GUIDELINE FOR THE ESTIMATION OF THE WITHDRAWAL PERIOD IN EDIBLE TISSUES

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1. Introduction

Consumer´s safety needs to be safeguarded through the asessment of all pharmacologically active substances aimed at food-producing animals. Withdrawal periods are determined with the aim of ensuring that residues of such substances in edible tissues are reduced to authorized concentrations.

While the Maximum Residue Limit (MRL) for a specific tissue is applied to the active principle, the withdrawal period is determined individually for each veterinary drug as part of the commercialization approval process.

The active principle included in the veterinary drug administered to food-producing animals is not necessarily the substance that will be present in edible products. The enzymatic systems or physiological fluids of an animal can act on the active principle that has been administered and produce new substances such as metabolites that can be as or more damaging for the consumer than the original active principle. Concentration of these substances in edible products of animal origin will be in relation to the speed and degree of absorption of the original pharmacological agent, the speed of metabolism and the excretion rate both of the original active principle and its metabolites. Therefore, the total residue of the pharmacological agent administered to treated animals will be composed of the original active principle, free metabolites and metabolites united to endogenous molecules.

Since the different components of the total residues may differ in their toxicological potentials, information about the chemical nature, quantity and persistence of the total residues in edible tissues of treated animals should be provided.

The simplest and most practical way of determining the withdrawal period has been to identify the time when, in all monitored tissues of all animals in the trial, concentrations are found below the MRL. In some cases, and when there´s a marked variability within the depletion data, a period of time is usually added as security factor.

In other cases, statistical methods have been used that, if generally accepted, they would generate a great opportunity of harmonization.

The choice of the statistical method to be applied is responsibility of the applicant and, in any case, it should be properly justified with adequate documents.

This guidance describes standardized procedures to establish the appropriate withdrawal period for each active principle associated to a pharmaceutical form, dose and route of administration proposed for a specific animal species, in order to warranty consumer´s safety.

2. Glossary

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Standard food basket: It is an estimate of the total amount of food of animal origin consumed daily by an adult weighting 60 kg. The basic food basket employs arbitrary figures of consumption, based on percentiles higher than the daily intake of products of animal origin.

The figures of daily consumption of food of animal origin are:

For mammals:

300 g. of muscle, 50 g. of fat or fat and skin, 100 g. of liver and 50 g. of kidney.

For birds:

300 g. of muscle, 90 g. of fat or fat and skin, 100 g. of liver and 10 g. of kidney.

For fish:

300 g. of muscle and skin in natural proportions.

Also an intake of 1.5 l. of milk, 100 g. of eggs and 20 g. of honey are considered.

The estimate of the risk of consumption of present residues in a food basket is calculated taking into account the ADI.

Active Pharmaceutical Ingredient –Drug substance (API): Any substance intended to cure, mitigate, treat, prevent or diagnose diseases in humans or animals.

Acceptable Daily Intake (ADI): It is the estimate of the residue, expressed in terms of weight per kilogram of body weight (kg/bw) that can be daily consumed during consumer´s whole life without noticeable risks to his health.

Confidence Interval: Range of values in which the value of a population parameter is expected to be found with certain degree of certainty.

Maximum Residue Limit (MRL): Maximum concentration of residues founded in an animal product or by product that not introduce any risk for the consumer safety, based on known facts at the time of its publication (CAMEVET definition).

Limit of Quantitation (LOQ): It is the smallest concentration of an analyte that can be quantified with a specific degree of accuracy and precision, within statistically defined limits.

Limit of Detection (LOD): It is the smallest concentration of an analyte from which it is possible to deduce the presence of the analyte in the test sample but it is not possible to quantify it, within statistically defined limits.

Tolerance limits: The extreme values of a series of values (interval) within which a determined percentage of the individuals of a determined population is expected to be found with certain degree of certainty.

Veterinary drug: Any chemical, biological, biotechnological substance or manufactured (elaborated) preparation administered either individually or collectively, directly or mixed with food, aimed at the prevention, cure or treatment of animal diseases.

Safety Period – Use restriction- Withdrawal Period – Elimination Period: Minimum period of time that must happen between the last application of a veterinary medicinal product to an animal, in the normal conditions of use, and the extraction of food products from that animal, to warranty that such food products do not contain residues in quantities that exceed the established maximum limits.

Veterinary Product (as defined by CAMEVET): A veterinary product is any chemical, biological, biotechnological substance or manufactured preparation administered either individually or collectively, directly or mixed with food or water, aimed at the prevention, cure or treatment of animal diseases, including additives, supplements, promoters, animal production enhancers, antiseptics, disinfectants for environmental use or for devices and ectoparasiticides and any other product that, used in animals and their habitat, protects, restores or modifies the organic and biological functions. It also encompasses products destined to enhancing animals' beauty.

Marker Residue: It is an analyte that is reliable to determine the presence of residues of a specific drug in the tissue. The marker residue can be a mother cell or any of its metabolites, degradation products or a combination of any of them. The marker residue can also be a chemical derivate of one or various components of the residue. The relationship between the marker residue and the concentration of the residues of interest in edible tissues must be known (marker residue/ residue of interest). The MRL reflects the maximum allowed concentration of the marker residue in the edible tissues.

Total Residue: The total residue of a drug in animal derived food consists of the parent drug together with all the metabolites and drug based products that remain in the food after administration of the drug to food producing animals.

The total sum of the residues normally includes all parent residues of the pharmacological agent (parent drug and its metabolites), and in most cases it is identical to the sum of residues determined by radiometric tissue depletion studies.

Residues of toxicological relevance: For the estimate of an exposure based on a toxicological ADI, the residue of relevance is the residue of toxicological relevance. This normally includes all the parent compounds and the molecule (parent drug and the metabolites) and in most cases it is identical to the sum of residues determined by radiometric tissue depletion studies. However, if a component of the residue or a fraction of the total residues is demonstrated to be toxicologically inactive, it is possible to deduct it from the total residue or any other fraction of residues that is not bioavailable by oral route or the metabolites known to be toxicologically inactive.

Residues of pharmacological relevance: For the estimate of an exposure based on a pharmacological ADI, the residue of relevance is the residue of pharmacological relevance. In general, the mother cell plus any other of its residues are considered. If there is lack of data on pharmacological activity of the total residue components, the total residues are presumed to present the same pharmacological activity as the parent drug.

Residues of microbiological relevance: For the estimate of an exposure based on a microbiological ADI, the residue of relevance is the residue of microbiological relevance. In most cases, it is identical to the residues determined in microbiological assays. In case of lack of

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such data, the total residue can be used, or alternatively the sum of the individual components known to present microbiological activity. Therefore, the microbiological activity of the total residue or of the metabolites and/or products of degradation is the same as the parent drug.

Injection site: It is the area of tissue where the veterinary medicinal product has been injected. The samples of tissue obtained from the injection sites for the conduction of residue studies should be representative of the edible tissue that is feasible of being selected in the slaughter procedures. The tissue sample should include muscular tissue, connective tissue and subcutaneous fat in natural proportions (cutting off the samples to eliminate the connective tissue and the fat attached to muscle are considered artificial procedures that differ from the real situation). Injection site should not include the skin portion that covers it, since it is not required for the residues analysis.

Tissue: Any edible animal tissue, including muscles and sub-products (Definitions established and adopted by the Joint FAO/WHO Expert Committee on Food Additives – JECFA).

3.   Scope of the guidance

This guidance is a recommendation for the estimation of appropriate withdrawal periods for edible tissues from food-producing animals receiving a specific veterinary medicinal product, in an attempt to harmonize the methodology internationally used for those purposes. This guideline also focuses on the determination of the withdrawal period through the statistical method based on the maximum residue limit (MRL), considered of first choice.
The evaluations of withdrawal periods in other animal products such as milk, eggs, honey and aquaculture products, which require a separate consideration, are not in the scope of this guidance.

The following procedures are described:

- – Statistical procedure based on MRL
- – Alternative procedure based on MRL
- – Pre-slaughter withdrawal period estimated from the residues in the injection site

The residue studies mentioned above should include the description and validation of analytical methods (see Guideline 2: "Guideline for the validation of analytical methods for the estimation of residues in biological matrices").

For the experimental design of the animal phase, see Guideline 1 "Technical guideline to conduct metabolism and residue kinetic studies of veterinary pharmacological agents in food-producing animals".

4.   Estimation of the withdrawal period

4.1. Statistical model

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The calculation of the withdrawal period by the statistical method is based on accepted, basic pharmacokinetic principles. According to the pharmacokinetic compartment model, the relationship between active principle concentration and time through absorption, distribution and depletion phases can be described by multi-exponential exponential terms. However, depletion of the substance and/or its metabolites from tissues follows a curve of tissue concentrations that follows a first order exponential decay, which can be adequately described by a one-compartment model with only one exponential term. The first order equation that describes the tissue depletion kinetics is the following:

$$C_t = C_0 \, e^{-kt}$$

where C_t is the tissue concentration at a given time, C_0 is the pre-exponential term, e is the base of the natural logarithms, k is the first order elimination rate constant and t is the time.

The term C_0 represents the intersection point at the y-axis at zero time; actually it is only a theoretical concentration needed for the adjustment of experimental data to one exponential term model. Being an equation that describes a decreasing exponential function, the constant takes a minus sign.

Linearity of the natural log plot of tissue concentrations versus time ($\log_e C_t$ vs t), provides evidence that the one exponential term model is applicable and that linear regression statistical analysis of the logarithmic transformed data can be considered a reliable method for the estimation of the withdrawal period. In that case, experimental data can be described by the first order equation, also known as general equation of a line given by the following expression:

$$y = a + bx$$

where y is the value at the ordinate or y- axis, x is the value at the abscissa or x-axis, a is the point where the line crosses the y-axis and b indicates the quantity at which y changes for each change unit at x. The value of a is known as the y-axis intercept in a chart and the value of b as slope intercept.

The procedure used to obtain the expected line is known as method of least squares, and the resulting line (best mean estimated values in each sampling point) is known as least square line.

4.1.1. Database

The analysis of linear regression requires experimental data which are independent from each other. Generally, tissue depletion data meet this requirement, since they originate from different individuals.

In case of counting with duplicated or triplicated measurements of tissue concentration from a single sample, the mean value will be used for the conduction of a linear regression analysis.

To avoid errors in the calculation of slope and intercept, each data point of tissue concentration should, if possible, originate from the same number of repeated sample measurements.

As a general recommendation and depending on the animal species, between 4 and 10 animals should be used per sampling point (slaughter day). A basic diagram would include the tissue

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concentration data from 16 (sixteen) animals, which would be slaughtered in groups of 4 (four) individuals in 4 (four) appropriately distributed slaughter days

Experimental data of tissue concentration falling above the limit of detection (LOD) and below the limit of quantitation (LOQ) do have informational value, if not strictly quantifiable value. Hence, if it is necessary to use them, they will be considered optional values and this fact should be properly justified.

Experimental data reported as below the LOD should be excluded from the analysis.

When all or some of the reported experimental data in a slaughter time are "optional" the possibility of excluding from the analysis the corresponding slaughter time should be considered. It should be borne in mind that three sampling points (slaughter time) taken during the terminal elimination phase and three samples (animals) per sampling point are required at least in order to perform a linear regression analysis.

Tissue concentration experimental data should be reported just like they were quantified, that is without any analytical method correction for recovery, and should be attached to supporting data involving recovery experiments and the correction value derived from them. In that case, prior to linear regression analysis, experimental data should be corrected with the value of correction for recovery before being logarithmically transformed. When the test is done using a calibration curve obtained from fortified samples before the extraction process, there is no need of correction for recovery.

4.1.2. Linear regression analysis assumptions

In order to conduct a linear regression analysis, the following basic assumptions need to be met:

- Homogeneity of variances (homoscedasticity) of the $\log_e$ of the experimental data in each sampling point (slaughter day).

- Linearity of the $\log_e$ of the experimental data versus time.

- Normal (Gaussian) distribution of the errors.

4.1.2.1. Homogeneity of variances (homoscedasticity)

It should be confirmed that variances of log_e of experimental data from the different slaughter days are homogeneous. Different statistical tests, such as Bartlett´s Test[1], Hartley´s Test[9] and Cochran´s Test[16], may be used for this purpose

4.1.2.2. Linearity of log_e of experimental data

A visual inspection of the $\log_e$ plot of experimental data versus time is usually sufficient to assure that there is a linear relationship among experimental data.

A deviation from linearity of $\log_e$ of experimental data in the first sampling points may indicate that the distribution process of the marker residues has not been concluded yet and these points should therefore be excluded from the analysis.

Deviations from linearity in the late sampling points may be due to concentrations below the LOD, so the kinetic process of tissue depletion should not be considered in these sampling points, and exclusion of these values from the statistical analysis is justified.

It should be taken into account that all other experimental data from the remaining sampling points should be preserved, unless their exclusion is properly justified.

For statistical assurance of the linearity of the regression line, an analysis of variance should be performed. The procedure consists of comparing the variation between group means and the estimated line with the variation between animals within the groups.

4.1.2.3. Normality of errors

Normal distribution of errors can be observed through visual inspection of the ordered residuals versus their cumulative frequency distribution on a normal probability scale.

Residuals are the differences between the observed values and their corresponding estimated values (differences between the logarithmically transformed value and the values estimated by the regression line).

A straight line indicates that the observed distribution of residuals is consistent with the assumption of a normal distribution. To verify the results of the residuals plot, the Shapiro-Wilk Test[13] can be applied. This test has proved to be efficient even with small samples.

The plot of cumulative frequency distribution of the residuals can be used as a highly sensitive test. Deviations from the straigth line indicate a non-normal distribution of the residuals, which can be due to:

- Deviations from normality of logarithmically transformed marker residue tissue concentrations data within one or more slaughter groups.

- Deviations from values estimated by linear regression (regression line).

- Non homogeinity of variances (heteroscedasticity).

- Outliers

In the submission of the experimental data using the standardized residuals (residual divided by the residual error Sy, x), an outlier could present a value <-4 o $> +4$, indicating that the residual is four standard deviations off the regression line. The use of this kind of data must be properly justified. The information of the animal that originated the sample should be taken into account.

4.1.3. Statistical procedure based on MRL

The withdrawal period should be calculated using data results estimated by the regression line. The withdrawal period is the time when the upper limit of a 95% tolerance interval estimated with a 95% confidence interval intercepts the value of the maximum residue limit (MRL).

If this time point does not correspond to a full day, the estimated withdrawal period will be rounded up to the following day. For example, if the estimated withdrawal period is 6.3 days, it will be fixed at 7 days.

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The value of the upper limit of a 95% tolerance interval at a 95% confidence interval is calculated through the non-central t distribution method described in the annex.

It is not valid to estimate the withdrawal period based on the absence of data from tissue concentration below the MRL. Therefore, to calculate it, experimental data with values below the MRL in at least the last sampling time should be available. Given this perspective, the LOQ of the analytical method always needs to be less than the MRL used. Preferentially, the LOQ should be at least half the MRL.

The withdrawal period can be estimated using the software WT1.4, recommended by the EMA, The withdrawal period can also be estimated using the method developed by Stange[14], proposed by the EMA[5]. It is methodologically easier to perform and provides results comparable to the method that uses the non-central t distribution.

4.1.4 Alternative procedure based on the MRL

This procedure, also known as "decision rule" is an alternative method when the experimental data available do not allow the use of the statistical model based on the MRL.

It is not possible to propose general recommendations for this procedure, since results will depend on sample size, animal slaughter time, biological sampling, variability of the experimental data and factors related to the analytical methodology.

The method is based on establishing a withdrawal period, at the time point when tissue concentrations of all animals are below the MRL value[7]. However, once this time has been estimated, a security span should be established to compensate for the biological uncertainty that represents the variability in tissue depletion kinetic.

The dimension of the security span will depend on various factors associated to the experimental design and the pharmacokinetic properties of the active principle under study.

Although it is not possible to give general recommendations applicable to all cases, an approximate guidance to calculate the duration of the security span is to increase in 10% - 30% the value of time in which all tissue concentrations are below the MRL. Another alternative is to increase the time mentioned by a value equivalent to 1-3- times the tissue depletion half-life.

4.2. Estimated withdrawal period based on injection site residues

In addition to the effect of the formulation, dose and frequency of administration on the duration of the withdrawal period, the latter is significantly influenced by the route of administration. Injectable formulations can present significantly slower residue elimination kinetics from the injection site than that evidenced with other edible tissues.

This phenomenon may be attributed to the design of slow-release systems, depot-forming formulations, physical and chemical properties of the molecule, use of the subcutaneous and/or intramuscular route of administration, or other factors related to the variability of the route of administration (eg: in the connective tissue between semitendinosus and semimembranosus muscles).

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Unlike other tissues, the exact localization of injection site samples to be analyzed can have a significant impact on the residue concentration found. Additionally, the metabolism and/or degradation of active ingredients at the injection site may cause the composition of the total residue to vary significantly from values found in other tissues.

In view of all of the above, from a pharmacological viewpoint, the injection site is not directly comparable with muscle or other edible tissue. Similarly, withdrawal periods established for muscle tissue distant from the injection site are not suitable to guarantee that residues at the injection site will have dropped to safe concentrations within a given period. It must be taken into account that the procedure for calculating MRLs includes a risk analysis of veterinary drug residue in food based on the ADI which takes into consideration dietary exposure to residue throughout a lifetime.

Consequently, injection site residues need to be considered specifically in relation to the risk to the consumers of the treated animals.

4.2.1. Interpretation

It must be taken into account that the injection site withdrawal period estimated according to this guideline should not necessarily be considered the definitive withdrawal period for the veterinary drug analyzed.

The injection site withdrawal period must be considered in comparison with withdrawal periods based on residue depletion in other edible tissues. Lastly, the withdrawal period selected for the veterinary drug in question must be duly justified.

4.2.2. General principles_{x |}

It is necessary to identify the injection site residues that are of interest. In the case of veterinary drugs that contain new active ingredients, residues relating to active molecules must be properly identified, including metabolites and degradation and/or conversion products with potential biological impact. This information is obtained from radiometric residue depletion studies (eg: total residue) or, where appropriate, from residue depletion studies directed at the toxicological, pharmacological and microbiological characterization of residues.

For veterinary drugs that contain known active ingredients whose injection site residue composition is known, radiometric residue depletion studies are not necessary. What is needed is an appraisal of the original active ingredient or of any other relevant component of the injection site residue (eg: marker residue). Information relating to the ratio of marker residue / total residue can be obtained from information available in literature.

4.2.3 Study design and sampling

We recommend using Guide nr. 1 G.F: "Technical Guide for conducting Metabolism and Kinetic Studies of Residues of Veterinary Pharmacological Ingredients in Food Producing Animals", item 4.6.2.

The injection site residue depletion study report must include a full description, detailed study design and experimental conditions, selection of the injection site for the product, injection technique used, instruments used, depth of injection (intramuscular), measures taken to allow the

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exact localization of the injection site at slaughter, detailed description of the sample taking technique, and sample conditioning.

4.2.4 Procedure

It must be taken into account that the ingestion of injection site residues, as mentioned above, is a sporadic event. Therefore, the determination of the withdrawal period must be based on parameters that allow the quantification of the short-term risk of dietary exposure. Depending on the characteristics of the active ingredient, the Acute Reference Dose (ARfD) is the most suitable concept. Nevertheless, in some cases it may be recommended to use a different reference parameter. Also, the ARfD is not available for many active ingredients. In these cases another reference limit must be proposed and justified, including: therapeutic human dose, maximum recommended intake (eg: vitamins), maximum tolerable intake (ej: minerals/trace elements), basal or natural levels for compounds that can be produced endogenously (eg: hormones). A security factor should be considered for any reference limit selected.
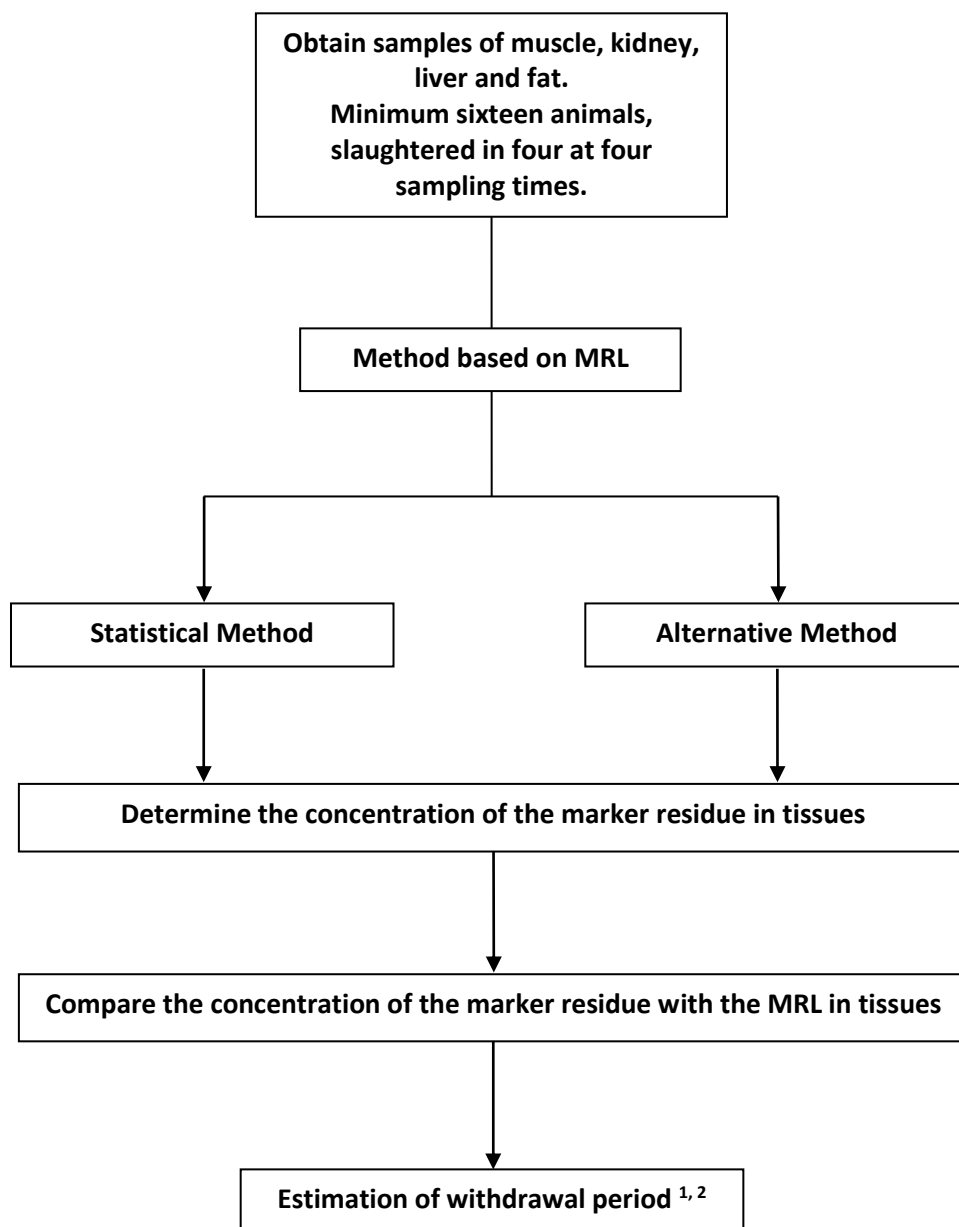
It is also possible to transform the MRL for muscle or fat (according to the animal species or the active ingredient in question) by applying a factor of 10 or more within an appropriate reference limit. The selection of any reference limit must be justified.

When this procedure is used, it is necessary to check that the marker residue is valid for predicting the target injection site residues.

Withdrawal periods must guarantee that the concentration of the residues has dropped below the chosen reference limit for the injection site within the specified period. It can be calculated as described in items 4.1.3 and 4.1.4 for edible tissues.

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COMPARATIVE PLOT OF SAMPLING AND ANALYSIS OF EDIBLE TISSUES AND ESTIMATION OF THE WITHDRAWAL PERIOD USING THE METHOD BASED ON THE MRL.

Obtain samples of muscle, kidney, liver and fat.
Minimum sixteen animals, slaughtered in four at four sampling times.

Method based on MRL

Statistical Method

Alternative Method

Determine the concentration of the marker residue in tissues

Compare the concentration of the marker residue with the MRL in tissues

Estimation of withdrawal period [1, 2]

1- The withdrawal period should be estimated for all edible tissues and will be calculated following the procedures proposed in this guideline. The longest withdrawal period will be considered OIE most appropriate one.
2- If the veterinary product is parenterally administered (IM – SC), the withdrawal period of the musle tissue will be replaced by the withdrawal period of tissues at the injection site.

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Registration of Veterinary Medicinal Products, GL 49 (MRK) - Method Used in Residue Depletion Studies. For consultation at step 4 - Draft 1.

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6. Annex

Statistical procedures

Bartlett´s Test

The Bartlett´s test[1] is used to check if a number of k samples come from populations presenting similar variances. Equality of variances across samples is called homogeneity of variances or homoscedasticity.

The use of Bartlett´s test is justified by the fact that many statistical tests, such as Student´s t-test of mean differences, or the variance analysis assume that variances across samples are equal.

The FDA[6, 7] recommends the use of this test due to its robustness, even though it is extremely sensitive to deviations from normality. On the other hand, this test can only be used when each group of data is equal to or higher than 5 (five). It presents the advantage of allowing the comparison of data groups of different sizes.

Bartlett´s test is used to check the nule hypothesis H_0 states that the population variances are equal, compared to the alternative hypothesis H_1 that states that at least two are different. If we count with k samples with a size of n_i samples and a S_i^2 variance, the statistical Bartlett test would be:

$$X^2 = \frac{(N-k)\ln(S_p^2) - \sum_{i=1}^{k}(n_i-1)\ln(S_i^2)}{1 + \frac{1}{3(k-1)}\left(\sum_{i=1}^{k}(\frac{1}{n_i-1}) - \frac{1}{N-k}\right)}$$

where: $N = \sum_{i=1}^{k} n_i$ an $S_p^2 = \frac{1}{N-k}\sum_i (n_i-1)S_i^2$ is the estimator of the total variance.

Bartlett`s statistical test has approximately a distribution X_{k-1}^2. The nule hypothesis is rejected when $X^2 > X_{k-1,\alpha}^2$, where $X_{k-1,\alpha}^2$, is the upper critical value for a distribution X_{k-1}^2.

Hartley´s Test

This test was developed in 1950 by Hartley[9] and is also known as the F_{max} test or Hartley´s F_{max} test. It is used in the analysis of variances to verify that different groups have similar variances, indispensable condition to make comparisons across groups through the application of parametric statistical test. The disadvantage of the test is that it can only be used to compare groups of data of the same size.

The test is based on the calculation of the relationship between the upper group variance (max s_j^2) and the lower group variance (min s_j^2). The resulting value is then compared to the critical value present in the $F_{max\,3,\,12}$ distribution table.

It is assumed that the groups present similar variances if the calculated value is lower than the critical value.

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The Hartley test assumes that the data of each group presents normal distribution and that the groups present the same number of individuals. This test, though convenient, is not very sensitive to deviations from normal distribution[10].

Cochran Test

This test, developed by William Gemmell Cochran[16] is the analysis of two randomized block designs, where the result of the comparison can only have two results. The Cochran test is also known as Cochran´s Q Test, it is a non-parametric statistical test.

The EMEA[5] considers it the best test, since it is simpler than Bartlett´s test. On the other hand, it is less seinsitive to deviations from normality than the latter and it can also be used to analyze groups of data of different sizes.

The test assumes that the number of experimental treatments is higher than two ($k>2$) and that the observations are arranged in a certain number of (b) blocks, as shown below:

	Treatment 1 (k_1)	Treatment 2 (k_2)	….	Treatment$_i$ (k_i)
Block 1 (b_1)	X_{11}	X_{12}	….	X_{1k}
Block 2 (b_1)	X_{21}	X_{22}	….	X_{2k}
Block 3 (b_1)	X_{31}	X_{32}	….	X_{3k}
Block 4 (b_1)	….	….	….	…..
Block$_i$ (b_i)	X_{b1}	X_{b2}	…..	X_{bk}

Cochran´s test is based on the nule hypothesis (H_0) that states that the treatments are equal and on the alternative hypothesis (H_1) which states that there is a difference across treatments.

Cochran´s statistical test is:

$$T = k\,(k-1) \sum_{j=1}^{k} \left(X_{\bullet j} - \frac{N}{k} \right)^2 \Big/ \sum_{i=1}^{b} X_{i\bullet}\,(k - X_{i\bullet})$$

where:

k is the number of treatments

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$X_{\cdot j}$ is the column total at j^{th} treatment

b is the number of blocks

$X_{i\cdot}$ is the total value of cells al $j^{\acute{e}simo}$ bloque$X_{\cdot j}$ is the total row at j^{th} block$N$ is the total value of all samples

The significance level of the critical region is given by:

$$T > X^2_{1-\alpha, k-1}$$

where $X^2_{1-\alpha, k-1}$ is the quantile $(1-\alpha)$ of chi-square distribution with k-1degree of freedom. The nule hypothesis is rejected if the statistical falls in the critical region.

Shapiro-Wilk Test

The test was published in 1965 by Samuel Shapiro and Martin Wilk[13], and is used to check the null hypothesis (H_0) that a sample $X_1,\ldots\ldots,X_n$ comes from a normally distributed population.

The test starts with a null hypothesis (H_0) which affirms that the experimental data come from a normally distributed population, and an alternative hypothesis (H_1) that states that the experimental data come from a not normally distributed population. The statistical test is:

$$W = \frac{\left(\sum_{i=1}^{n} a_i x_{(i)}\right)^2}{\sum_{i=1}^{n}(x_i - \overline{x})^2}$$

where:

$x_{(i)}$ is the ith order statistic, i.e.: the smallest number of the sample.

$\overline{x}$ is the sample mean.

The constants a_i are given by the following equation:

$$(a_1,\ldots,a_n) = \frac{m^\top V^{-1}}{(m^\top V^{-1}V^{-1}m)^{1/2}}$$

where:

$$m = (m_1,\ldots,m_n)^\top$$

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$m_1,….,m_n$ are the expected values of the order statistics of independent and identically distributed random variables, sampled from the standard normal distribution and V is the covariance matrix of those order statistics.

The null hypothesis is rejected when the value statistic (W) is lower than the chosen alpha level (0.05).   In case the statistic (W) is higher than the chosen alpha level, then the null hypothesis cannot be rejected and experimental data are assumed to be from a normally distributed population.

Calculation of the upper limit of the tolerance interval

The FDA proposes to estimate the upper limit of the tolerance interval at 99% with a 95% confidence interval. However, an objection to this criterion is the excessive extrapolation of the values of tolerance interval, since many times this intersects the MRL value posteriorly to the values of the last detected tissue concentrations. This excessive extrapolation can result in an inadequate estimation of the withdrawal time. The EMEA[5] proposes a 95% tolerance interval, which minimizes the extrapolation problem and provides a more realistic estimation of the withdrawal period.

Procedure of non-central t distribution (FDA)

The upper limit of the tolerance interval at any time is calculated with the following equation:

$$T(y) = a + b.t + k.s. \left[\frac{1}{n} \cdot \left(\frac{t - \bar{xt}}{\sum t_i - \bar{xt}} \right)^2 \right]^{0.05}$$

where:

$k = $ 95th percentile of a non- central"t" distribution with a non-central "d" parameter and a degree of freedom equal to S^2.

$$d = \frac{z}{\left[\frac{1}{n} \cdot \left(\frac{t - \bar{xt}}{\sum t_i - \bar{xt}} \right)^2 \right]^{0.05}}$$

$z = $ 95th percentile of a normalized standard distribution.

In order to calculate the "k" value, see D.B. Owen, *Handbook of Statistical Tables, Addison-Wesley, Reading, Massachusetts* (1962)[11,] and employ "d" value and the table of factors for the

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calculation of critical values for the non-central "*t*" distribution with a 95% percentile (0, 95) and "*n*" degree of freedom.

The upper limit of the tolerance interval is calculated as the antilogarithm of the calculated value. Check that the estimated value does not exceed the MRL value. If that happens, *t* value should be increased and the calculation procedure should be repeated until the estimated value is lower than the MRL, in that case *t* value is consistent with the withdrawal period.

Stange equation (EMEA)

The calculation of the upper limit of a 95% tolerance interval with a 95% confidence interval can be also be performed following the procedure reported by Stange[14], as described below:

$$Ty = a + bt + k_T s_{y.x}$$

where:

Ty = upper limit of the tolerance interval at a determined sampling time.

a = point where the straight line crosses the ordinate- axis

b = slope of the straight line

t = time.

$$k_T = \frac{\sqrt{(2n-4)}}{(2n-4)^* - u_{1-\alpha}^2} \left[\sqrt{(2n-4)^*}\, u_{1-\gamma} + u_{1-\alpha} W_n \right]$$

$$W_n = \sqrt{ u_{1-\gamma}^2 + \left[(2n-4)^* - u_{1-\alpha}^2 \right] \left[\frac{1}{n} + \frac{(x-\bar{x})^2}{S_{xx}} \right] }$$

$$s_{xx} = \sum x_i^2 - \frac{1}{n} \left(\sum x_i \right)^2$$

The respective standard normal distribution statistical values are:

- For 1-α = u$_{1-\alpha}$ = 1,6449
- For 1-α = u$_{1-\alpha}$ = 1,6449

$S_{y, x}$ = residual error, ()* = (2n-5) according to Graf et al.[7].

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The correction proposed by Graaf[8] (using the term (2n-5) instead of (2n-4), results in a slightly higher tolerance interval limit. According to Stange, the equation is valid for a value $n \approx 10$, while Graf increases the validity of the estimation to a value $n \approx 20$.

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