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Veterinary Drug Residue Control in the European Union*

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A b s t r a c t: Veterinary drug residue control in the European Union is an important step to reduce the harmful effect for consumers in relation with their exposure to residues from veterinary treatment. After an initial risk assessment by competent authority in charge of approval of veterinary drugs, the European legal framework defines the responsibility of the different stakeholders and the way to monitor and control the veterinary drug residues in animal production by the competent authorities. At the European level, it defines the rules of different actors (inspection services, laboratories) in charge of this control as well as the specification of methods and analytical laboratories performance. The concept of compliant and non compliant samples from an analytical point of view is defined precisely by regulation. The control is regularly improved by the development of validated analytical methods, rapid method dissemination, regular training and follow the rapid improvement of analytical technologies and scientific knowledge.

Key Words: *Veterinary drugs, residue, analytical methods, legal framework, validation*

KONTROLA REZIDUA VETERINARSKIH LEKOVA U EVROPSKOJ UNIJI

S a d r ž a j: Kontrola rezidua veterinarskih lekova u Evropskoj uniji predstavlja značajan korak ka smanjenju štetnih efekata za potrošače u pogledu njihove izloženosti reziduama od veterinarskog tretmana. Nakon početne procene rizika od strane kompetentnog tela zaduženog za odobravanje veterinarskih lekova, evropski pravni okvir definiše odgovornost različitih interesnih grupa i način na koji se prate i kontrolišu rezidue veterinarskih lekova u stočarskoj proizvodnji od strane kompetentnih organa. Na nivou Evrope, definiše pravila različitih faktora (inspekcijske službe, laboratorije) zadužene za ovu vrstu kontrole, kao i za specifikovanje metoda i načina rada analitičkih laboratorija. Koncepcija odgovarajućih i neodgovarajućih uzoraka sa analitičke tačke gledišta precizno je definisana propisima. Kontrola se redovno poboljšava razvojem vrednovanih analitičkih metoda, brzom diseminacijom metoda, redovnom obukom i prati brz napredak analitičkih tehnologija i naučnog znanja.

Ključne reči: *veterinarski lekovi, rezidue, analitički metodi, pravni okvir, validacija*

Veterinary drugs are regulated in the European Union according to the legal framework defined in the Directive 2001/82/EC. Before approval by competent authority, each new veterinary drug is assessed for its quality, safety and efficacy. To protect consumer health, each drug used in food producing animal is assessed for the risk in relation with residues according to the rules establishing maximum residue limits for veterinary medicinal products in foodstuffs of animal origin (**www.emea.eu.int**). The evaluation procedure is laid out in Council Regulation (EC) 2377/90 of 26 June 1990.

Good agricultural practices about the use of veterinary medicinal products are based on the use by responsible professional supervised by veterinarians. Good veterinary practices defined the principle of choice of treatment after diagnosis, scientific assessment of the best therapeutic choice, respect of dosage regimen and application of withdrawal time. Control of residues according to Directive 96/23/EC is a way to evaluate regularly the respect of good veterinary practices and to detect and regulate any deviation in veterinary drugs usage. It is a part of the risk management framework organised by competent authority and professionals organised according to the principles of White Paper on Food Safety (2000).

For the purpose of this review we will put most of our examples about the progress in veterinary drug residue control in the class of antimicrobials. Few of them will be taken with other therapeutic drugs as example of the way to improve an analytical strategy of control. After a review of the European legislation, we will present the organisation of

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control laboratories, the recommendations for their management, the types of methods used and the level of performance reached for some controls. At the end, the perspective in the residue control will be discussed.

1 European legislation

1.1 Regulation 2377/90/CE

According to the legal definition, treatment residues are defined as the parent drugs and/or metabolites in edible animal tissues (muscle, fat, liver and kidney) and animal products (milk, eggs, honey).

In the EU, the use of veterinary drugs is regulated through Council Regulation 2377/90/EC (1990). This regulation describes the procedure for the establishment of maximum residue limits (MRs) for veterinary medicinal products in foodstuffs of animal origin. Its Annexes present the following information:

- Annex I includes substances for which final MRLs have been established;
- Annex II includes substances for which no MRLs are necessarz because their residues are not considered to present a public health risk from the levels used;
- Annex III includes substances with provisional MRLs. These are established for a defined period of time, when not all requirements for the establishment of MRL, have yet been fully addressed;
- Annex IV includes substances for which no MRL could be established because of uncertainty about the risk which its residues pose in relation with their toxicological profile, or lack of data. The administration of substances listed in this Annex IV to food-producing species is prohibited.

The substances listed in Annex I, II and III are allowed for use in veterinary medicinal products for food-producing species for the animal species indicated and according to the conditions established.

Prescription rules by veterinarians are defined by Member States according to the community code relating to veterinary medicinal products (2004). According to the European regulations, veterinarians can prescribe antimicrobials under their control according to the cascade principles which means that a drug only approved for cattle for example, could be administered to another mammalian species if the veterinarian judged this prescription necessary for the animal health and considered that he has no other alternative in its therapeutic armentarium. In this case the MRL defined for the species listed in the market authorization is extrapolated to the other species. In this case, the veterinarian is responsible of the withdrawal time determination and will be responsible in case of non compliant result determined by residue control.

1.2 Directive 96/23/EC

Council Directive 96/23/EC regulates the residue control of pharmacologically active compounds, i.e., environmental contaminants, dyes, chemical elements, etc. in products of animal origin and live animals.

This Directive divides all residues into Group A compounds, which comprise prohibited substances defined by Council Directive 96/22 and Annex IV of Regulation 2377/90 and Group B compounds. The Group B comprises all registered veterinary drugs in conformity with Annexes I and III and other substances and contaminants. Antibacterial compounds including sulphonamides and quinolones belong to the group B1. Group B2 contains other veterinary drugs such as anthelmintics, anticoccidials including nitroimidazoles, carbamates and pyrethroids, sedatives, non-steroidal anti-inflammatory drugs and other pharmacologically active substances. Dyes which can be used for therapeutic purpose (i.e. malachite green) belong to the group B3e. Directive 96/23/EC includes the control of food-producing animals as well as their primary products like meat and aquaculture products. The commission decision 97/747/EC defines the sampling frequency for milk, eggs, rabbit meat and the meat of wild game and farmed game and honey. This means that samples are taken from the live animal at the producing farms as well as from carcasses in the slaughterhouse. Directive 96/23/EC also establishes National Surveillance Programmes for the monitoring of residues.

Control for Group A is more critical, because of public-health concern. Relatively large numbers of samples have to be analysed and more stringent criteria have to be used in view of the serious implications of non compliant results for public health and legal action to be taken by competent authority. Control can be performed in foodstuffs. But residue of treatment could be also found in other animal matrices such as urine, faeces, tissue (retina, skin) or hair. These matrices can be used for control purposes according to Directive 96/23/EC.

Control for Group B is important to verify the application of good veterinary and good agricultural practices and the evaluation of consumer exposure

to contaminants. For veterinary drug residue, it is one of the control steps implemented by the European legislation. It is a part of the inspection system established to insure the consumer safety. Matrices (muscle, milk, kidney, liver, fat or skin+fat, milk, eggs, honey) used for control are defined by the regulation 2377/90/CE according to defined MRL.

Directive 96/23/CE lays down that samples collected for the National Surveillance Programme have to be analysed in accredited laboratories. The laboratory must fulfil the ISO17025 guide for quality assurance management system. According to the principle of quality assurance, laboratory must be regularly evaluated by independent body recognised by European Accreditation Body.

Accordingly, an extensive network of analytical residue laboratories has been created for the purpose of residue inspections. This hierarchical system comprises three levels: Routine and/or Field Laboratories, one National Reference Laboratory (NRL) by group of compounds, and, at the top, four Community Reference Laboratories (CRLs) which are located in Germany, France, Italy and The Netherlands. The four CRLs work under contract for the General Directorate "Health Protection" and are responsible of a group of compounds. Antimicrobial and dyes are under the supervision of AFSSA Fougères (France), LERMVD. Other veterinary drugs are under supervision of BVL, Berlin (Germany), except for sedatives which are under responsibility of RIVM, Wageningen (Netherlands). Main tasks of CRLs are to improve the network performance level by dissemination of robust analytical methods, organization of training, exchange of information with NRL, yearly evaluation of the methods used in the national residue monitoring plan and organisation of proficiency tests.

1.3 Decision 2002/657/EC

Decision 657/2002/EC (2002) defines the performance criteria for analytical residue methods. As a result of advances in analytical chemistry since the adoption of Directive 96/23/EC the concept of routine methods and reference methods has been superseded by criteria approach, in which performance criteria and procedures for the validation of screening and confirmatory methods are established. The Decision defines the criteria to verify for the validation of screening and confirmatory methods used for official control of residue. It defines the notion of compliant and non-compliant samples according to the substance group defined in Directive 96/23/CE.

For Group A substances, the principle of control applied is the concept of "zero tolerance". But this concept of zero tolerance is dependant on the performance of the screening and confirmatory methods used by each control laboratory. To improve the efficiency of the control in the European Union, to harmonize the level of consumer protection in the Community and to guide the network of laboratories to validate their analytical methods at a same level of performance, the progressive establishment of minimum required performance limits (MRPL) of analytical method for substances for which no permitted limit has been established was introduced. The minimum required performance limit (MRPL) is the minimum content of an analyte in a sample that has to be detected and confirmed by an official laboratory in charge of control. The first MRPLs were published in Annex II of Commission Decision 2003/181/EC (2003) for chloramphenicol and nitrofurans. According to the decision, a sample is declared "non compliant" if the analytical results fulfilled identification points criteria (see below).

For Group B substances, the validation principles for screening and confirmatory methods around the MRL are defined. A sample is declared "non compliant" if the residue concentration is higher than the maximum residue level and analytical results fulfilled identification points criteria (see below). Unfortunately, use of some veterinary drugs is limited to the approved indication. Some species limitations can exist in this indication such as "not approved for laying hens" or "not approved for treatment of lactating cows". The presence of residues of some veterinary drugs could be the result of intentional use which can be a lack of respect of good veterinary practice (e.g. milk residues due to misidentification of treated animal) or result of cross-contamination (e.g. feed cross-contamination in poultry production) (*Glenn Kennedy, Cannavan et al*., 2000).

Next to the general performance requirements, e.g., detection level, selectivity and specificity, the decision 2002/657/CE defines the techniques which can be used for confirmatory purposes.

Group A substances must be confirmed only with LC or GC method coupled with mass spectrometric detection or infra-red spectrometric detection. Group B substances can be confirmed by the techniques recommended for Group A and other analytical techniques such as LC-full-scan DAD, LC -fluorescence for molecules that exhibit native fluorescence and to molecules that exhibit fluorescence after either transformation or derivatisation, 2-D TLC – full-scan UV/VIS, GC-ECD if two

columns of different polarity are used, LC-immunogram if at least two different chromatographic systems or a second, independent detection method are used, LC-UV/VIS (single wavelength) only if at least two different chromatographic systems or second independent detection method are used. The criteria for the method validation are defined.

Decision 657/2002/EC defines additional requirements for confirmatory methods based on mass spectrometric methods by introducing the concept of *identification points* (IPs) and defining criteria for ion intensities. During confirmatory analysis a specific number of IPs has to be collected. For the confirmation of the identity of Group A substances, minimum of four IPs is required while for the confirmation of the identity of Group B substances, minimum of three IPs is required. The number of IPs earned by a specific analysis depends on the technique used. The relative *ion intensities* or ion ratios are another important aspect for confirmatory methods and maximum allowable variation tolerances have been laid down.

2 Monitoring programme

2.1 Sample

For monitoring drugs having a MRL, animal tissues such as liver, kidney, muscle, milk or fat are selected as target edible tissue. The MRL is defined taking account of the acceptable daily intake for humans defined by the toxicological experts after review of the safety dossier, and the pharmacokinetics of the drug in the animal species for which the drug is designed. Target tissue and marker residue for control are choosing to reflect the overall residue (including metabolites) depletion curves and allow the national production and import control. The definition of the maximum residue level takes into account safety factor. If the dosage regimen indicated for the drug and the withdrawal time are followed, the drug concentrations will be likely below or equal to the MRL. The same approach is used to define marker residue and MRL in animal production (milk, eggs).

For monitoring drugs from Group A, animal tissues can be sampled at slaughterhouses. Specific target tissue such as retina could also be used which is the case for control of nitroimidazoles in some member states (*Polzer, Stachel et al*., 2004; *Cooper, McCracken et al*., 2005). The control could be also performed in farm with live animals using blood, urine, faeces or hair.

Another group of samples frequently used to monitor Group A or B substances are animal feed and drinking water. Feed is a difficult matrix because drug extraction is not easy and feed composition could vary from batch to batch regarding proteins and carbohydrates origin. However, feed drug concentrations are usually much higher (1–10 mg/kg) than in animal tissues (1–100 g/kg); consequently drugs can be more easily detected. For Group B substance, feed control will be linked with the review in farm of drug registration book. Management options based on analytical results must discriminate between non-authorized use and feed or water cross-contamination.

2.2 Methods

2.2.1 Analytical strategy

The residue control needs to define an analytical strategy based on the combination or not of screening and confirmatory methods. In the field of 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).

The strategy definition will also take into account the number of samples to monitor for a group of substances and for a type of matrices. Due to large variation of animal production between member states, country size and history of laboratory organisation, available resources and technical expertises, national strategy differs widely from the choice to sub contracting few analysis to another member-state to large laboratory network more or less specialized by techniques in function of national or regional resources.

Selecting a suitable method of residue analysis will depend on the problem at hand, on the final goal, as well as on the laboratory resources and national budget available for this control. If laboratory have to monitor a large sample series, sample throughput will be an important criterion since effective workload by sample and speed are important parameters to optimize. In this situation, a screening method is selected because high sample throughput and speed are the characteristics of such a method. For the group A substances, the selectivity of the method is the main criterion and must avoid false non-compliant results at the minimum level of performance required. In this situation, a confirmatory method is necessary to provide full or complementary information enabling confirmation of substance identification. In some cases, at the beginning of

new control, screening methods could not be available and sophisticated physico-chemical methods can be used.

For the group B substances, in function of the sample throughput, the choice would depend on the control strategy.

If the sample number to analyse for a family of compounds is low, routine laboratory can use a method able to perform screening and confirmatory steps. The total analysis can be performed in one confirmatory run or by two steps. In this case, during a first run, analyst will check a batch of samples for signal detection. If a positive signal is recorded in a sample, a run will be performed to quantify and confirm the analyte by comparison with the maximum residue limit. Physico-chemical methods developed to analyse a large number of compounds in different matrices are recommended to limit the analytical cost and the work load for the quality management of several different methods.

If the sample number is high, routine laboratories could perform a simple and robust screening method able to detect non-compliant samples (i.e. with concentration higher than MRL) with a low false compliant rate. In case of positive results, quantification and confirmation can be performed by another method in the same laboratory or in a more specialized one. Physico-chemical method based on planar chromatography can be performed with a limited laboratory capacity and a high throughput (*Juhel-Gaugain and Abjean*, 1998).

In the case of antimicrobial drugs, the analytical strategy is a little bit more complex due to the history of this control and availability of a lot of simple screening methods based on bacterial growth inhibitory effect developed before publication of MRL value. Moreover, the susceptibility of germ test vary between antimicrobial classes and also between compounds belonging to the same class (*Gaudin, Maris et al*., 2004). Similar variability exists for receptor test or immunological ones with different value of cross reactivity between compounds.

2.2.2 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). In the meantime, the presence of antimicrobial activity in meat was also detected using inhibitory test (*Mitchell, Griffiths et al*., 1998). Different inhibitory tests were developed to screen milk, muscle, kidney, eggs, fish (*Popelka, Nagy et al*., 2004). They are based on inhibition of bacterial growth which can be detected by a pH change, visible by use of a pH dependant dye or by the inhibitory zone around a piece of meat or a milk deposit on a bacterial culture on agar plates. Inhibitory test detects an antimicrobial activity which is dependant on test organism susceptibility to antibiotics and growth conditions. 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 able to produce medium and bacterial suspension (*Gaudin, Maris et al.*, 2004). They are widely used to perform residue control and self control by industry. Unfortunately, most of them were developed before publication of antimicrobial MRLs and their performance differ widely between antimicrobial families. Able to detect an inhibitory activity, cross reactivity is widespread between antimicrobial families in function of germ susceptibility. They are valid to detect a limited number of compounds below their respective MRL, they could also give a positive response with other drugs at concentration higher than their MRL (*Gaudin, Maris et al*., 2004).

Other screening tests were also developed, based on knowledge of antibacterial activity of some antibiotic family. They use a bacterial receptor as target for antibacterial binding (*Charm and Chi*, 1988). They are based on detection of competition between free drugs in sample and labelled drugs. Some of them are valid for few compounds in regards to MRL and have cross-reactivity with other drugs of the same family at concentration higher than the MRL. They could be used effectively as screening test for an antimicrobial group or as post-screening test.

In order to detect antimicrobial residues in animal products, bioassay techniques are widely used as screening methods. These methods generally do not distinguish between members of a class of antibiotics, but provide a semi-quantitative estimate of 'total' residues detected by their inhibitory effect. Some of these methods are available as kits, easy to perform with a low cost. In regard to the MRL, bioassay methods are valid for the screening of few drugs such as betalactam representatives. According to the Decision 2002/657/CE, a screening method should be valid to detect 95 % of non-compliant sample at level of interest (i.e. MRL). Then a method can be considered valid for some compounds and not for the others, limiting its scope. Their cross-reactivity with other antimicrobial family at concentrations higher than MRLs allow detection of true non-compliant samples. The use of several plates expands the screening capacity of the method

(*Myllyniemi, Nuotio et al*., 2001; *Gaudin, Maris et al*., 2004). Combined with post screening methods and confirmatory methods, they can be used in the effective analytical strategy for control of antimicrobial residues if they are used in residue control plan for inhibitory effect, performed in parallel with control plan designed for an antimicrobial class.

Immuno- and Receptor- assay

Some screening tests are also developed using antibodies 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 MRL, these tests are valid for a list of compounds. Some ELISA tests were designed specifically for antimicrobials belonging to the group A such as chloramphenicol (*Gaudin, Cadieu et al.*, 2003) or nitrofurans metabolites (*Cooper, Elliott et al*., 2004).

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 betalactams, sulphonamides and tetracyclines. Effective kits are developed for some aminoglycosides and are used to screen these compounds in kidney or urine by some member states. NRLs used them also as post screening test after a positive inhibitory test to give information about the chemical family of the residue. New technologies to detect molecular interaction such as plasmon resonance (*Gaudin, Fontaine et al*., 2001), fluorescence or electrochemical (*Pellegrini, Carpico et al.*, 2004) monitoring improve the performance of screening test and allow high throughput by simultaneous analysis of multiple samples or fast on-line analysis.

Planar chromatography

Physico-chemical approach such as planar chromatography can be used alone for the screening inside a chemical family such as quinolones (*Juhel-Gaugain and Abjean*, 1998). It can also combined to detect a biological signal (*Choma*, 2006).

2.2.3 Confirmatory methods

As defined by the Decision 2002/657/EC, confirmatory methods are based on physico-chemical techniques. The most popular for antimicrobials are based on a sample extraction, using liquid extraction and liquid solid extraction, liquid chromatography and detection by UV-Vis spectrometry or fluorimetric detection or mass spectrometry. Liquid extraction (LE) comprises conventional liquid-liquid extraction as well as the liquid extraction of homogenized tissues such as liver, kidney and meat, referred to as liquid-phase extraction. Liquid-Solid extraction (LSE) is almost always performed in the form of solid-phase extraction and in few cases by application of matrix solid-phase dispersion (*McCracken, Spence et al*., 2000), immunoaffinity extraction (*Godfrey*, 1998) and molecular imprinted polymers (*Fernandez-Gonzalez, Guardia et al.*, 2006). In many instances LE and LSE were used in combination: after analyte isolation by means of LE, the drugs were subsequently enriched by using a suitable solid phase extraction procedure.

According to the Decision 2002/657/CE, confirmation of Group A antimicrobials requires the use of mass-spectrometry. The first confirmatory method for chloramphenicol was based on gas chromatrography coupled with mass spectrometry (GC--MS). Today, GC/MS is rapidly superseded by liquid chromatography coupled with mass spectrometry (LC/MSn) such as tandem mass spectrometry by triple-stage quadrupole (LC/MSMS) and ion-trap (LC/ITMSn). The history of control of chloramphenicol residue is a good example of the continuous decrease of the limit of detection with the progress of analytical technology (*Impens, Reybroeck et al.*, 2003).

The rapid development of new hybrid technology where a single quadrupole is combined with a ion-trap (*Heller and Nochetto*, 2004) or a time-of-flight instrument (*Hernando, Mezcua et al*., 2006) allows accurate mass measurement and enhances selectivity compared to the other types of tandem MS machine.

For the group B, the list of confirmatory methods available is wider. 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, Zhang et al.*, 2006), tetracyclines (*Samanidou, Nikolaidou et al.*, 2007) or quinolones (*Yorke and Froc*, 2000). The introduction of mass spectrometry for the control of Group A allowed the development of methods applicable for family of compounds difficult to analyse with classical detection, as aminoglycosides (*Isoherranen and Soback*, 1999). Most of the ongoing developments are to develop simultaneous analysis of di-

fferent compounds belonging to different antimicrobial families, (*Heller and Nochetto*, 2004; *Hernando, Mezcua et al*., 2006) or anthelmintics classes (*De Ruyck, Daeseleire et al*., 2002) combined in one LC-MSn method. Simultaneous analysis of compounds from different veterinary medicine classes is also possible (*Hernando, Mezcua et al*., 2006).

The analytical methods development follows the technological progress in mass spectrometry. Initially, the methods frequently used mass detection technique which was single stage quadrupole MS. Today, most of them are based on triple-stage quadrupole and ion-trap MS. Their excellent selected reaction monitoring, based selectivity, are preferably used for confirmatory purposes.

A relatively new, and extremely powerful, technique is Q-ToF-MS, where a single quadrupole is combined with a time-of-flight (ToF) instrument (*Hernando, Mezcua et al.*, 2006). The accurate mass measurement of the ToF-MS ensures a distinctly enhanced selectivity compared with the other two types of tandem MS machine.

2.2.4 Method conception and validation

The challenge for a laboratory in charge of antimicrobial residue control is the need to use different validation methods and to proof its capacity to apply them routinely during accreditation body evaluation.

The Decision 2002/657/CE was introduced to follow the rapid development of analytical science in the field of residue and contaminants control. By the criteria approach, the schedule of conception, development and validation is only dependant on the laboratory network and independent of a normalisation process. The goals for the control method are defined by European legislation (MRL, MRPL) as well as the criteria to fulfil. Then, the development of new methods is rapid and efficient, as demonstrated over the last 10 years. The development of some new methods (nitrofurans, anticoccidials) was supported by the European (PCRD) research programme and efficiently disseminated through the CRL/NRL/routine laboratory network to be applied routinely, few months after introduction.

The story of nitrofuran residue shows the efficiency of the combination of development of new concept of control based on the knowledge of drug metabolism and improvement of analytical methods. Furazolidone, furaltadone, nitrofurazone and nitrofurantoin are nitrofuran antibacterial agents which have been widely used as veterinary medicine for the treatment of gastrointestinal infections (bacterial enteritis caused by *Escherichia coli* and *Salmonella*) in cattle, pigs and poultry.

Toxicological studies have shown that furazolidone is a mutagenic and genotoxic drug. According to principles guiding the 2377/90 regulations and because the different nitrofurans share the same structural chemical pattern suspected to be responsible for the mutagenic effect, use of nitrofuran antimicrobials in food-producing animals has been prohibited within the EU since 1997.

Researches to understand pharmacokinetics and toxicity of nitrofurans demonstrated that protein bound residue are produced after administration. Structural identification of bound-residue was performed after release from protein under acidic conditions (*McCracken and Kennedy*, 1997). A research programme, named FoodBrand funded by the EU, allowed development new analytical methods and demonstrated the long half-life of bound residue (*Boenke*, 2002).

During 2002, the nitrofuran bound residues were found in poultry and shellfish imported into the EU using the new method developed within the research programme. Standards of bound metabolites and methods were disseminated in the CRL/NRL network to improve the control, and MRPLs for nirofuran metabolites in poultry meat and aquaculture products were set at 1 μg/kg (2003).

In two years, almost all NRLs used LC method coupled with mass spectrometry for screening and confirmatory analysis of nitrofurans, and problems of illegal use were detected in few member states.

Recently, ELISA methods were introduced to screen two nitrofuran metabolites (AOZ and AMOZ) in tissues (*Franek, Diblikova et al*., 2006).

It was demonstrated that SEM and AOZ can be contaminants of processed food provided by other sources such as caps of baby food jars (*Ginn, Wilson et al*., 2006), flours or purification columns used in egg protein production. This assumed careful interpretation of the results obtained with processed food. But, according to our knowledge, it was not demonstrated that other natural products, approved drugs or additives can conduct to this bound metabolites in live animals excepted for semicarbazide in shell fish at very low concentrations. Recently, methods were expanded to nifursol and nitrofuran previously used as feed additives (*Verdon, Couedor et al*., 2007).

In case of non-compliant results for AOZ and SEM, the CRL recommend to investigate the nitrofuran source taking account of the possible release of old medication in the farm environment.

2.2.5 Interlaboratory studies

One of the functions of CRL is to organize comparative tests for the benefit of NRLs. Three kinds of interlaboratories can be planned.

Proficiency tests are regularly performed in order to assess the ability of these laboratories to analyse residues in animal products. Participation in proficiency testing scheme is requested by the accreditation bodies as it is a good way to assess the reliability of the results. The organization and statistical analysis of these studies were performed within a quality assurance system according to international, national guidelines and internal procedures (*Juhel-Gaugain, Sanders et al.*, 1998; *Juhel-Gaugain, Fourmond et al*., 2005; *Hurtaud-Pessel, Verdon et al*., 2006). Proficiency tests are effective tools to improve the overall performance of the veterinary drugs residue control, reducing the inter-laboratory variability.

The validation of new methods can also need organisation of an interlaboratory study to determine accuracy and precision (*Verdon, Couedor et al.*, 2002).

The production of certified reference materials needs also interlaboratory study to define precisely the specification of the materials (*Juhel-Gaugain, McEvoy et al*., 2000).

3 Conclusion

Veterinary medicines contain a large list of different chemical compounds belonging to different therapeutic and chemical families. Over the last ten years, physico-chemical analytical technology progress was greatly improved. Today, almost all veterinary drug residues could be quantified and confirmed at the level of interest (MRL) in most of the national reference laboratories. For unauthorised compounds, control efficiency needs to determine a goal for the analyst. The publication of MRPL improves the control efficiency facilitating the validation steps even if it opens discussion around the "zero tolerance" concept. It is important to keep in mind that this minimum required performance level could be easily modified by risk managers if necessary.

The EU regulatory framework defines the duty and taks of risk managers and risk assessors. The analytical laboratories network plays an important task in the communication between them. The EU regulatory framework defines for the laboratory management, the professional goals to reach and the way to prove their analytical capabilities.

The future of this activity will follow two parallel pathways. First, the development will continue with effective multi-analyte, multi-matrices screening and confirmatory methods dedicated to authorized compounds (*Heller, Nochetto et al*., 2006) and to known unauthorized ones. The major problem for the laboratories for the next years is the management of the validation workload for this combined method. Moreover, this task needs the continuous improvement of the European consensus about the validation concept which is a complex issue.

The second pathway will follow the detection of biological effect due to unknown compounds or combination of pharmacological compounds at low levels. In the area of antimicrobial residue control, 40 years of use of biological screening methods demonstrate existence of inhibitory effect without confirmation of presence of a compound in the list of approved drugs.

The development of new biological tools resulting from genomics and proteomics research opens the way to new biological screening methods (*Hoogenboom, Hamers et al.*, 1999; *Schumacher, Van Den Hauwe et al*., 2003). Concurrently, the rapid development of hybrid MS technology opens the way for new methods able to identify and confirm presence of a compound without any prior information (*De Wasch, Van Hoof et al*., 2003). It would be a new progress in the identification of natural biological active compounds in animals or illegal use of unapproved veterinary drugs.

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