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STUDIES TO EVALUATE THE SAFETY OF RESIDUES OF VETERINARY DRUGS IN HUMAN FOOD: GENERAL APPROACH TO ESTABLISH A MICROBIOLOGICAL ADI

Revision at Step 9

Recommended for Consultation
at Step 4 of the VICH Process
on 24 February 2011
by the VICH Steering Committee

This Guideline has been developed by the appropriate VICH Expert Working Group and is subject to consultation by the parties, in accordance with the VICH Process. At Step 7 of the Process the final draft will be recommended for adoption to the regulatory bodies of the European Union, Japan and the USA.

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GENERAL APPROACH TO ESTABLISH A MICROBIOLOGICAL ADI

1. INTRODUCTION

1.1. Objectives of the guideline

A variety of toxicological evaluations are performed to establish the safety of veterinary drug residues in human food. An issue that needs to be addressed for veterinary antimicrobial drugs is the safety of their residues on the human intestinal flora. The objectives of this guideline are (1) to outline the steps in determining the need for establishing a microbiological acceptable daily intake (ADI); (2) to recommend test systems and methods for determining no-observable adverse effect concentrations (NOAECs) and no-observable adverse effect levels (NOAELs) for the endpoints of health concern; and (3) to recommend a procedure to derive a microbiological ADI. It is recognized that different tests may be useful. The experience gained with the recommended tests may result in future modifications to this guideline and its recommendations.

1.2. Background

The intestinal flora plays an important role in maintaining and protecting the health of individuals. This flora provides important functions to the host such as (1) metabolizing endogenous and exogenous compounds and dietary components; (2) producing compounds that are later absorbed; and (3) protecting against the invasion and colonization by pathogenic microorganisms.

Ingested antimicrobial drugs can potentially alter the ecology of the intestinal flora. They may reach the colon due to incomplete absorption or may be absorbed, circulated and then excreted via bile or secreted through the intestinal mucosa.

The microbiological endpoints of current public health concern that should be considered when establishing a microbiological ADI are:

Disruption of the colonization barrier: The colonization barrier is a function of the normal intestinal flora that limits colonization of the colon by exogenous microorganisms, as well as overgrowth of indigenous, potentially pathogenic microorganisms. The capacity of some antimicrobial drugs to disrupt this barrier is well established and known to have human health consequences.

Increase of the population(s) of resistant bacteria: For the purposes of this guideline, resistance is defined as the increase of the population(s) of bacteria in the intestinal tract that is (are) insensitive to the test drug or other antimicrobial drugs. This effect may be due either to the acquisition of resistance by organisms which were previously sensitive or to a relative increase in the proportion of organisms that are already less sensitive to the drug.

> An extensive literature review did not reveal reports of human health effects (e.g. prolonged antimicrobial therapy, prolonged hospital stay, predisposition to infection, and treatment failure, etc.) that occur as a result of changes in the proportion of antimicrobial resistant bacteria in the normal human intestinal flora. However, based on the understanding of microbial ecology, such effects cannot be excluded.

Although the effect of antimicrobial residues in food on the human intestinal flora has been a concern for many years, a harmonized approach to determine the threshold dose that might adversely disturb the flora has not been established. International regulatory bodies have used a formula-based approach for determining microbiological ADIs for antimicrobial drugs. These formulae take into consideration relevant data including minimum inhibitory concentration (MIC) data against human intestinal bacteria. Due to the complexity of the intestinal flora, uncertainty factors have been traditionally included in the formula. However, the use of uncertainty factors results in conservative estimates and it was considered that more relevant test systems should be developed that would allow a more realistic estimate of a microbiological ADI possibly without the use of these factors.

The present guideline is an attempt to address the complexity of the human intestinal flora and reduce uncertainty when determining microbiological ADIs. The guideline outlines a process for determining the need for a microbiological ADI and discusses test systems that take into account the complexity of the human intestinal flora. These test systems could be used for addressing the effects of antimicrobial drug residues on human intestinal flora for regulatory purposes.

Since further research is needed to confirm the reliability and validity of all test systems discussed in this guideline (see Appendix A), this guideline does not recommend any one particular system for use in regulatory decision-making. Instead, this guideline provides recommendations for a harmonized approach to establish a microbiological ADI and offers test options rather than specifying a testing regimen.

1.3. Scope of the guideline

This document provides guidance for assessing the human food safety of residues from veterinary antimicrobial drugs with regard to effects on the human intestinal flora. However, it does not limit the choice of studies that may be performed to establish the safety of residues in human food with respect to adverse effects on human intestinal flora. This guidance does not preclude the possibility of alternative approaches that may offer an equivalent assurance of safety, including scientifically based reasons as to why microbiological testing may not need to be provided.

2. GUIDELINE

If a drug intended for use in food-producing animals has antimicrobial activity, the safety of its residues needs to be addressed with respect to the human intestinal flora. Derivation of a microbiological ADI is only necessary if residues reach the human colon and remain microbiologically active.

2.1. Steps in determining the need for a microbiological ADI

When determining the need for a microbiological ADI, the following sequence of steps is recommended. The data may be obtained experimentally or from other appropriate sources such as scientific literature.

Step 1. Are residues of the drug, and (or) its metabolites, microbiologically active against representatives of the human intestinal flora?

- Recommended data:
  - MIC data, obtained by standard test methods, from the following relevant genera of intestinal bacteria (*E. coli*, and species of *Bacteroides, Bifidobacterium, Clostridium, Enterococcus, Eubacterium* (*Collinsella*), *Fusobacterium, Lactobacillus, Peptostreptococcus/Peptococcus).*

  - It is recognized that the understanding of the relative importance of these microorganisms is incomplete and that the taxonomic status of these organisms can change. The selection of organisms should take into account current scientific knowledge.

- If no information is available, assume that the compound and (or) its metabolites are microbiologically active.

Step 2. Do residues enter the human colon?

- Recommended data:
  - Absorption, distribution, metabolism, excretion (ADME), bioavailability, or similar data may provide information on the percentage of the ingested residue that enters the colon.
  - If no information is available in humans, use appropriate animal data. If there is no available information, assume that 100% of the ingested residue enters the colon.

Step 3. Do the residues entering the human colon remain microbiologically active?

- Recommended data:
  - Data demonstrating loss of microbiological activity from *in vitro* inactivation studies of the drug incubated with feces or data from *in vivo* studies evaluating the drug's microbiological activity in feces or colon content of animals.

If the answer to any of questions in steps 1, 2, or 3 is "no", then the ADI will not be based on microbiological endpoints and the remaining steps need not be addressed.

Step 4. Assess whether there is any scientific justification to eliminate the need for testing either one or both endpoints of concern. Take into account available information regarding colonization barrier disruption and resistance emergence for the drug. If a decision cannot be made based on the available information, both endpoints need to be examined.

Step 5. Determine the NOAECs/NOAELs for the endpoint(s) of concern as established in step 4. The most appropriate NOAEC/NOAEL is used to determine the microbiological ADI.

2.2. Recommendations for determining NOAECs and NOAELs for the endpoints of concern

2.2.1. Disruption of the colonization barrier

2.2.1.1. Detection of colonization barrier disruption

Changes in bacterial populations are indirect indicators of potential disruption of the colonization barrier. These changes can be monitored by various enumeration techniques in a variety of test systems. A more direct indicator of barrier disruption is the colonization or overgrowth of an intestinal ecosystem by a pathogen. *In vivo* test systems or complex *in vitro* test systems (e.g. fed-batch, continuous, or semi-continuous culture systems) have the potential to evaluate barrier disruption as evidenced by colonization of a challenge organism added to the test system.

Challenge organisms (e.g. *Salmonella, Clostridium*) should be insensitive to the test drug. Inoculation schemes with the challenge organisms should take into account the timing of the challenge relative to drug treatment, the number of organisms per challenge dose, and the number of times that the test system is challenged.

2.2.1.2. Test systems and study design

2.2.1.2.1. *In vitro* tests

The use of MICs to assess the potential for a drug to disrupt the colonization barrier does not take into account the complexity of the human intestinal flora. Therefore, the MIC_{50} of the most relevant genus/genera for which the drug is active (see Section 2.1.) results in a conservative estimate of a NOAEC for disruption of the colonization barrier. The NOAEC estimate is conservative because, among other reasons, the inoculum density is orders of magnitude lower than the bacterial population in the intestinal tract[1]. Therefore, it may be considered as an option to establish an ADI. The isolates should be obtained from multiple healthy individuals, and include a minimum of 10 isolates from each of the genera listed in Section 2.1.

Each MIC test of a pure culture of a relevant isolate provides data for a single strain of a species. Other *in vitro* test systems provide information for hundreds of bacterial species (>10^8 bacterial cells/g) for each fecal inoculum. Each inoculum can be tested in replicate to determine treatment effects. Based on all the above, *in vitro* systems using fecal batch cultures are inherently more robust and relevant than the MIC test system.

Other test systems discussed below, which model the intestinal flora, may result in a more appropriate NOAEC and possibly a higher ADI.

Fecal slurries provide a simple test system to derive a NOAEC for disruption of the colonization barrier following short-term exposure to the drug and may be appropriate for dose-titration studies. The slurries can be monitored for changes in bacterial populations and the production of short chain fatty acids. These two response variables, when monitored together, can be used as indirect indicators of barrier disruption. The NOAEC derived from this test system may prove to be a conservative estimate of barrier disruption.

Semi-continuous, continuous and fed-batch cultures of fecal inocula may be appropriate to evaluate disruption of the colonization barrier following prolonged exposure to the drug. However, exploratory work using continuous and semi-continuous cultures has given various NOAECs for barrier disruption because of differences in protocols. As a consequence, study designs should take into account the issues raised in Appendix A.

In the case of fecal slurries, semi-continuous and continuous cultures, and fed-batch cultures of fecal inocula, there are unresolved issues such as the impact of fecal inocula (individual variation and gender), dilution rate, duration of drug exposure, and reproducibility of the tests.

2.2.1.2.2. *In vivo* tests

In vivo test systems using human flora-associated (HFA) and conventional laboratory animals may be suitable for the assessment of disruption of the colonization barrier. Compared to conventional laboratory animals, the intestinal flora of HFA animals possesses greater similarity to the human intestinal flora, both in terms of the range of bacterial populations and metabolic activity. However, the intestinal flora derived from humans may not be stable in the HFA animals. The relative importance of the stability of the implanted flora and the specific composition of the flora is unknown. For technical reasons, the conventional laboratory animal can be tested in higher numbers, which allows a more robust statistical analysis of the results.

Study design should take into account factors such as animal species, gender, inoculum variability among donors, number of animals per group, diet, randomization of treatment groups, minimization/elimination of coprophagy, housing of animals within an isolator, cross contamination within the isolator and route of drug administration (e.g. gavage, drinking water). Germ-free animals should be inoculated in sequence, first with a *Bacteroides fragilis* strain, followed by the fecal inoculum.

2.2.2. Increase in the population(s) of resistant bacteria in the human colon (as defined in Section 1.2.)

The guidance below highlights the considerations that need to be taken into account when addressing this endpoint.

2.2.2.1. Detection of changes in the population of resistant bacteria

Studies to evaluate the emergence of resistance should take into account the organisms of concern in the intestinal tract and the documented resistance mechanisms to the drug class. Preliminary information regarding the prevalence of resistance in the human intestinal flora, such as daily variation within individuals and the variation among individuals can be useful in developing criteria for evaluating resistance emergence. MIC distributions of sensitive and known resistant organisms of concern can provide a basis to determine what drug concentration should be used in the selective agar media to enumerate resistant organisms in the fecal samples. Since drug activity against an organism can vary with test conditions, the MIC of the organism growing on selective medium should be compared to the MIC determined by standard methods (e.g., National Committee for Clinical Laboratory Standards [NCCLS][2,3]). Changes in the proportions of resistant organisms during pre-treatment, treatment and post-treatment periods can be evaluated by enumeration techniques on media with and without the antimicrobial drug, applying phenotypic and molecular methodologies.

Changes in antimicrobial resistance can be influenced by factors other than drug exposure (e.g. animal stress) which should be taken into consideration in animal test systems.

2.2.2.2. Test systems and study design

2.2.2.2.1. *In vitro* tests

The duration of exposure required for resistance to develop in a population of bacteria can be dependent on the drug, the nature of the resistance mechanisms, and how it evolves in nature (e.g. by gene transfer between cells, by gene mutations). For these reasons acute studies of pure cultures to assess the endpoint are not considered to be appropriate. Therefore, MIC tests cannot be used to determine a NOAEC for increases in resistant populations.

Defined cultures may provide useful information to determine the potential for a resistant population to emerge due to mutation in an isolate and/or gene transfer among isolates. However, these test systems are not designed to evaluate changes in resistant populations and are not recommended.

Tests systems using short-term exposure of fecal slurries to a drug are not recommended for resistance emergence testing because the duration of the test is inadequate to assess changes in resistant populations.

Continuous and semi-continuous cultures and fed-batch cultures of fecal inocula provide a means to evaluate long-term exposure of bacteria to the drug. Refer to Appendix A for issues that must be addressed regarding study conduct and data evaluation.

2.2.2.2.2. *In vivo* tests

Changes in resistant populations can be assessed in HFA-rodents. General study design and supporting protocol should follow the recommendations stated in 2.2.1.2.2. The test system supports a complex flora, and would be a source of genetic resistance determinants. The system accommodates more replication than the continuous or semi-continuous culture systems, but less than fed-batch cultures. The variability of the HFA-rodent test has not been assessed; however it is useful for identifying gender differences. There are also advantages to conducting resistance studies in conventional laboratory animals.

HFA-rodents and conventional animals provide means to evaluate the potential for resistance emergence following long-term exposure of bacteria to the drug. Refer to Appendix A for issues that must be addressed for study conduct and data evaluation.

2.3. General recommendations

- Fecal samples or bacterial isolates from human donors should be obtained from healthy subjects with no known exposure to antimicrobial agents for at least 3 months.

- In the case of *in vivo* tests, the test species selected for testing should allow for (1) maximum independent replication; (2) sufficient quantity of feces to be collected for analyses; and (3) minimal coprophagy. Evaluation of both genders should be considered unless data demonstrate that only one gender is appropriate.

- Statistical issues need to be addressed when designing studies of antimicrobial residues (see Appendix B).

- The pre-validation and validation process, such as that being developed by OECD since 1996[4], should be considered for subsequent validation of test systems to assess the effects of antimicrobial drugs on human intestinal flora. The process should be adapted and modified for this use depending on the test system being validated.

- Study designs should take into account unresolved issues of the effects of storage and incubation conditions on fecal inocula.

2.4. Derivation of a microbiological ADI

When more than one value can be determined for the microbiological ADI, in accordance with the methods discussed below, the most appropriate value (relevant to humans) should be used.

2.4.1. Disruption of the colonization barrier

2.4.1.1. Derivation of an ADI from *in vitro* data

If the endpoint of concern is disruption of the colonization barrier, the ADI may be derived from MIC data, fecal slurries, semi-continuous, continuous, and fed-batch culture test systems.

ADI derived from MIC data:

$$ADI = \frac{MIC_{calc} \times \text{Mass of Colon Content (220 g/day)}}{\text{Fraction of oral dose available to microorganisms} \times \text{60 kg person}}$$

MIC_{calc}: The MIC_{calc} is derived from the lower 90% confidence limit for the mean MIC_{50} of the relevant genera for which the drug is active, as described in Appendix C.

ADI derived from other *in vitro* test systems:

$$ADI = \frac{NOAEC \times \text{Mass of Colon Content (220 g/day)}}{\text{Fraction of oral dose available to microorganisms} \times \text{60 kg person}}$$

NOAEC: The NOAEC derived from the lower 90% confidence limit for the mean NOAEC from *in vitro* systems should be used to account for the variability of the data. Therefore, in this formula uncertainty factors are not generally needed to determine the microbiological ADI.

Mass of colon content: The 220 g value is based on the colon content measured from accident victims.

Fraction of an oral dose available for microorganisms: The fraction of an oral dose available for colonic microorganisms should be based on *in vivo* measurements for the drug administered orally. Alternatively, if sufficient data are available, the fraction of the dose available for colonic microorganisms can be calculated as 1 minus the fraction (of an

oral dose) excreted in urine. Human data are preferred, but in its absence, non-ruminant animal data are acceptable. In the absence of data to the contrary, it is assumed that metabolites have antimicrobial activity equal to the parent compound. The fraction may be lowered if the applicant provides quantitative *in vitro* or *in vivo* data to show that the drug is inactivated during transit through the intestine.

2.4.1.2. Derivation of an ADI from *in vivo* data

The microbiological ADI is the NOAEL divided by the uncertainty factor.

Uncertainty factors for *in vivo* studies should be assigned as appropriate, taking into consideration the class of compound, the protocol, numbers of donors, and sensitivity of the measured outcome variables.

2.4.2. Increase in the population(s) of resistant bacteria

2.4.2.1. Derivation of an ADI from *in vitro* data

If the endpoint of concern is an increase in the population(s) of resistant bacteria, NOAECs derived from semi-continuous, continuous, and fed-batch culture test systems may be used to establish a microbiological ADI.

$$\text{ADI} = \frac{\text{NOAEC} \times \text{Mass of Colon Content (220 g/day)}}{\text{Fraction of oral dose available to microorganisms} \times \text{60 kg person}}$$

NOAEC: The NOAEC derived from the lower 90% confidence limit for the mean NOAEC from *in vitro* systems should be used to account for the variability of the data. Therefore, in this formula uncertainty factors are not generally needed to determine the microbiological ADI. However, where there are concerns arising from inadequacies in the quality or quantity of *in vitro* data used in determining the NOAEC, the incorporation of an uncertainty factor may be warranted.

2.4.2.2. Derivation of an ADI from *in vivo* data

The microbiological ADI is the NOAEL divided by the uncertainty factor.

Uncertainty factors for *in vivo* studies should be assigned as appropriate, taking into consideration the class of compound, the protocol, numbers of donors, and sensitivity of the measured outcome variables.

3. GLOSSARY

The glossary includes terminology referred to in the Appendices as well as in the text.

Acceptable Daily Intake (ADI)	An estimate of the amount of a substance, expressed on a body weight basis, that can be ingested daily over a lifetime without appreciable risk to human health.
Antimicrobial Activity	The effect of an antimicrobial agent on a bacterial population.
Antimicrobial Agent	A drug substance that is either biologically derived or chemically produced with antimicrobial activity as its major effect.
Balanced Design	A statistical design is balanced if each combination of values or levels of all factors in the design (treatment factors, factors of interest such as gender, or blocking factors) have the same number of experimental units or replicates. A partially balanced design is not balanced, but combinations of treatments and other factors occur in a regular way such that the analysis remains relatively simple.
Batch Culture	A culture where neither substrate nor waste products are removed until completion of incubation, normally incubated for short periods, generally up to 24 hours.
Blocking Factor	An experimental factor whose values or levels define groups of experimental units that are similar or that can be expected to respond in a similar manner. Systematic variation among blocks can be removed from the estimate of error in the statistical analysis, resulting in greater precision. An example is a cage containing several animals, which are the experimental units, or an isolator containing several cages.
Challenge organism	An organism added experimentally to a test system to evaluate colonization barrier disruption.
Colonization	The establishment of microorganisms in the intestinal tract.
Colonization Barrier	A function of the normal intestinal flora that limits colonization of the colon by exogenous microorganisms, as well as overgrowth of indigenous, potentially pathogenic microorganisms.

Complete Design	A statistical design is complete if all combinations of factors or groups in the design have at least one observation. An incomplete design is one in which no observations are made for some combinations of factors.
Continuous Culture	A culture maintaining continuous growth of microorganisms by the simultaneous supply of nutrient and removal of spent medium, maintaining a constant microbial load within a fixed incubation volume.
Conventional Laboratory Animal	A laboratory animal with its natural indigenous intestinal flora.
Coprophagy	The ingestion of feces.
Defined Culture	A microbial culture in which all microbial species are known.
Dilution (Flow) Rate	The rate of supply and removal of medium from a continuous culture system. Dilution rate controls the microbial growth rate within a continuous culture system.
Donor (Fecal) Inocula	Fecal flora obtained from human volunteers and used to inoculate the test system. Fecal flora is considered to be equivalent to the intestinal flora.
Drug Residue	The drug, including all derivatives, metabolites and degradation products that persists in or on food.
Experimental Unit	The standard subject to which a treatment is applied and a measurement is made. Examples include a whole animal or a specific organ or tissue, a cage containing several animals, a cell culture.
Factorial Design	An experimental design that involves combinations of a number of factors, including a treatment factor, each having two or more values or levels. Other factors may include stratification (e.g. gender) or blocking factors (e.g. cage). Typically the outcome variable is measured on a number of experimental units at each combination of levels of the various factors. The statistical analysis of the data involves a multifactorial analysis of variance.
Fecal Slurry	Human feces or fecal solids minimally diluted in anaerobic buffer.
Fed-Batch Culture	A batch culture fed continuously or semi-continuously with nutrient medium. Portions of the fed batch culture can be

	withdrawn at pre-determined intervals. A constant culture volume is not maintained.
Human Flora-Associated (HFA) Animal	A germ-free host animal implanted with human fecal flora.
Interaction Effect	Treatment effects that are modified by the presence of other factors. For example, the effect of a treatment may be greater or less in males than females, or may change over time.
Intestinal Flora	The normal microbial flora of the colon.
Minimum Inhibitory Concentration (MIC)	The lowest concentration of an antimicrobial compound that inhibits growth of the test organism as determined by standardized test procedures.
MIC_{50}	The concentration of an antimicrobial compound at which 50% of the tested isolates within a relevant genus are inhibited.
Microbiological ADI	An ADI established on the basis of microbiological data.
No-Observable Adverse Effect Concentration (NOAEC)	The highest concentration that was not observed to cause any adverse effect in a particular study.
No-Observable Adverse Effect Level (NOAEL)	The highest administered dose that was not observed to cause any adverse effect in a particular study.
Outcome Variable	A specific parameter measured in an experiment. Specific outcome variables must be defined as part of the protocol, and are the measurements actually made in the study.
Semi-continuous Culture	A culture where substrate and/or waste products are added and/or removed in a semi-continuous manner maintaining a fixed incubation volume.
Short Chain Fatty Acid	The volatile fatty acids that are produced by the intestinal flora. The principal acids are acetic, propionic and butyric.
Solid Phase	The particulate matter in an *in vitro* test system.
Systematic Variation	Factors that affect outcome variables. Such variation is systematic in the sense that it represents an effect that is reliably present. Systematic variation is distinguished from random variation, which is not predictable. Systematic variation may be caused by factors that are of interest, such as gender, or by factors such as the particular isolator, which are not.

Test System	A method used to determine the effects of antimicrobial residues on the human intestinal flora.
Uncertainty Factor	A correction factor that takes into account the characteristics of the test data as described in 2.4.1.2., 2.4.2.1. and 2.4.2.2.

4. REFERENCES

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APPENDIX A

Issues that Need to be Investigated in Developing Test Systems and Data Interpretation

1. Experimental Conditions

Data generated for continuous flow, semi-continuous flow and fed-batch studies will be affected by the growth conditions (e.g., growth medium, pH, dilution rate). Different bacterial species may have different growth rates under the experimental conditions used for the test system. If the dilution rate of the culture exceeds the growth rate of a bacterial species, then this species ultimately will be eliminated from the test culture. The test system should be designed to maximize the retention of the different bacteria, and maintain the complexity of the initial inoculum.

Test antimicrobial agents can affect growth rates of various bacterial groups. This may lead to loss of components of the mixed culture by a reduction of growth rate below that of the dilution rate used in the test system, which might cause some components of the flora to be washed out of the culture. This may be minimized by developing test conditions with lower dilution rates.

Antimicrobial susceptibility is influenced by the physical condition of the exposed organisms, which will be influenced by the growth conditions used in the test system. Based on the above, further work is needed to determine the impact of different growth conditions on the NOAECs derived for colonization barrier disruption and the increase in the population of resistant bacteria.

A number of factors should be considered in protocols for *in vivo* test systems. For example, cross-contamination is a major issue when performing animal studies within a germ-free isolator. The protocol should be designed to minimize cross-contamination.

2. Inoculum

The composition of the intestinal flora may vary among individuals with respect to bacterial groups and resistant organisms. The bacterial populations are relatively stable within a single individual, but this is not necessarily the case for resistant bacterial groups.

Multiple donors should be used to account for differences in flora between individuals. Pooled inocula do not account for differences in flora between individuals. Therefore, test systems that use fecal inocula obtained from individual donors are preferred to determine the effect of antimicrobial residues on the intestinal flora. In addition, the composition of the donor inocula should be taken into account when interpreting study results.

3. Study duration

The optimum incubation time to monitor for changes in bacterial populations in fecal batch cultures needs to be determined. Likewise, in the case of complex long-term in vitro or in vivo test systems, it is important to determine the period during which the integrity and complexity of the intestinal flora remains stable and representative of the intestinal flora.

APPENDIX B

Statistical Issues to be Considered When Designing Studies of Antimicrobial Residues

Two broad endpoints of current public health concern were identified, disruption of the colonization barrier and increases in population(s) of resistant bacteria. The experimental design must depend on which of these is to be addressed and should take account of the particular outcome variables. A design paradigm for these test systems involves choice of the test system, application of treatments and follow-up of the system over time. The choice of test system depends on the characteristics of the human intestinal tract that must be represented by the test system. Since the MIC tests are simple in design, many of the issues discussed below do not apply to this method.

The experimental unit is a central component of the study design. For an *in vivo* test system, for example, the unit may be an individual animal or an entire cage. If cages are grouped within isolators, some or all of the treatments to different cages within each isolator can be applied. In this case the isolator becomes a blocking factor, since cages within the same isolator would be expected to respond in a similar fashion. The use of blocking factors is an important tool for reducing systematic variation. A related question is whether there are other systematic factors such as gender that must be included, that is, whether a factorial design must be used. If there are multiple factors, then the design involves choices of what combinations of these should be included. It is important that this be done in such a way that the resulting design is balanced. In a complete, balanced design, all combinations are represented, and occur the same number of times. It is also possible to have incomplete designs, as well as various kinds of partial balance. For such designs the analysis of variance may be required, but such designs can be useful when, for example, experimental resources are limited. An example of an incomplete design is the standard two period cross-over design.

It must be decided how the treatments should be applied to the experimental units. In some cases a two-stage treatment, involving a drug treatment and a bacterial challenge, may be necessary. There should be at least three antimicrobial treatment groups in addition to appropriate control groups. The choice of antimicrobial treatment levels will depend on the desired range of doses, but should cover both effect and no-effect levels. The duration and the method of drug administration will depend on the test system. An important aspect of some studies is the evolution of effects over time, and repeated measurement of outcome variables may be required. Common issues are the timing and spacing of the measurements and bias caused by missing data.

Control of random variation due to biological variability and to measurement error depends on the number of experimental units and number of samples. This number can be determined from previous knowledge of the test system and outcome variables, either from past experience or through a sample size computation, which should be employed where possible. Sufficient replication should be included to allow precise measurement of treatment effects and appropriate interaction effects, e.g., treatment effects that change

over time. In some studies, it may be important to examine such interaction effects as part of the statistical analysis. Another type of replication is the pooling of fecal samples from animals in a single cage or the pooling of fecal samples from different donors. In both cases, we have the benefits of averaging, but not the ability to estimate variability among replicates. Pooling may obscure individual effects (of treatment and/or inoculum), and thus its use must be considered in terms of study objectives.

APPENDIX C

Calculation of MIC_{calc}

The MIC_{CALC} is derived from the lower 90% confidence limit for the mean MIC_{50} of the most relevant genera for which the drug is active. The lower 90% confidence limit is calculated using log transformed data. Thus the mean and standard deviation are calculated using the log transformed MIC_{50} values. This also implies that the lower 90% confidence limit needs to be back-transformed to obtain the correct value. The formula for the confidence limit is:

$$lower\ 90\%\ CL = Mean\ MIC_{50} - \frac{StdDev}{\sqrt{n}} \times t_{0.10,df}$$

where: ***Mean MIC_{50}*** is the mean of the log transformed MIC_{50} values,
Std Dev is the standard deviation of the log transformed MIC_{50} values,
n is the number of MIC_{50} values used in the calculations,
$\mathbf{t_{0.10,df}}$ is the 90th percentile from a central t-distribution with df degrees of freedom, and df = n-1.

Examine the MIC_{50} of relevant genera (see Section 2.1). The MIC_{calc} is based on a summary value of those genera which are not inherently resistant to the compound. Thus the MIC_{calc} is based on MIC_{50} of those genera for which the compound is active. Ensure that all MIC_{50} values are not characterized as "</=", so they may be used in the calculation of the MIC_{calc}.

Example Calculation

Any base log transformation of MIC_{50} values can be used. However, if 2-fold dilutions of drug are used in the MIC testing procedure, a base 2 log transformation conveniently will provide integer values for the calculation. In the following example, the MIC_{50} values were transformed as follows:

$$Log_2(MIC_{50}) - Log_2(minimum(MIC_{50})/2)$$

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Example calculation of MIC_{calc}								
Bifidobacterium	*Eubacterium*	*Clostridium*	*Bacteroides*	*Fusobacterium*	*Enterococcus*	*Escherichia coli*	*Peptococcus/ Peptostreptococcus*	*Lactobacillus*
MIC_{50}								
0.03125	0.25	0.25	8.0	32	2.0	>128	0.25	1.0
$Log_2(MIC_{50}) - Log_2(0.03125/2)$								
1	4	4	9	11	7	R*	4	6
Mean ($Log_2(MIC_{50}) - Log_2(0.03125/2)$) = 5.75								
StdDev ($Log_2(MIC_{50}) - Log_2(0.03125/2)$) = 3.196								
$t_{0.10,7}$ = 1.415								
Lower 90% Confidence Limit = 5.75 – 3.196/sqrt(8)*1.415 = 4.15								
Back-transforming to the MIC scale = $2^{(4.15 + \log2(0.03125/2))}$ = 0.277								
MIC_{calc} = 0.277								
* MIC_{50} values of inherently resistant genera are not included in the calculation								

APPENDIX D

Supplement to Section 2 Regarding the Determination of the Fraction of Oral Dose Available to Microorganisms

1. Introduction

VICH GL 36 has been implemented since 2005. Having gained experience in working with the guideline, regulators from all VICH regions agreed that additional guidance and clarity were needed regarding *in vivo* and *in vitro* testing methods to determine the fraction of oral dose available to microorganisms.
This Appendix is based on review of new data, scientific literature, and information from disclosed sponsor submissions.

This Appendix contains three sections: a table of examples of test systems for the assessment of the fraction of oral dose available to microorganisms, general considerations regarding methodological aspects of the implementation of these test systems, and a description of how the test systems could be used in determining the fraction of oral dose available to microorganisms.

2. Examples of Test Systems for the Assessment of the Fraction of Oral Dose Available to Microorganisms

Various *in vitro* and *in vivo* test systems could be used separately and in combination to determine the fraction of oral dose available to microorganisms. The table below provides examples of such test systems, the type of data generated and considerations relevant to their use.

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Examples of Test Systems and Assay Methodology for the Assessment of the Fraction of Oral Dose Available to Microorganisms*		
Test System	**Type of Data Generated**	**Considerations**
	***In Vivo* Test Systems**	
Human and (or) animal absorption, distribution, metabolism and excretion (ADME) studies	- Concentration of administered drug (and metabolites) in urine and (or) feces - Metabolite profile of administered drug in urine and (or) feces - Percentage of administered drug entering the colon	- Data from oral (not parental) route of dosing should be used. - Oral dose levels given to the animals and duration of dosing may be considered. - Data for drug candidate are preferred, although data from humans dosed orally with a drug analog of the same class may provide supportive information. -When human ADME data are not available, ADME data from animals can be used. - Residue depletion studies in the target species may provide information about fecal metabolite profiles and (or) drug available to colonic microorganisms. - Data derived from chemical or radiolabel assays may be complemented by data from microbiological assays to determine the percentage of oral dose available to microorganisms.
Experimental animals dosed orally to determine drug available to colonic microorganisms	- Concentrations of drug in feces or intestinal contents determined by microbiological and (or) chemical assays - Metabolite profile in feces or intestinal contents	- Oral dose levels given to the animals and duration of dosing may be considered. - Human flora-associated rodents and conventional animals may be considered. - Ruminants and avian species are not appropriate.

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***In Vitro* Test Systems**		
Drug added to fecal slurries to determine fraction of drug available to microorganisms	-Concentration (mass per unit volume) of free drug in the test system -Percentage of added drug that is bound -Amount of added drug that is metabolized in the fecal slurries	- The experimental design should include considerations of incubation, sampling time points for kinetics, drug concentrations to be tested, fecal parameters such as non-sterilized and sterilized feces, and other test conditions. - Assays include both determination of microbiological activity and chemical analysis of the drug (see Microbiological and Chemical Assay Methodologies). - Incubation of non-sterile fecal slurries can be used to determine drug degradation.
Microbiological Assay Methodology		
Microbiological assays to measure microbiological activity of drug concentrations in fecal samples or fecal slurry incubations	- Quantification of microbial growth or inhibition of growth to measure free drug concentrations	- For quantitative microbiological assays, the choice of the indicator bacterial strain should take into account the method used and the spectrum of activity of the drug. - Testing could include, for example, bacterial enumeration, MIC, killing curves, most probable number, detection of minimal disruption concentration, detection of indicator metabolic substances and molecular methods.
Chemical Assay Methodology		
Chemical, radioisotopic, and (or) immunological assays of drug concentrations in fecal samples or fecal slurry incubations	- Quantification of total and free drug concentrations - Quantification of drug and metabolites	- Chemical analytical assays (e.g., Gas Chromatography, High Performance Liquid Chromatography (HPLC), HPLC-Mass Spectrophotometry), radioisotopic assays and (or) immunological assays could be used to detect and quantitate the drug and potential metabolites in fecal slurries.
*This is not a comprehensive list of test system options. One or more test systems could be used, as appropriate to the drug, to address the fraction of oral dose available to microorganisms.		

3. Methodological Aspects of Test Systems

This section provides general considerations regarding the experimental conditions used in designing and conducting studies to determine the fraction of oral dose available to microorganisms.

a) Dose and concentration of drug:

- Dose and drug concentration range to be used in the test systems and the experimental objective should be justified.
- Dose and drug concentrations for testing should include levels that are expected with residue ingestion, as well as higher levels.

b) Fecal parameters:

- Source and number of fecal samples:
  - Donors should be healthy with no known exposure to antimicrobial agents for at least 3 months before fecal collection (see Section 2.3 of the guideline).
  - Variability among donors (e.g., age, sex, diet) is inherent, and the implications of donor variability for experimental design should be taken into account. The number of fecal donors should be based on the experimental objective, and a minimum of six donors are recommended (Figure 1).
  - It is recommended that fresh samples (first motion of the day) should be processed within the day of collection. Anaerobic storage for up to 72 hours at refrigerator temperatures is acceptable.

- Physical characterization of fecal samples (e.g., fecal viscosity, water content, pH, and solid content) is recommended. This information may be useful in interpreting variability in subsequent study results.

- Fecal concentrations:
  - At least one fecal concentration should be considered. A 25% fecal preparation (1 part fecal sample + 3 parts diluent) is recommended as representative of colon contents.

- Diluent used to prepare fecal slurries:
  - The chemical components used in diluting fecal material should be standardized to minimize variability.
  - An anaerobic buffer that is based on minimal salts should be used.

- Fecal incubation:
  - Consider an initial experiment using a minimum of two donor samples to determine an appropriate protocol. This should include a relevant range of residue concentrations, incubation time and sampling at multiple time points, so as to enable kinetic calculations.

- The data for a minimum of six donors should be used for the final determination of the fraction of oral dose available to microorganisms.

- Use of non-sterile or sterile fecal samples:
  - Consider the impact of sterilization of feces on drug binding to fecal suspensions in initial studies using a chemical assay.
  - Non-sterilized feces should be used where possible when conducting *in vitro* drug-binding/inactivation studies. Small differences between binding to non-sterilized and sterilized fecal suspensions may allow further studies to be based on sterilized feces only.

c) Methods to quantitatively determine the fraction of the microbiologically active drug available to microorganisms:
- While either microbiological or chemical assays may be used in these experiments, justification of the specific type of assay should be provided. If chemical assays are used, they should be bridged to the microbiological activity
- The strain of the indicator bacterial species will depend on the spectrum of activity of the drug.
- The sensitivity and reproducibility of the assays should be considered.
- Study controls should be considered according to the test system used.

d) Reversibility of observed drug binding:
- A time course approach is recommended which will reveal possible reversibility of drug binding.
- Further work to define the mechanism of binding is not essential for the purpose of establishing the fraction of oral dose available to microorganisms.

4. Description of How Test Systems Could Be Used in Determining the Fraction of Oral Dose Available to Microorganisms

In vivo and *in vitro* approaches, using different test systems considered applicable to determine the fraction of oral dose available to microorganisms, were identified and reviewed. Conceptual approaches of their application in deriving this fraction are outlined below and illustrated in Figure 1.

APPROACH 1: *In vivo* test systems. Animals dosed with the drug, followed by one of the following options:
- Option A: chemical extraction and analysis of the intestinal content and (or) feces to determine the total drug concentration, is used to establish the fraction of oral dose available to microorganisms.

- Option B: both chemical and microbiological activity assays of the intestinal content and (or) feces of dosed animals is used to establish the fraction of oral dose available to microorganisms.

APPROACH 2. *In vitro* test systems. This approach comprises two steps (Phase A and B) using *in vitro* fecal slurry test systems (see Figure 1). Phase A is an initial experiment, with fecal samples from two donors, used to identify the incubation times and relevant range of added drug concentrations sampling at multiple time points. This phase includes both chemical and microbiological assays. Phase B is conducted based on results from Phase A with samples from four additional donors, and uses microbiological assays. The data for all six donors are used for the final determination of the fraction of oral dose available to microorganisms.

APPROACH 3: Approach 1[Option A] + Approach 2. This approach combines both *in vivo* studies and *in vitro* studies.

Figure 1. Schematic Representation of Test Systems to Determine the Fraction of Oral Dose Available to Microorganisms