed by solvent extraction of the analyte, and this solution is subjected to MS determination. Alternatively the TLC plate, once developed, can be placed on a stage inside the mass spectrometer. The plate can be scanned or the spots can be imaged. The method is applicable to an extensive range of compounds (McGrane, 2000), yet the unavailability of commercial interfaces, which allow direct detection of analytes on the TLC plate, currently presents a problem. The main disadvantages of TLC are the need of sample preparation (extraction, filtration, etc.) and high cost (only one thin-layer plate per residue searched) (Toldra and Reig, 2006).

**High Performance Liquid Chromatography**

High Performance Liquid Chromatography (HPLC) with UV detection was introduced in the middle 70s, but the first instruments were expensive and not robust (Toldra and Reig, 2006). UV detection also doesn’t match the specificity and limits of detection needed for group A substances (substances having anabolic effect). However, for the quantitative determination of group B substances (Veterinary drugs) UV detection and post column derivatization were often used. Typical detection of multi-residues in samples is relatively simple and rapid, requires preliminary clean-up through solid-phase extraction followed by filtration before injection into reversed phase HPLC with diode array detection. This procedure has been applied to meat for detection of antimicrobial like quinolones, sulphonamides, ß-lactams, macrolide and tetracycline, also anabolic and corticosteroids veterinary drugs (Kirbi et al., 2005).

The important advantages of HPLC are that, it takes a short time (few min/sample) to obtain the results, has high sensitivity and specificity depending on detector, high automatisation leading to high productivity and the possible receipt of more information from spectra when using diode array detector. The disadvantages include initial investment (equipment), need of expertise, and need of sample preparation (extraction and filtration, addition of internal standard, etc.) (Toldra and Reig, 2006).

**Confirmatory Methods**

Until the last decade of the 20th century, the main instrumental techniques used for veterinary drug residue analysis were liquid chromatography (LC) using ultraviolet detection (UV), diode array detection (DAD) and fluorescent detection (FLD), and gas chromatography (GC) using flame ionisation detection and electron capture detection (Berendsen, 2013).
For several therapeutic classes, robust and accurate methods, based on LC-UV-Vis and LC-Fluo, were developed and validated for quantification and confirmation (Stolker and Brinkman, 2005). For example, several methods, based on UV-Vis or fluorimetric detection have been validated and published to analyse sulphonamides in different matrices at a concentration range in line with MRLs (Wang et al., 2006), tetracyclines (Samanidou and Nisyriou, 2008).

In the last decade fast switching (<10ms) triple quadruple instruments became available. This facilitated the development of HPLC methods in which a larger number of compounds were detected within one run. These methods include large numbers of compounds that belong to different antibiotic groups (Stolker and Brinkman, 2005; Samanidou and Nisyriou, 2008; Schneider et al., 2010; Martos et al., 2010; Blasco et al., 2011) and even to different classes like veterinary drugs and pesticides (Bogiali and Di Corcia, 2009).

The development of high resolution LC (HRLC) featuring sub-2 µm stationary phase particles delivered a higher chromatographic resolution and higher sample throughput, because separation can be carried out with the same or better chromatographic resolution within a shorter time frame (Kaufmann et al., 2008). This innovation resulted in further development of multi-compound and multi-class methods containing over 250 compounds belonging to different compound groups (Mol et al., 2008; Aguilera-Luis et al., 2012; Tang et al., 2012).

Meanwhile, with developments in the detection limits of flight mass spectrometry (TOF/MS) and the development of orbitrap instruments, highly selective, HR-MS detection techniques became routinely available (Stolker et al., 2007). HR-MS is a full scan technique; in theory, an indefinite number of compounds that can be ionised by the selected ionisation technique can be analysed simultaneously within a certain m/z range. Several methods using these techniques for the analysis of, among others, antibiotics have been published (Peters et al., 2009; 2010; Deng et al., 2011; Filigenzi et al., 2011).

Conclusion

The veterinary drugs are administered to prevent or treat diseases or to promote growth. Depending on the specific compound and its application, the use of veterinary drugs is regulated by international and national legislations. Public perception and the growing awareness of this issue have raised the interest among regulators to check for such residues. The development, implementation and validation of suitable analytical methods are indispensable to put legislation into practice and to have appropriate means for effective veterinary drug residue control. Reliability of results is pivotal, as false negative results are jeopardizing consumer protection, whereas false positive results provoke financial loss and ruin reputation of food producers.

References


