#3

Guidance for Industry

GENERAL PRINCIPLES FOR EVALUATING THE SAFETY OF COMPOUNDS USED IN FOOD-PRODUCING ANIMALS

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Additional copies of this guidance document may be requested from the Communications Staff (HFV-12), Center for Veterinary Medicine, Food and Drug Administration, 7519 Standish Place, Rockville, MD 20855 and may be viewed on the Internet at **http://www.fda.gov/cvm**.

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Center for Veterinary Medicine
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GENERAL PRINCIPLES FOR EVALUATING THE SAFETY OF COMPOUNDS USED IN FOOD-PRODUCING ANIMALS

FDA is required by the general safety provisions of sections 409, 512, and 706 of the Federal Food, Drug, and Cosmetic Act (the act) to determine whether each food additive, new animal drug, or color additive proposed for use in food-producing animals is safe for those animals and whether the edible products derived from treated animals are safe. The pertinent regulations implementing the statutory provisions are found at 21 CFR part 70, 21 CFR 514.1, and 21 CFR Part 570.

The sponsor of the compound is required to furnish to FDA the scientific data necessary for demonstrating that the residues of the sponsored compound in the edible products of treated animals are safe. FDA has developed a series of guidances to inform sponsors of the scientific data that FDA believes will provide an acceptable basis for determining the safety of the compound. The individual guidances are listed below.

   I.   Guidance For Metabolism Studies And For Selection Of Residues For Toxicological Testing

  II.   Guidance For Toxicological Testing

 III.   Guidance For Establishing A Safe Concentration

  IV.   Guidance For Approval Of A Method Of Analysis For Residues

   V.   Guidance For Establishing A Withdrawal Period

  VI.   Guidance For New Animal Drugs and Food Additives Derived From A Fermentation

 VII.   Guidance For The Human Food Safety Evaluation Of Bound Residues Derived From Carcinogenic New Animal Drugs

Although sections 409, 512, and 706 of the act and their implementing regulation vary slightly in wording, they have a common purpose- assuring the safety of the residues that people will consume from tissues of treated animals. Therefore, FDA believes that the same testing requirements should

apply to a new animal drug or a food or color additive used in food-producing animals. When evaluating the safety of a new animals drug, section 512(d)(2) of the act directs that FDA:

> "shall consider, among other relevant factors, (A) the probable consumption of such drug and of any substance formed in or on food because of the use of such drug, (B) the cumulative effect on man or animal of such drug, taking into account any chemically or pharmacologically related substance, (C) safety factors which in the opinion of experts, qualified by scientific training and experience to evaluate the safety of such drugs, and (D) whether the conditions of use prescribed, recommended, or suggested in the proposed labeling are reasonably certain to be followed in practice."

The guidances describe studies that the sponsor may conduct to meet these statutory provisions. FDA describes in sections I and IV appropriate scientific studies for obtaining information on the probable consumption of the sponsored compound and its residues. FDA describes in sections II and III appropriate scientific studies for obtaining information on the toxicity (cumulative effect) of the sponsored compound and its residues and the safety factors normally used. FDA describes in section VI appropriate studies for showing the safety of "biomass" products. Finally, FDA describes in section VII the studies necessary to perform the risk assessment on bound residues of carcinogenic veterinary drugs.

FDA's guidance documents, including this guidance, do not establish legally enforceable responsibilities. Instead, guidances describe the Agency's current thinking on a topic and should be viewed only as recommendations, unless specific regulatory or statutory requirements are cited. The use of the word "should" in Agency guidances means that something is suggested or recommended, but not required.

I. GUIDANCE FOR METABOLISM STUDIES AND FOR SELECTION OF RESIDUES FOR TOXICOLOGICAL TESTING

A. INTRODUCTION

Section 512(d)(2) of the act explicitly provides that FDA consider the safety of any substance formed in or on food by a sponsored compound before approving its use. The compound administered to food-producing animals (target animals) is not necessarily the substance present in the edible products from these target animals. The enzymatic systems or physiological fluids of an animal can act upon a compound administered to the animal and produce new substances (metabolites and degradation products of the sponsored compound). The amount of these substances in edible animal products will be a complex function of the rate and extent of absorption of the parent compound, the rate and extent of the metabolism of the absorbed parent compound, and the rate of excretion of parent compound and metabolites.

The total residue of a sponsored compound in treated animals will consist of parent compound, free metabolites, and metabolites that are covalently bound to endogenous molecules. The relative and absolute amounts of each residue will vary among the tissues with the amount of the compound given

and the time following the last administration of the sponsored compound to the animal. Because different components of the total residue may possess dissimilar toxicological potentials, the sponsor should develop information on the amount, persistence, and chemical nature of the-total residue in the edible products of treated target animals. The sponsor should also develop information on the metabolism of the compound in the species of laboratory animal used for the toxicological testing.

FDA needs the same type of information on metabolism of the sponsored compound in target and laboratory animals for both suspect and non-suspect carcinogens. However, after a chronic bioassay demonstrates that a compound is a carcinogen, FDA may ask the sponsor to obtain more information on the carcinogenic potential of individual metabolites. Such information could include more complete structural elucidation, *in vitro* genetic toxicity testing, and chronic bioassays for carcinogenicity.

B. TOTAL RESIDUE DEPLETION STUDY

The sponsor should measure the depletion of total drug-related residue in edible tissues of target animals at times after the last administration of the compound. For large animals, the edible tissues are muscle, liver, kidney, fat, and, where appropriate, milk. For poultry, the edible tissues are muscle, liver, skin with adhering fat, and, where appropriate, eggs. For an injectable compound, the sponsor should also measure the depletion of residues remaining at the injection site. To facilitate this determination, the sponsor should shave and permanently mark a circular area on the hide and inject the compound at the center of this area. At sacrifice, the sponsor should homogenize approximately 500 g of tissue from the area and measure its residue concentration. (The dimensions for a cylinder containing 500 g of tissue are, for an intramuscular injection, 10 cm in diameter and 6 cm in depth, and, for a subcutaneous injection, 15 cm in diameter and 2.5 cm in depth.)

The sponsor may use these tissues for the identification and quantification of parent drug and individual metabolites as described in Section C below. FDA may use these data to establish a withdrawal period for INAD purposes. FDA will use other experimental data, as discussed in another guidance, to establish a withdrawal period for NADA purposes.

Radiotracer methodology is currently the most useful technique for determining the total drug-related residue. Carbon-14 is the isotope most widely used because there is usually no problem with intermolecular exchange of the label. Ordinarily, the sponsor performs the depletion study by administering radiolabeled drug to a sufficient number of previously unmedicated animals to permit the serial sacrifice of groups of animals at intervals after the last treatment.

The administered compound should have a high radiopurity because radiolabeled contaminants may result in artifacts that could give the appearance of persistent drug residues. The sponsor should choose the site(s) of the radiolabel to assure that portions of the parent compound that are likely to be of toxicological concern are adequately labeled. For example, in the situation where two chemical moieties of the parent compound are likely to be of toxicological concern and a metabolic process may chemically separate the two moieties, then the sponsor should determine the depletion of both moieties. The sponsor could conduct a single study by administering equimolar amounts of the compound with each moiety individually labeled, two studies by separately administering the compound with each moiety individually labeled, or a single study by administering a dual-labeled (for example, Carbon-14 and Tritium) compound.

The sponsor should choose the specific activity for the radiolabeled compound high enough to demonstrate that the concentration of total residue at the last sacrifice time is below the expected permitted concentration. Ordinarily for non-carcinogens, this concentration is near 100 parts per billion (ppb), but for a carcinogen, the concentration may be below 1 ppb. The sponsor is urged to consult with FDA on the appropriate specific activity before designing a total residue depletion study. The specific activity of the administered compound need not be the same for each time point. For example, the sponsor could use low specific activity material in animals sacrificed at short withdrawal times and higher specific activity material in animals sacrificed at long withdrawal times.

The sponsor should conduct the total residue depletion study in previously unmedicated animals that are representative of the proposed target population. The metabolism of a compound may vary according to a number of parameters including species, sex, age, dose, and duration of treatment. The differences observed due to these parameters any include the rate of production and excretion of metabolites and their chemical nature. For this reason FDA will generally require a radiotracer study in each distinct species for which the sponsor is seeking approval. If a product is intended for use in both male and female animals, then the sponsor should use animals of both sexes in the study because males and females of a species can metabolize chemicals at a different rate. Because the enzyme type and concentration in young animals may not be representative of the adult animal, the sponsor should conduct the study in animals that are representative of the target population, that is, in neonates, prepubertal animals, or sexually mature animals. In those instances when a drug's use will be extended from one production class to another within a species, FDA generally will accept an abbreviated total residue depletion study to support approval in the new production class. Sponsors should discuss plans for the abbreviated study with FDA.

The dose should be the highest intended treatment level and should model exposure received by target animals. For example, if a drug is given once to an animal for a specific therapeutic effect, then a single dose of radiolabeled drug is appropriate exposure. When the sponsor is requesting prolonged treatment and is also requesting a zero withdrawal period, the sponsor should supply data to demonstrate that the residue concentration has approximately reached steady state. In other cases, FDA will generally accept that a seven day dosing regimen is adequate for the purposes of the residue depletion studies. However, the sponsor should use a twelve day dosing regimen for laying hens to approximate the time required for complete development of the yolk. Where the data do not demonstrate that a steady state concentration is attained, the sponsor should show that there is no new metabolite being formed as a result of prolonged treatment. For example, the sponsor could demonstrate that the metabolite pattern and relative proportions of metabolites in tissues have stabilized.

When measuring the depletion of the total residue in edible tissue, the sponsor should sacrifice groups of at least three animals at zero withdrawal and usually at three later times. If the sponsor is requesting a zero withdrawal period, the sponsor should 'dose six animals for a sufficient time previously demonstrated to achieve a steady state concentration of residue and then sacrifice the animals at zero time. For other special cases, the sponsor is urged to consult with FDA before designing a study. If the sponsor intends to use the dosed tissue for metabolite identification, the sponsor may wish to include additional animals in the study. For purposes of the residue depletion study, zero withdrawal for tissues of large animals is considered eight to twelve hours after the last treatment; for lactating

animals, zero withdrawal for milk is considered twelve hours after the last treatment. For tissues of poultry, zero withdrawal is considered six hours after the last treatment.

The sponsor should present the results of these experiments in a format that will facilitate FDA review and should include supporting raw data. The sponsor should provide:

- The specific activity of parent drug.

- The radiopurity of parent drug (98% or greater is recommended) with supporting chromatograms from at least two different chromatographic systems and a determination of microbiological activity if appropriate.

- A full description of methodology and of the statistical and/or mathematical approach including sample calculations.

- A practical demonstration of the limit of detection of parent drug through fortification of each of the edible tissues. In the absence of this demonstration, FDA will use twice the background counting rate as a nominal limit of detection.

- The sampling procedure and sample size. FDA recommends that the whole organ or representative portions be homogenized and the analysis be done on an aliquot.

- Data from duplicate analyses of each tissue sample derived from treated animals. For each experimentally derived data point, results should be identified with an individual male or female animal and be presented in CPM, in DPM, and in parent drug equivalents per gram of wet tissue.

C. METABOLISM STUDIES IN TARGET ANIMALS

The sponsor should provide information on the metabolic fate of the compound in the edible tissues of the target animal dosed at the maximum use level requested. Often the published literature contains information on the metabolism of a closely related chemical. Such information is useful but usually cannot replace the experimental observation of the metabolic fate of the sponsored chemical in target

The sponsor may use the tissues from the total residue depletion study for these studies. If the tissues are stored frozen for long periods of time, FDA may ask the sponsor to demonstrate that the metabolites are stable in the frozen state, such as by demonstrating that metabolite profiles do not change. If suitable tissue is not available, the sponsor should conduct a total residue depletion study as discussed above in Section B. The sponsor should develop procedures for extraction, fractionation, separation, and isolation of metabolites that will facilitate the comparison of metabolite profiles for each tissue and for later comparison of these profiles with the metabolite profiles derived from laboratory animals. Unless human food safety questions arise requiring examination of the other edible tissues, the sponsor will need to collect data only in the target tissue.

1. Structural Identification of Metabolites

Structural identification of major metabolites may be necessary depending on the degree of toxicological concern for the parent compound and its potential metabolites. FDA will consider a metabolite to be a major metabolite if, at the time the concentration of total residue peaks (normally zero withdrawal), either (a) it is present in an amount greater than 10% of the total residue in an edible tissue, or (b) its concentration exceeds 0.1 ppm. In some cases, chemical characterization rather than unequivocal structural identification for a major metabolite will be sufficient. For example, if chromatographic evidence demonstrates that a metabolite and its conjugate are present in tissue, then FDA will not consider structural identification of the particular conjugate to be necessary for evaluating the safety of the compound. Similarly, the sponsor may use chromatographic evidence to demonstrate that the same major metabolite occurs in more than one tissue. FDA will normally not require structural identification or chemical characterization of minor metabolites.

The sponsor may isolate sufficient quantities of metabolites for structural identification by a variety of techniques. The sponsor may isolate metabolites from excreta. Another example of an acceptable approach is the research procedure described by Paulson and Struble (Ref. 1) and Bakke *et al.* Ref. 2), summarized below.

The sponsor treats a group of animals with cold drug at or near the use level for a period of time, then administers from one to several large doses (5x to 20x) of radiolabeled drug, and slaughters the animals six to twelve hours after the final dose. The sponsor then uses purification and separation techniques to obtain individual metabolites for structural determination.

2. Persistence of Metabolites

The sponsor should determine the concentration and relative percent of the parent compound and individual metabolites in edible tissue(s) at a series of times after the last administration of the compound. Because the residues that persist to the expected withdrawal period will be consumed by people, these are the residues of toxicological concern. FDA will normally require an evaluation of their toxicological potentials in laboratory animals (see sections D and E below).

3. Covalently Bound Residues

In some cases the sponsor may be unable to extract the total radiolabeled residue. The non-extractable material usually represents two general classes of compounds, endogenous components derived from a portion or the radiolabeled compound of covalently bound residues derived from the reaction between a metabolite of the compound and cellular macromolecules. If the sponsor shows that a portion of the total residue results from the incorporation of radiolabel into endogenous compounds (for example, amino acids, fatty acids, carbohydrates, nucleotides), FDA will subtract that portion from the total residue because it is not of toxicological concern. FDA will consider the covalently bound residue to be of toxicological concern, but will not consider it to be more toxic than the parent compound. Unless the covalently bound residue represents a major portion of the residue and the information is needed for analytical methodology, FDA will not require that the sponsor attempt structural identification.

If the total residue concentration (that is, free metabolites plus covalently bound residue) is below the permitted concentration of residue in edible tissue at the requested withdrawal time, then this residue is shown to be safe within the meaning of the act, and FDA will not ask for additional safety testing on the covalently bound residue. However, if the concentration of covalently bound residue exceeds the permitted concentration of residue, then FDA cannot approve the compound until the sponsor provides additional data to demonstrate safety.

If the parent compound is not a carcinogen, FDA will discount from the residue of toxicological concern that portion of the covalently bound residue that the sponsor demonstrates is not bioavailable, provided that a substantial portion (for example, 50%) of the covalently bound residue is not bioavailable. FDA will adjust the total residue based on the relative bioavailability of the parent compound and the covalently bound residue. The experimental technique described by Gallo-Torres (Ref. 3) is an example of an acceptable protocol. However, if the parent compound is a demonstrated carcinogen, FDA will normally not accept bioavailability data alone to discount the covalently bound residue from carcinogenic concern. In any specific case the sponsor has the option of proposing to FDA an experimental approach to demonstrate that the covalently bound residue is not of carcinogenic concern.

4. Reporting of Data

The sponsor should submit data from the metabolism studies in a format that will facilitate FDA review and should include supporting raw data. A flow chart of known or postulated metabolic pathways is usually helpful. The sponsor should provide:

- The concentration of the total residue in each edible tissue, and milk or eggs where applicable, at a series of times following administration of the product.

- Information on the extractability of the total residue with no treatment, conditions of varying pH, or following denaturation, chemical and/or enzymatic hydrolysis.

- Chromatographic profiles of the metabolites extracted from edible tissue(s) and, if applicable, excreta. FDA recommends high resolution chromatographic methods. FDA also recommends rechromatography after treatment of the extract with conjugate-hydrolyzing enzymes or dilute acid.

- Structural identification of the major metabolites.

- Data on bioavailability of covalently bound residues, if appropriate.

D. METABOLISM STUDIES IN LABORATORY ANIMALS

The purpose of these studies is to determine whether the metabolites that people will consume from tissues of target animals are also produced by metabolism in the laboratory animals used for the toxicological testing. The sponsor should conduct these studies for compounds being tested for carcinogenicity as well as those being tested for other toxicological endpoints. To facilitate evaluation

of the data, the sponsor should use the same procedures for chromatography and chemical characterization as those employed in the metabolism study in the target animals. Qualitative information on metabolites is sufficient. FDA will use this information to determine what metabolites, if any, need separate toxicological testing (see section E below).

The sponsor should give laboratory animals a sufficient number of daily doses of the radiolabeled test compound to ensure that it undergoes all relevant metabolic events, including those associated with enzyme induction. The sponsor should consult with FDA prior to dose selection.

The sponsor should try to conduct toxicological studies in the laboratory animals whose profile of metabolites most closely resembles that observed in target animals. However, other factors also need to be considered. For example, knowledge that certain laboratory animals are especially sensitive to the parent compound or its chemical class may dictate their choice, even though the metabolite profile of other laboratory animals may more closely model that of the target animals. FDA does require that the strain of test species chosen for the comparative metabolism work be the one used for the toxicological testing.

E. SELECTION OF METABOLITES FOR TOXICOLOGICAL TESTING

FDA may ask for separate toxicological studies on a metabolite if it is not tested through autoexposure and it is likely to have toxicological potency significantly greater than the parent compound. FDA will normally conclude that autoexposure provides an adequate test of the toxicity of the sponsored compound if laboratory animals produce the metabolites that collectively comprise over 90% of the residue that people will consume from tissues of treated target animals. Failing that, FDA will use the information obtained from target animals on the concentration, persistence, and chemical structure or characterization of that metabolite to determine whether separate toxicological testing is desirable.

F. IDENTIFICATION OF TARGET TISSUE AND MARKER RESIDUE

The target tissue is the edible tissue selected to monitor for the total residue in the target animal. The target tissue is usually, but not necessarily, the last tissue in which residues deplete to the permitted concentration. A marker residue is a residue whose concentration is in a known relationship to the concentration of the total residue in the last tissue to deplete to its permitted concentration. The marker residue can be the sponsored compound, any of its metabolites, or a combination of the residues for which a common assay can be developed. The target tissue and marker residue are selected so that the absence of marker residue above a designated concentration R(M) will confirm that each edible tissue has a concentration of total residue at or below its permitted concentration.

When a compound is to be used in milk- or egg-producing animals, milk or eggs may be a target tissue in addition to one tissue selected to monitor for residues in the edible carcass because milk or eggs enter the food supply independently. In these cases, it may be necessary to select a marker residue for milk or eggs that is different from the marker residue selected for the target tissue representing the edible carcass.

Application of the concepts of marker residue and target tissue requires an experimental determination of the quantitative relationships among the residues that might serve as the marker residue in each of

the various edible tissues that might serve as the target tissue. Because these relationships may change with time, the sponsor should measure the depletion of potential marker residues in potential target tissues starting after the last treatment with the sponsored compound and continuing until the residue has reached the permitted concentration for that tissue.

The sponsor may use the results from the total residue depletion study and metabolism study to determine the marker residue, target tissue, and R(M). If a new depletion study is advisable, the desired experimental parameters are as outlined for the total residue depletion study in section B. (A radiotracer depletion study provides an initial estimate of the R(M). The final R(M) is determined with the regulatory assay.)

G. REFERENCES

1. Paulson, G. and C. Struble (1980), I. A unique deaminated metabolite of sulfamethazine [4-amino-N-(4,6-dimethyl-2-pyridinyl) benzensulfonamide] in swine, Life Sci. 27:1811-1817.

2. Bakke, J. E., V- J. Feil, C. E. Price, and R. G. Zaylskie (1976), Metabolism of Carbon-14 crufomate (4-t-butyl-2-chlorophenyl methyl methylphosphoramidate) by the sheep, Biomed. Mass. Spectrom. 3:299-315

3. Gallo-Torres, H. E. (1977), Methodology for the determination of bioavailability of labeled residues, J. Tox and Environ. Health 2:827-845

II. GUIDANCE FOR TOXICOLOGICAL TESTING

(Section B, Testing Generally Needed for Sponsored Compounds, has been updated to remove outdated information and refers the readers to other available guidance on toxicological testing.)

A. INTRODUCTION

For compounds used in food-producing animals, we are concerned with intermittent and chronic exposure of people to relatively low concentrations of residues. We tailor the type of toxicological testing needed for a demonstration of safety for a specific compound by considering its proposed use in animal husbandry, the probable exposure of people to the parent compound and its metabolites (residues) under its conditions of use, the possible biological effects of the residues as deduced by structure-activity relationships, and their effects as observed in biological systems.

The purpose of the toxicological studies is to define the biological effect(s) of the sponsored compound and its quantitative limits. We normally ask for testing of the sponsored drug substance. In addition, we may ask for separate testing of a metabolite and/or excipient when such testing is necessary to define adequately the biological effect of the sponsored drug product.

B. TESTING GENERALLY NEEDED FOR SPONSORED COMPOUNDS

A recommended testing approach to assure the safety of human food derived from animals treated with veterinary drugs is outlined in Guidance for Industry (GFI) #149 "Studies to Evaluate the Safety of Residues of Veterinary Drugs in Human Food: General Approach to Testing" (May 18, 2004). GFI #149 references additional human food safety toxicology guidance documents, as listed in the following table.

CVM GFI# and Date	VICH GL #	Title
149 May 18, 2004	33	Studies to Evaluate the Safety of Residues of Veterinary Drugs in Human Food: General Approach to Testing
147 November 12, 2003	31	Studies to Evaluate the Safety of Residues of Veterinary Drugs in Human Food: Repeat-Dose (90-Day) Toxicity Testing
160 February 4, 2005	37	Studies to Evaluate the Safety of Residues of Veterinary Drugs in Human Food: Repeat-Dose (Chronic) Toxicity Testing
115 January 3, 2002	22	Safety Studies for Veterinary Drug Residues in Human Food: Reproduction Toxicity Testing
148 March 16, 2004	32	Studies to Evaluate the Safety of Residues of Veterinary Drugs in Human Food: Developmental Toxicity Testing
159 February 10,2005	36	Studies to Evaluate the Safety of Residues of Veterinary Drugs in Human Food: General Approach to Establish a Microbiological ADI
116 January 3, 2002	23	Studies to Evaluate the Safety of Residues of Veterinary Drugs in Human Food: Genotoxicity Testing

In all cases, we recommend that you contact us for clarification of experimental protocol details or if you have any other questions about the details of any particular study.

If the testing shows that the sponsored compound is a carcinogen, we will apply the "no-residue" requirement of section 409(c)(3)(A), 512(d)(1)(H), or 706(b)(5)(B) of the act as operationally defined in 21 CFR subpart E of Part 500. We will calculate the concentration of residue giving no significant risk of cancer, S(O), from the tumor data using a statistical extrapolation procedure. Because the mechanism of carcinogenesis may not be sufficiently understood, the mathematical procedures for extrapolation may not have a fully adequate biological rationale. In the absence of information establishing the mechanism of carcinogenesis for a particular chemical, we will use a non-threshold, linear-at-low dose extrapolation procedure that determines the upper limit of the risk. We will use the linear interpolation procedure of Gaylor and Kodell (D. W. Gaylor and R. L. Kodell, "Linear Interpolation Algorithm for Low Dose Risk Assessment of Toxic Substances", Journal of Environmental Pathology and Toxicology, 4:305-312, 1980) as modified by Farmer, Kodell, and Gaylor (J. H. Farmer, R. L. Kodell, and D. W. Gaylor, "Estimation and Extrapolation of Tumor Probabilities from a Mouse Bioassay with Survival/Sacrifice Components", Risk Analysis, 2:27-34, 1982). In the extrapolation, we will use the upper 95 percent confidence limit on the tumor data and a permitted maximum lifetime risk to the test animal of 1 in 1 million.

We will use an alternative procedure if the sponsor presents sufficient information to demonstrate that a different extrapolation procedure is more appropriate.

For other toxicological endpoints, we will calculate the acceptable daily intake (ADI) from the results of the study illustrating the most sensitive endpoint in the most appropriate species. The ADI is the highest dose used in the study that demonstrates a no-observed-effect-level (NOEL) divided by an appropriate safety factor. As a general rule, we will use the safety factors indicated below for the various types of studies identified.

Type of Study	Safety Factor
Chronic	100
Reproduction/Teratology	(100 or 1000; 100 for a clear indication of maternal toxicity,1000 for other effects)
90-Day	1000

However, it should be noted that the actual safety factor that is applied to a NOEL in the calculation of an ADI is dependent on an interplay between the type of study, the species of animal used in the study, and the endpoint observed in the study.

C. TESTING NEEDED FOR SEX STEROIDS

The studies we recommend for a showing of safety for a sex steroid, considering both carcinogenicity and the other toxicological endpoints associated with these compounds, are described below.

Although not all sex steroids are demonstrated carcinogens, current evidence supports our conclusion that all endogenous sex steroids and synthetic compounds with similar biological activity should be regarded as suspect carcinogens.

1. Endogenous Sex Steroids

For the endogenous sex steroids and their simple ester derivatives, we have concluded that safety can be assured without the need for additional animal data because the compounds are endogenous in people and in food-producing animals. Therefore, an individual is exposed to rather large quantities of these compounds by *de novo* synthesis, and to much lesser quantities from food-producing animals that are not treated with these substances. Therefore, we have concluded that no additional physiological effect will occur in individuals chronically ingesting animal tissues that contain an increase of endogenous sex steroids from exogenous sources equal to 1% or less of the amount in micrograms produced by daily synthesis in the segment of the population with the lowest daily production. We believe that the 1% value is supported by scientific evidence, is reasonable, and reflects sound public health policy. For estradiol and progesterone, prepubertal boys provide the baseline benchmark. For testosterone, prepubertal girls provide the baseline benchmark. The daily production values and the calculated increase permitted above the amount naturally present in untreated target animals are listed below.

	Daily Production (micrograms)	**Permitted Increased Exposure (micrograms)**
Estradiol	6	0.06
Progesterone	150	1.50
Testosterone	32	0.32

When a sponsor can demonstrate with a suitable assay that, under the proposed conditions of use the concentration of residue of the endogenous sex steroid in treated food-producing animals is such that the actual increase in exposure of people will not exceed the permitted increase, then the compound is shown to be safe within the meaning of the Federal Food Drug and Cosmetic Act.

2. Synthetic Sex Steroids

We have concluded that animal testing is necessary for establishing the safety of a synthetic sex steroid. We recommend the studies described in Section A under GFI #149.

In addition, we recommend a 180-day study in rhesus monkeys or other suitable subhuman primates that includes a 90-day observation period before the 90-day dosing period, and a dose that gives no observed hormonal response. We recommend that the study assess the effect of the sponsored compound on ovulation, the duration of the menstrual cycle, and changes in the concentration in the blood of the gonadotropins (luteinizing hormone, LH, and follicle stimulating hormone, FSH) and of the endogenous sex steroids (estradiol, estrone, and progesterone). Because of the complexity of this type of study, we recommend that you contact us in advance of the study execution in order to reach agreement on the details of the study protocol.

We recommend chronic bioassays for oncogenicity in two rodent species if either the genetic toxicity tests are positive, or data from the other bioassays indicates a preneoplastic lesion in other than an endocrine sensitive tissue.

In the absence of a carcinogenic response or if tumors are observed only in endocrine sensitive tissue, we will calculate the acceptable daily intake (ADI) from the results of the most sensitive endpoint in the most appropriate species. We will normally use the safety factors indicated in in section B above. Also, we normally use a safety factor of 100 for the study in subhuman primates.

If a carcinogenic response is observed in a nonendocrine-sensitive tissue, we will determine the dose that will satisfy the "no-residue" requirement of the act using the tumor data from that tissue and a statistical extrapolation procedure.

III. Guidance For Establishing A Safe Concentration

A. CALCULATING THE ACCEPTABLE DAILY INTAKE (ADI)

As described in guidance II, the toxicology tests are designed to determine the dose at which the compound produces an adverse effect and a dose which produces no observed effect (NOEL). If the drug is not a carcinogen, the NOEL of the most sensitive effect in the most sensitive species divided by a safety factor is used to determine an acceptable daily intake (ADI) for drug residues. If the sponsor provides strong scientific data that this NOEL is not predictive of human toxicity, the FDA will use a more appropriate NOEL for establishing the ADI. Therefore, the ADI is calculated by dividing the NOEL obtained in the toxicology study with the most appropriate species by a safety factor.

B. CONSUMPTION VALUES

FDA assumes that an individual eats more edible muscle than organ tissue. FDA accounts for this difference in consumption when calculating the safe concentration for drug residues. The consumption values (grams consumed) for edible muscle and organ tissue are described in Table 1. These consumption values will be applied across all species, because it is assumed that when an individual consumes a full portion of a meat product from one species, they will not consume a full portion of a meat product from another species.

Table 1: Consumption Values

Edible Product	Grams Consumed
Muscle	300
Liver	100
Kidney	50
Fat	50

The Center will continue to regulate milk and eggs as independent commodities, that is, these products are consumed in addition to the consumption of the edible muscle or organ tissues. FDA assumes that on a daily basis a person consumes a full portion of milk in addition to the full portion edible muscle or organ tissue. The intake estimate for milk is 1.5 L. For eggs, the intake estimate will be changed to 100 g. Again, the FDA assumes that on a daily basis a person consumes a full portion of eggs in addition to the consumption of muscle or organ tissue.

C. ESTABLISHING THE SAFE CONCENTRATION

FDA will calculate the safe concentration for each edible tissue using the acceptable daily intake (ADI), the weight in kg of an average adult (60 kg), and the amount of the product eaten per day in grams.

Safe concentration ADI ug/kg/day) x 60 kg

=   _____

(ppm)   grams consumed/day.

For products approved in lactating dairy cows, and for products approved in lactating dairy cows and other meat-producing species; a part of the ADI (generally one-half) will be reserved for milk. The safe concentration for milk equals the ADI reserved for milk (µg/kg/day) times 60 kg divided by 1500 ml/day. The remaining part of the ADI will be partitioned among the animals tissues as described above. For products approved in both laying hens and other animals, a part of the ADI (usually one-fifth) will be reserved for eggs. The safe concentration for eggs equals the ADI reserved for eggs (µg/kg/day) times 60 kg divided by 100 g/day.

FDA will apply these consumption values to determine the safe concentration in organ tissue for most new animal drug products. Based on an appropriate scientific justification, alternate consumption values may be used for calculating the safe concentration. FDA will establish a lower safe concentration than that calculated from the toxicological data, if the calculated safe concentration is higher than that needed to support the intended use in target animals (see 21 CFR 556.1(b)).

IV. Guidance For Approval Of A Method Of Analysis For Residues

A. INTRODUCTION

Before approving a carcinogenic or noncarcinogenic compound for use in food-producing animals, FDA generally asks that the sponsor provide an acceptable analytical method (either chemical or biological) capable of reliably measuring the marker residue to ensure that the total residue of toxicological concern is not exceeded. The determination of the total residue of toxicological concern can be accomplished by: (a) choosing a marker residue, (b) establishing a quantitative relationship between the marker residue and total residue of toxicological concern, and (c) calculating the maximum permitted concentration of marker residue, R(M), in the target tissue (the tissue used to monitor for total drug residues in all tissues) to ensure that the total residue of toxicological concert does not exceed the permitted concentration.

The process of obtaining approval of a method consists of three steps:

Step 1.   Method development by the sponsor and demonstration that the method satisfies the acceptability criteria (part B of this guidance).

Step 2.   FDA desk review of the sponsor's data (part C I of this guidance) to determine suitability of the method for interlaboratory study.

Step 3.   Interlaboratory Study (part C 2 of this guidance) to determine whether the method performs as claimed and thus can be used as a practicable and reliable regulatory tool.

Because methods which appear marginally acceptable after desk review often do not pass the interlaboratory validation trials, sponsors are urged to develop methods that are rugged, and exceed rather than meet the minimal standards of acceptability.

B. EVALUATION CRITERIA

Any method will be characterized by a set of attributes that determine its applicability: *specificity* (what is being measured), *precision* (the variability of the measurement), and *systematic error* (or bias, measured as recovery).

1. Specificity

Specificity is the ability of a method to respond only to the substance being measured. The proposed method must provide for identification of the compound being measured. Certain instrumental techniques such as infrared spectroscopy or mass spectrometry may be sufficiently specific by themselves. If the method is not sufficiently specific, then a confirmatory or identification procedure will be needed. In those cases, the regulatory method will have two components, the "determinative" procedure to quantify a given compound and the "confirmatory" procedure to verify the identity of the

compound. Other techniques can achieve comparable specificity if used in combination. For example, specificity may be verified by thin-layer chromatography, element-specific gas liquid chromatography, formation of characteristic derivatives and rechromatography, and characteristic relative retention times with several different chromatographic systems of different polarity. Such procedures must be applicable at the designated marker residue concentration R(M).

The sponsor should take into account the possible presence of other compounds approved for use in the same target species and should demonstrate that they will not interfere with the determination of the sponsored compound by the proposed method.

2. Precision

Precision is an important quantitative performance characteristic of a method. It is a measure of the variability of repetitive measurements. Contributions of variability from numerous sources affect precision, but the major components are those from different laboratories (reproducibility) and those from within a laboratory (repeatability). Precision is usually expressed as a standard deviation, but an even more useful term is the relative standard deviation (or coefficient of variation) because it is relatively constant over a considerable concentration range.

The variability finally achieved in the sponsor's laboratory after considerable experience usually is less than that achievable by less experienced laboratories who may later use the method. The final version of the method should be optimized by such procedures as ruggedness testing (W. J. Youden and E. H. Steiner, *Statistical Manual of the AOAC,* Association of Official Analytical Chemists, Box 540 Benjamin Franklin Station, Washington, DC 1975, p. 33). If a method cannot achieve a suitable degree of repeatability in the sponsor's laboratory, it cannot be expected to do any better in other laboratories. The method should be performed by an analyst not involved in the development of the method to verify the adequacy of the method's description and the identification of critical parameters.

The within laboratory coefficient of variation should not exceed 10% where the designated concentration of marker residue R(M) is greater than or equal to 0.1 ppm. Where the designated concentration of marker residue R(M) is less than 0.1 ppm, the within laboratory coefficient of variation should not exceed 20%.

3. Systematic Error

Systematic error, or bias, is the difference of the measured value from the true, assigned, or accepted value. It is generally expressed as the percent recovery of added analyte, realizing, in the case of residue analysis, that analyte added to a sample may not behave in the same manner as the same analyte biologically incurred. At relatively high concentrations, recoveries are expected to approach 100%. At lower concentrations, and particularly with methods involving a number of steps which may include extractions, solvent transfers, and adsorption chromatography, recoveries may be lower.

Assuming acceptable precision, an average recovery of 80 to 110% should be obtained when the designated concentration of marker residue R(M) is 0.1 ppm or greater. An average recovery of 60 to 110% will be accepted when the designated concentration of marker residue R(M) is less than 0.1 ppm. Correction factors are not ordinarily acceptable unless they are an inherent part of the procedure,

as in definitive radioisotope dilution procedures. Since higher recoveries (greater than 110%) may indicate a lack of specificity, FDA will ask for an explanation for such values. FDA will accept lower recovery values than those presented above for methods employing internal standards.

4. Collateral Criteria

The method should: (a) utilize commercially available reagents, supplies, instruments (except that new or unusual reagents or standards may be supplied by the sponsor on request); (b) be capable of being performed by reasonably experienced analysts: (c) be capable of being completed within reasonable time periods consistent with regulatory objectives (usually no more than 48 hours of total elapsed time); (d) not need unique instrumentation, large quantities of solvents, reagents, and supplies which would render the method economically impractical; and (e) be capable of being performed safely.

There are several other indications of satisfactory performance that may be helpful in determining if the method is acceptable. These factors may include requirements for a linear calibration (standard) curve and analytical (recovery) curve; effectiveness of extraction; the effect (or noneffect) of specific potential interferences; adequate sensitivity (slope of the calibration curve) and resolution; adequately low and constant blanks; and stability studies.

In practical regulatory use, samples are examined without benefit of treatment history. For interpretation of the analytical response observed in analyses of actual samples, the response for the marker residue should be clearly resolved from any other responses present and should be readily distinguishable above the background signal. (For example, in a gas chromatographic determination, the marker residue peak(s) should be resolved and clearly recognizable from other peaks. At the designated concentration of marker residue, R(M), the response should be a peak at least 20 mm in height and at least 10 times greater than the variability of the background response.)

C. SPECIFIC DATA NEEDED

1. Sponsor's Petition

In development of regulatory methods, sponsors should collect data from three types of samples: (a) "control" target tissue from untreated animals, (b) "fortified" target tissue containing known concentrations of the marker residue added to the sample of control target tissue, and (c) "dosed' target tissue from animals of the target species that have been treated with the drug. The sponsor should determine the baseline (background) response of the method and its variability. The sponsor should demonstrate that the proposed method can satisfactorily recover and identify known amounts of the marker residue which have been added to the target tissue. Finally, the sponsor should demonstrate that the proposed method can satisfactorily recover the biologically incurred marker residue.

In presenting a petition for approval of a proposed regulatory method, the sponsor should provide:

* A complete description of the method including sampling, preparation of analytical samples, storage conditions, reagents, instrumentation, standards, and identification of critical steps and stopping places.

* Quality control criteria that may be needed to verify
and maintain method performance.

* A typical standard curve prepared from marker residue of known purity.

* A typical analytical curve prepared by fortifying (spiking) control tissue with marker residue and
observing the resulting analytical responses.

* Data derived from control, fortified, and dosed tissue showing that the method meetsthe specificity,
precision, and systematic error attributes.

* Relevant worksheets, calculations, statistical analyses, spectrograms, chromatograms, etc., from the
analyses of control, fortified and dosed target tissue.

* Using the proposed determinative procedure of the method, results of analyses of the following
samples (as a minimum):

- 5 control tissues
- 5 control tissues fortified with marker residue at 0.5X R(M)
- 5 control tissues fortified with marker residue at 1X R(M)
- 5 control tissues fortified with marker residue at 2X R(M)
- 5 dosed tissues containing biologically incurred total residue at approximately its permitted
concentration.

* Using the proposed confirmatory procedure, results of analyses to verify the identity of the marker
residue with the following samples (as a minimum):

- 5 control tissues to ensure the absence of false positives
- 5 control tissues fortified with marker residue at R(M)
- 5 dosed tissues containing biologically incurred total residue at approximately its permitted
concentration.

2. Interlaboratory Study

After the regulatory method has passed desk review (section 1, above), FDA will conduct an
interlaboratory trial of the proposed method to verify that the regulatory method can be employed as a
practicable and reliable regulatory tool. At a minimum, the method will be tested in two FDA
laboratories and one USDA laboratory using target tissues supplied by the sponsor.

Each of the three laboratories will analyze the following samples by the determinative procedure:

* 5 control tissues
* 5 control tissues fortified with marker residue at 0.5X R(M)
* 5 control tissues fortified with marker residue at 1X R(M)
* 5 control tissues fortified with marker residue at 2X R(M)

* 5 dosed tissues containing biologically incurred total residue at approximately its permitted concentration.

If a separate procedure is necessary to confirm the identity of the marker residue, each of the three laboratories will analyze the following samples:

* 5 control tissues
* 5 control tissues fortified with marker residue at R(M)
* 5 dosed tissues containing biologically incurred total residue at approximately its permitted concentration.

(Whenever possible the confirmatory procedure should be performed on the same extract used for the determinative portion of the method.)

Alternatively, the sponsor may elect to conduct a collaborative study of the proposed method under the auspices of an organization such as the Association of Official Analytical Chemists (AOAC). The study would include the three aforementioned government laboratories and the sponsor's laboratory. The sponsor would be responsible for obtaining the additional collaborators and for supplying the participating laboratories with all the necessary target tissues (control, fortified, and dosed). A minimum of six laboratories would be needed to constitute a collaborative study. The number of tissue samples analyzed by each participating laboratory can be reduced from five to a minimum of two for each group listed above.

V. Guidance For Establishing A Withdrawal Period

A. INTRODUCTION

The withdrawal period or the milk discard time is the interval between the time of the last administration of a sponsored compound and the time when the animal can be safely slaughtered for food or the milk can be safely consumed. (For convenience this guidance will use the "withdrawal period" to refer to both the withdrawal period and the milk discard time.) The recommended withdrawal period, if followed, should (1) provide a high degree of assurance to the producer that his animals or milk will be in compliance with applicable regulations, (2) be compatible with livestock management practices, and (3) be reasonably certain to be followed. If the producer follows the recommended withdrawal period, the consumer has a high degree of assurance that the edible products from treated animals are safe.

This guidance describes a procedure for establishing a withdrawal period that is based on a statistical tolerance limit procedure (Ref. 1). The withdrawal period is determined when the tolerance limit on the residue concentration is at or below the permitted concentration. A tolerance limit provides an interval within which a given percentile of the population lies, with a given confidence that the interval does contain that percentile of the population. FDA will use the 99th percentile of the population and the 95 percent confidence level.

If the calculated withdrawal period is a fraction of a day or milking, FDA will establish the withdrawal period as the next day or milking. The sponsor can obtain information-from the Center for Veterinary Medicine on the maximum withdrawal period that is generally considered practical for a specific drug use. If the sponsor proposes a withdrawal period that exceeds the general limit, the sponsor should submit information to justify that the proposed withdrawal period will be followed and will not jeopardize the safe and effective use of the drug.

B. OBTAINING THE RESIDUE DATA

The sponsor will obtain residue data from animals as a function of time after the last treatment with the compound. The sponsor should design the study so that the last phase of the depletion curve closest to the established tolerance can be used to calculate the tolerance limit. For the residue study in tissue, sufficient data are generally provided from residue data from the target tissue of 20 animals with five animals being slaughtered at each of four evenly distributed time points. For the residue study in milk, we believe sufficient data will be provided by 20 animals with milk collected from all animals at evenly spaced time points. FDA requires that the sponsor collect the residue data with the same assay that will be submitted for validation. For milk, the sponsor should conduct triplicate assays for each data point beginning with a separate milk sample. If more than a single value is used for the residue data in tissue, the procedure in Appendix A should be modified to account for the reduced variation of the residue data points due to multiple assays.

Animals used in field trials provide the best source of residue data because these animals are representative of the proposed target population. Failing that, the sponsor should design an experiment that simulates the conditions of use for the drug paying particular attention to the proposed dosing regimen, normal husbandry conditions, animal gender, and animal maturity. Animals treated in the studies and/or their milk may be marketable if protocols have been accepted by FDA.

C. STATISTICAL ANALYSIS OF THE RESIDUE DATA

The assumptions for the statistical analysis are that during the phase of the depletion closest to the established tolerance the measurements on the animals are independent from each other; the residue assays are independent from each other and from the animal in question; the depletion of the Ln concentration of residue is linear with time; and the measured Ln concentrations of residue are distributed normally and have a constant variation over time. For a residue study in tissues, one straight line is fit to all suitable data by the method of least-squares (Ref. 2). For a residue study in milk, straight lines are fit to the data of each animal separately. The withdrawal period is then established from these lines. Sample calculations are provided in the attached Appendix A for data from residue study in tissue and in Appendix B for data from a residue study in milk.

In contrast to the carcasses which are individually sampled by USDA in the residue monitoring program, milk from an entire dairy herd is pooled in the bulk tank before sampling in a compliance program. Therefore, the statistical procedure for calculating the milk discard time must contain a term for the number of cows contributing milk to the bulk tank. To approximate the size of a small dairy operation, FDA will use 10 for that value. In a situation where an individual cow is treated for mastitis, FDA will assume that at a maximum one-third of the milk in the bulk tank would come from treated cows. In a situation where an entire dairy herd is treated, such as with an anthelmintic, FDA will not allow that correction.

D. REFERENCES:

1. D.B. Owen, *A Survey of Properties and Applications of the Non-central t-Distribution*, Technometrics 10, 445 (1968).

2. N. R. Draper and H. Smith, *Applied Regression Analysis*, Wiley, New York (1966).

APPENDIX A

Residue Data from Tissue

The results of a depletion study constructed from data involving 25 animals are listed below. Tissue samples were taken from five animals at each of the five slaughter times and analyzed for residues. The sponsor validated the analytical method for the compound to a concentration of 4.5 ppb although the lower limit of detection was 0.9 ppb. The permitted concentration for the marker residue (Rm) is 9.0 ppb.

Days withdrawn (t)	Concentration (ppb)	Ln Concentration
3	27.9	3.329
3	31.5	3.450
3	26.6	3.279
3	36.9	3.609
3	32.9	3.492
5	19.8	2.986
5	22.5	3.114

5	26.6	3.279
5	19.8	2.986
5	30.6	3.421
7	17.1	2.839
7	18.0	2.891
7	11.3	2.421
7	31.5	3.450
7	13.5	2.603
10	13.5	2.603
10	12.2	3.498
10	10.8	2.380
10	10.8	2.380
10	5 0	1.600
14	3.6	1.281
14	5.4	1.687
14	6.8	1.910
14	5.4	1.687
14	7.2	1.974

1. Regression Analysis

STEP 1. Determine which time points fall on the linear part of the depletion curve for the phase of the curve closest to the R(M)

Generally plotting the data on semi-logarithmic paper, time versus concentration, is helpful in detecting points that do not fall on the linear part of the curve. It is not uncommon for points very close to the zero time point to be inappropriate because they may be from a different phase of the depletion curve. The other area where points often must be excluded is when all or most of the measurements are below the limit of measurement. In the example here, the assay method is validated to one concentration but appears to have a lower acceptable limit of detection. The one value at 14 days of 3.6 ppb is below the validated limit of 4.5 ppb but was kept because it was above the lower limit of detection of 0.9 ppb.

Often the decision to keep values is not so clear. Generally values that are indicated as "not detectable", or zero, or "less than the limit of detection" are excluded because they are likely to bias the estimation of error and to appear as departure from an otherwise appropriate model. Sometimes a time point has some valid observations and some that need to be excluded. A general rule is to use a time point only if there are at least three acceptable observations. Otherwise the whole time point should be excluded. If the study is properly planned, there should still be sufficient data to determine an appropriate regression line through the linear part of the depletion curve.

STEP 2. Determine that the variances of Ln concentrations at each slaughter time are constant.

No matter what form the depletion model assumes, the variances at each slaughter time should be constant. Recall that because this is a log-linear model, variances of the Ln concentration should be tested. Computations for using Bartlett's test for heterogeneity are listed below. Bartlett's test is not the

only one available for this purpose, nor is it always the best. Here, however, FDA takes its non-significant P-value (P = 0.234) as support that the variances are constant.

Bartlett's Test Computation (Chi Square Test)

$(s(i))^2$ = Variance of Ln concentration in (i)th group
g = Number of withdrawal groups = 5
f = Common number of degrees of freedom for variance estimates = 4

Days Withdrawn	$(s(i))^2$	$Ln(s(i))^2$
3	.0173	-4.0570
5	.0363	-3.3159
7	.1518	-1.8852
10	.1584	-1.8426
14	.0737	-2.6078

M= (sum of f(i)) Ln (sum of f(i) $s(i)^2$/Sum of f(i)) - sum of f(i) Ln $s(i)^2$ = 6.1119

C = 1 + (1/(3(g-1)))(sum of 1/f(i) - 1/sum of f(i)) = 1.1

Chi^2 (g-1) = M/C = 5.56: P = 0.234

STEP 3. Check the assumption of log-linearity.

The test of linearity is a standard analysis of variance procedure. Its application to these data is summarized below. The test for departure from linearity, judged non-significant (P>0.25), supports the assumption that the data describe a linear process. If the departure from linearity is substantially more significant than 0.25, check again for points that do not belong to the linear part of the depletion curve as described in step 1. An unusually large or small observation at one time point can also produce a poor lack of fit. A discussion of possible explanations for departure from linearity is appropriate.

Summary of Analysis of Variance

DEPENDENT VARIABLE: Ln CONCENTRATION

INDEPENDENT VARIABLE: DAY WITHDRAWN

Source	df	SS	MS	F	P-VALUE
Linearity	1	9.5550	9.5550		
Departure	3	0.0185	0.0062	0.0705	>0.975

Error   20   1.7497 0.0875
F = MS (Departure)/ MS (Error) = 0.0705

STEP 4. Compute the necessary quantities for determining the withdrawal period from the linear regression.

Obtain an estimation of intercept and slope by fitting a regression line with Ln concentration and day withdrawn as the independent variable. Note that the degrees of freedom for error are n-2=23 rather than 20 as in step 3 because variation due to departure, judged above as not significant, is pooled with error.

a = INTERCEPT = 3.93266
b = SLOPE = -0.15983
s^2 = RESIDUAL MEAN SQUARE = 0.076879
DF = RESIDUAL DEGREES OF FREEDOM = 23
n = NUMBER OF ANIMALS = 25
mean of t = MEAN DAYS WITHDRAWN = 7.8
sum of $(t(i) - \text{mean of } t)^2$ = BETWEEN DAYS SS = 374

2. Calculating The Tolerance Limit
The tolerance limit at any time t is
$T(y) = a + bt + ks[1/n + (t - \text{mean of } t)^2/\text{sum of } (t(i) - \text{mean of } t)^2]^{.05}$
k = the 95th percentile of the non-central t-distribution with non-centrality parameter d and degrees of freedom equal to those of S^2
$d = z/[1/n + (t - \text{mean of } t)^2/\text{sum of } (t(i) - \text{mean of } t)^2]^{.05}$
z = the 99th percentile of the standard normal distribution

The sponsor should determine the withdrawal period as follows:

STEP 1. Fix a candidate withdrawal period, in this case 14 days, and calculate d.

$d = 2.3264/(0.1428)^{.05} = 6.1566$

STEP 2. Calculate k. See D. B. Owen, *Handbook of Statistical Tables,* Addison-Wesley, Reading,. Massachusetts (1962). Using the value of d and a table of factors for computing critical values of the non-central t-distribution with Pr = 0.95 and 23 degrees of freedom, k is 8.9248. (There is statistical software available for computing the non-central t-values. For SAS users, the TINV function is available through the SUGI Supplemental Library User's Guide under "Nine Functions for Probability Distributions." Subroutines are also available through International Mathematical and Statistical Library. There may be other sources as well.)

STEP 3. Calculate the tolerance limit and its antilog. Check to see if the antilog exceeds the permitted value. If so, increase t and repeat the calculation. If not, then t is the recommended withdrawal period.

$\exp[3.93266 + (-0.15983)(14) + (8.9248)(0.27727)(0.3779)] = 13.88$

Because the tolerance limit of the residue concentration exceeds the permitted concentration of 9 ppb, the calculation is repeated using 15, 16, 17, and 18 days as the candidate withdrawal period. The tolerance limit at 18 days is 7.86 ppb. Therefore, 18 days is the assigned withdrawal period.

APPENDIX B

Residue Data from Milk

For ease of presentation, this example will use data from the simulated results of a residue depletion study in milk from 10 animals only, rather than the recommended 20 animals. The drug product is for. the treatment of mastitis. Milk samples were obtained from each cow every 12 hours from 12 to 48 hours after the last dose. The results of the triplicate analysis for the marker residue in milk are given below. The validated limit of the method is 0.005 ppm and the limit of detection is 0.001 ppm. The permitted concentration for the marker residue R(M) is 0.0061 ppm.

Cow	---Concentration (ppm)---- time (hours)				------Ln Concentration------ time (hours)			
#	12	24	36	48	12	24	36	48
1	12.74	0.987	0.0417	0.0069	2.54	-0.01	-3.18	-4.98
	11.71	0.877	0.0806	0.0042	2.46	-0.13	-2.52	-5.48
	13.92	1.306	0.0582	0.0078	2.63	0.27	-2.84	-4.86
2	10.17	0.880	0.0494	0.0034	2.32	-0.13	-3.01	-5.68
	9.52	0.429	0.0646	0.0028	2.25	-0.85	-2.74	-5.87
	5.65	0.671	0.0718	0.0027	1.73	-0.40	-2.63	-5.91
3	20.77	6.025	0.3679	0.0944	3.03	1.80	-1.00	-2.36
	15.97	2.968	0.3988	0.0480	2.77	1.09	-0.92	-3.04
	22.73	5.129	0.6256	0.0781	3.12	1.63	-0.47	-2.55
4	6.56	0.602	0.0510	0.0053	1.88	-0.51	-2.98	-5.24
	8.37	1.209	0.0418	0.0025	2.12	0.19	-3.17	-6.00
	17.59	0.741	0.0389	0.0019	2.87	-0.30	-3.25	-6.30
5	13.20	1.474	0.1960	0.0150	2.58	0.39	-1.63	-4.20
	27.57	2.720	0.2232	0.0117	3.32	1.00	-1.50	-4.45
	17.49	2.243	0.3275	0.0186	2.86	0.81	-1.12	-3.99
6	18.03	1.524	0.1533	0.0203	2.89	0.42	-1.88	-3.90
	19.59	1.472	0.1599	0.0222	2.98	0.39	-1.83	-3.81
	30.77	1.881	0.2645	0.0199	3.43	0.63	-1.33	-3.92
7	17.29	1.042	0.1861	0.0238	2.85	0.04	-1.68	-3.74
	18.25	2.605	0.1808	0.0189	2.90	0.96	-1.71	-3.97
	14.81	1.480	0.1325	0.0188	2.70	0.39	-2.02	-3.97
8	14.85	0.502	0.0234	0.0013	2.70	-0.69	-3.75	-6.66
	18.37	0.987	0.0446	0.0019	2.91	-0.01	-3.11	-6.29
	13.15	0.580	0.0216	0.0018	2.58	-0.54	-3.83	-6.34

9	9.88	0.388	0.0220	0.0018	2.29	-0.95	-3.82	-6.33
	18.61	0.715	0.0328	0.0023	2.92	-0.34	-3.42	-6.07
	6.89	0.476	0.0288	0.0030	1.93	-0.74	-3.55	-5.81
10	13.47	1.528	0.1549	0.0138	2.60	0.42	-1.86	-4.28
	16.70	1.580	0.1341	0.0081	2.82	0.46	-2.01	-4.82
	19.81	1.575	0.0858	0.0067	2.99	0.45	-2.46	-5.00

Step 1. Fit a Linear Regression for the Data from Each Animal

The Ln concentration of residue of each separate assay for each animal is plotted versus time. These plots are helpful in determining which time points are in the final linear phase of the depletion curve closest to the established tolerance. For each animal only those points that fall on this final depletion phase should be used for subsequent calculations.

Fit a straight line to each animal's data by the least squares method. The residual sums of squares are partitioned into the "pure error" sums of squares and the "lack of fit" sums of squares. An F test is used for the final determination of the points to be included in the regression. The mean square for "pure error" is taken as an estimate for assay variance. Pooling error terms is not appropriate because the F test is used to determine which time points to include in the subsequent analysis of the data. For each animal only those consecutive data points that lie on the linear portion of the curve should be used. The determination should be done on an animal by animal basis. In this example all time points may be used for all animals. The results of the calculations and the appropriate degrees of freedom, DF, are shown in the table below.

Cow	Intercept	Slope	Residual	
#	a(i)	b(i)	(SS)	(DF)
1	5.12	-0.215	0.730	10
2	4.78	-0.218	0.865	10
3	5.05	-0.160	1.106	10
4	5.11	-0.228	1.516	10
5	5.39	-0.196	1.072	10
6	5.27	-0.192	0.563	10
7	5.00	-0.187	0.611	10
8	5.73	-0.255	0.777	10
9	5.08	-0.236	1.185	10
10	5.37	-0.209	0.590	10

Cow	Pure Error		Lack of Fit		F	P-value
#	(SS)	(DF)	(SS)	(DF)		
1	0.530	8	0.200	2	1.51	0.28

2	0.575	8	0.291	2	2.02	0.19
3	0.749	8	0.357	2	1.90	0.21
4	1.419	8	0.097	2	0.27	0.77
5	0.723	8	0.349	2	1.93	0.21
6	0.392	8	0.171	2	1.75	0.23
7	0.557	8	0.053	2	0.38	0.69
8	0.705	8	0.073	2	0.41	0.68
9	0.917	8	0.268	2	1.17	0.36
10	0.544	8	0.045	2	0.33	0.73

The estimate for mean square "pure error" (s^2) is 0.0889

Step 2. Estimate the Mean and Sample Variance of the Predicted Values

Calculate the mean intercept (mean of a) and mean slope (mean of b). In this example mean of a = 5.19 and mean of b = -0.210. The value predicted by the regression equation for animal i at time t is denoted by $y(i,t) = a(i) + b(i)t$. The sample mean of the $y(i, t)$'s is calculated by mean of $y(.,t)$ = mean of a + mean of bt. The sample variance of the $y(i,t)$'s, $s^2 y(i,t)'$ is calculated next. We pick a tentative withdrawal time t and calculate mean of $y(.,t)$ and $s^2 y(i,t)$.

At t = 48 hours $y(.,48) = -4.86$ and $s^2 y(i, 48) = 1.52$. Note, the calculation of $S^2 y(i,t)$ must be performed anew for each time t, or use the sample variance of a(i) and b(i) as well as the sample covariance of a(i) with b(i).

Step 3. Estimate the Between Animal Variance

The estimated sample variance calculated in step 2 is the sum of the between animal variance and the variance due to estimation of the intercepts and slopes. The between animal variance can be determined by subtraction once the variance due to estimation of the intercepts and slopes is determined.

For each animal we can estimate the variance of the predicted value y(i,t) based solely upon the regression data of that animal. We denote this estimate by $s^2 reg(i,t)$. The mean square for "pure error" should be used in this calculation in place of the unknown population variance (Ref. 2, formula 1.4.6). We then calculate the mean of the variance over all cows sum of $s^2 reg(i\,t)/n$. The between animal variance is then estimated by $s^2 y(i,t)$ - sum of $s^2 reg(i,t)/n$

In our example at 48 hours for cow #l, $s^2 reg(1.48) = 0.0154$, averaging over the values for the ten cows gives 0.0207. Therefore, at 48 hours the estimate for the between animal variance is 1.50 (1.52 - 0.0207).

Step 4. Estimate the Non-Centrality Parameter

The non-centrality parameter, d, for a residue depletion study in tissue is a function of the known time points used in the regression and the proposed withdrawal period. Unfortunately, for a residue depletions study in milk, the noncentrality parameter is a function of the true assay variance and the true between animal variance. Because the "true" values are unknown, an estimate of the non-centrality parameter is made using sample estimates in place of the "true" values. An estimate of the assay variance was calculated in step I (mean square "pure error") and an estimate of the between animal variance was calculated in step 3 (s^2 y(i,t) - sum of s^2

d = z [((s^2 y(i,t) - sum of s^2 reg(i,t)/n)/m) + s^2) / (s^2 y(i,t)/n)]^.05

z = the 99th percentile of the standard normal distribution = 2.32635

m = the minimum number of treated animals contributing milk to the bulk tank that may be tested. FDA has chosen 10 as the value to be used in this calculation. This value is not related to the sample size of 10 in this example.

s^2 = the sample assay variance calculated in step 1 (mean square for "pure error.")

n = the sample size = 10 in this example.

Evaluating the expression for this example gives d = 2.92.

Step 5. Calculate the 95th Percentile of the Non-Central t-Distribution

The next step is the calculation of the 95th percentile of the non-central t-distribution (k) with n-1 degrees of freedom and non-centrality factor, d.

Tables that can be used for this calculation are found in D. B. Owen, *Handbook of Statistical Tables.* Addison-Wesley, Reading, Massachusetts (1962). For SAS users, the TINV function documented under "Nine Functions for Probability Distributions" in *SUGI Supplemental Library User's Guide* performs this calculation for a non-negative non-centrality parameter. A program for the IBM PC and compatibles is available from CVM.

In this example at 48 hours, k = 5.76.

Step 6. Calculate the Desired Tolerance Limit

The desired tolerance limit at any time t is T(t) = mean of y(.,t) + k(s^2 y(i,t)/n)^0.5

In this example the drug product is used to treat mastitis and FDA has decided that no more than one-third of the milk in a bulk tank will come from treated animals. Therefore, we wish the 99% tolerance limit to be below 3 times the permitted residue concentration. The withdrawal time t must therefore obey the rule

Ln (3 times permitted concentration) >= T(t).

In this example, Ln (3 times 0.0061) = -4.02. The tolerance limit at 48 hours is -2.62. Since the inequality does not hold, choose another time and repeat calculations starting at step 2 above. We discover that at t = 60 hours, -4.02 >= -4.70, so the withdrawal period is 60 hours.

VI. GUIDANCE FOR NEW ANIMAL DRUGS AND FOOD ADDITIVES DERIVED FROM A FERMENTATION

I. Purpose and Applicability

The guidance describes how the sponsor of a new animal drug or a food additive derived from a fermentation can meet the statutory requirement that the sponsor show the safety of the compound before FDA can approve its use in food-producing animals. The testing and evaluation criteria described in this guidance are applicable to a new product that is derived from a fermentation that produces a drug and that is administered as a complex mixture. FDA is primarily concerned with new fermentations about which there is little information on the toxicity from the substances produced by the microorganism. The products derived from these fermentations may contain toxic components that are not readily isolated and identified and that might remain as residues in edible animal products.

Unless new evidence shows a concern for safety from uncharacterized substances, FDA will not consider these testing and evaluation criteria to be applicable to an animal drug that is presently being legally administered as a complex mixture, nor to a feed ingredient that is presently marketed under the restrictions of FDA Compliance Policy Guide # 7126.31. (The feed ingredient is derived from the production of a regulated antibiotic, but is marketed without a drug claim; each ton of feed ingredient contains less than two grams of antibiotic; each ton of finished feed contains less than three pounds of the feed ingredient.)

FDA also will not routinely consider these testing and evaluation criteria to be applicable to a new food additive that is derived from a by-product of the fermentation industry not producing drugs unless evidence shows a concern for safety due to uncharacterized substances in these products. This latter group presently includes such products as brewers dried or wet grains or condensed solubles, distillers dried or condensed solubles, extracted fermentation press cake or solubles from enzyme or organic acid producers, and yeast harvests from beer or ale mash.

II. Need for the Guidance

FDA, over the years, has approved for use in animal feed a number of antibiotics that are marketed as unpurified or partially purified products. The tolerances for most of these products were established from bioassays performed on the pure antibiotic. This regulatory approach assumes that the antibiotic is the most toxic substance in the product. FDA no longer considers this regulatory approach adequate. The drug portion of these products is typically 1-25%, leaving 75-99% of the product uncharacterized. Most of the uncharacterized portion will be normal constituents of animal cells (e.g., carbohydrates, amino acids, fatty acids, nucleotides, etc.) and is considered safe. However, the production of toxins by microorganisms is well known (1-4). The microorganism could produce both a useful product and a harmful toxin. The quantity of these substances could vary from changes in culture conditions (e.g. substrate, temperature, pH, rate of aeration) or from changes in the genetic integrity of the organism. The fermentation could become contaminated with a competing microorganism that produces a toxin. Finally, toxic components could be produced or introduced during processing of the material for marketing.

The criteria and procedures described in the other guidances in this series assume that the sponsored product is extensively characterized and that any component can be readily isolated, identified, and subjected to the testing described. However, the sponsored products addressed in this guidance are complex mixtures that may contain the organism, its cellular debris and metabolic products, and residual substrate and nonsubstrate material. This guidance provides an acceptable procedure for evaluating the safety of these products, but alternatives are possible (see 21 CFR 10.90). For example, the sponsor may choose to characterize the product chemically and to seek approval after testing those components that raise toxicological concerns.

III. Safety Evaluation of the Sponsored Product

The evaluation of the sponsored product under this guidance consists of (1) a safety evaluation of the purified drug by the currently applied criteria as described in other guidances, and (2) a toxicological evaluation of the uncharacterized portion of the sponsored product as described in this guidance.

A. Testing of the Uncharacterized Portion

The sponsor will normally conduct this testing on the product proposed for marketing. The product should be derived from at least a pilot factory fermentation (preferably from a production factory fermentation) and must be from the same lot or the same pooled lot. Sponsors are encouraged to submit detailed protocols to FDA for review and comment before initiating the testing.

The sponsor may select either of the following series of tests:

SERIES 1.

* A 180-day feeding study within a rodent species (usually the rat). An acceptable study will include serial sacrifices at 60 and 120 days. If necessary to satisfy the sponsor's statutory obligation to show a product's safety, FDA may require a longer feeding study when evidence indicates a potential for late occurring toxicity.

* A 90-day feeding study in a non-rodent mammalian species (usually the dog). If necessary to satisfy the sponsor's statutory obligation to show a product's safety, FDA may require a longer feeding study when evidence indicates a potential for late occurring toxicity.

* A 2-generation reproduction study with a teratology component in rats. If there is an indication of toxicity at lower doses, higher incidences, or greater intensity in the second generation as compared to the first, then the study should include a third generation.

*Genetic toxicity tests in the presence and absence of metabolic activation on the aqueous and organic solvent extractables of the sponsored product and on the residues occurring in the urine of the food-producing animals (target animals) fed the sponsored product at the highest practical level. If this testing shows a positive response then the sponsor must conduct chronic

bioassays in two rodent species to resolve questions concerning carcinogenicity and thus to show safety as required by law.

SERIES 2.

* Chronic bioassays for oncogenicity in two rodent species.

* A 90-day feeding study in a non-rodent mammalian species (usually the dog). If necessary to satisfy the sponsor's statutory obligation to show a product's safety, FDA may require a longer feeding study when evidence indicates a potential for late occurring toxicity.

* A 2-generation reproduction study with a teratology component in rats. If there is an indication of toxicity at lower doses, higher incidences, or greater intensity in the second generation as compared to the first, than the study should include a third generation.

In the feeding studies, the sponsor should use a dose high enough to provide information on the potential for toxicity from the uncharacterized components in the sponsored product. FDA will consider this condition satisfied if the highest of several doses used gives a response from the purified drug or if the sponsored product comprises 10% of the total diet and no toxicity is observed. FDA believes that the sponsored product needs to be fed at a dietary concentration greater than the 5% limit for non-nutritive substances recommended by the National Cancer Institute (5) because many components of the sponsored product will be nutritive; however, FDA is not suggesting that the sponsored product be fed at the 20% limit, recommended for nutritive substances, to reduce the probability that a toxic response will be observed due to nutritional or salt imbalances. The usual 90-day study is extended to 180-days and the serial sacrifices are included to increase the sensitivity of the assay.

The sponsor may choose to use genetic toxicity tests to assess the presence of or the formation of potentially carcinogenic residues from the uncharacterized components in the sponsored product. The results of these tests do not provide direct evidence of carcinogenicity, but the tests are useful because of the high correlation shown between a compound's ability to cause adverse data in genetic toxicity tests and its ability to cause cancer in animals. The tests recommended are discussed in the GUIDANCE FOR THRESHOLD ASSESSMENT. FDA will make a regulatory decision from a collective evaluation of these data, and not from the result of a single assay.

FDA would prefer that the sponsor conduct separate genetic toxicity tests on each residue that is present in the edible tissue of the target animals. This testing is clearly not feasible for these sponsored products. Individually testing the extractables of the sponsored product and the residues in the urine of target animals provide the most feasible and direct testing of the residues that people will consume. Mutagens have been detected by testing residues in urine (6-11). If there are technical reasons preventing the testing of the extractables or the urine sample, the sponsor must conduct chronic bioassays in two rodent species (Series 2) to assess the carcinogenic potential of the sponsored product and thus to show safety as required by law.

A suggested general procedure for preparation of the extractables is:

STEP 1. Extract an homogenized sample of the sponsored product with an aqueous solvent (an excess of water, or preferably, a water-menthol mixture). Remove insoluble material by filtration or centrifugation. Remove the solvent by evaporation under reduced pressure.

STEP 2. Extract the residual solids from the aqueous extraction with an appropriate organic solvent such as ethyl acetate. Remove the insoluble material by filtration or centrifugation. Remove the solvent by evaporation under reduced pressure.

STEP 3. Dissolve a known amount of the residue in a minimum volume of a solvent that is compatible with the test system. Sterilize each solution (filter sterilization is recommended).

A suggested general procedure for sample preparation from urine is:

Step 1. Collect 24-hour urine samples from at least three animals per sex before feeding the sponsored product. The pooled sample is the negative control; the positive control is this urine sample plus a substance known to give a positive response.

STEP 2. Feed the sponsored product to the animals for two weeks at a dose higher than the proposed dose of the product, but at a dose lower than that which induces toxicity. In order to avoid false positives, care should be taken to assure that the only dietary variable is the sponsored product.

Step 3. Collect 24-hour urine samples from the dosed animals. Treat the pooled sample with enzymes such as beta-glucuronidase and aryl sulfatase to release conjugated substances. Evaporate the sample to dryness under reduced pressure.

Step 4. Dissolve a known amount of the residue in a minimum volume of a solvent that is compatible with the test system and sterilize the solution (filter sterilization is recommended).

B. Evaluation of Results

FDA will determine from comparing the results of testing the purified drug and the product proposed for marketing whether all the toxicity is attributable to the purified drug. If FDA concludes that the data do not demonstrate any additional toxicity from the uncharacterized portion, FDA will use the results of the testing conducted on the purified drug to establish the ADI.

If FDA concludes that the data demonstrate additional toxicity from the uncharacterized portion of the sponsored product, FDA may use the results of this testing to establish the ADI. For example, assume that the no-observed effect level when the purified drug was tested was established at a dose of 400 ppm. Assume also that when the product proposed for marketing was tested, the no--observed effect level was established when the dose of the drug was 100 ppm. This result would suggest that another toxic substance was present in the uncharacterized portion. In this specific case, FDA will calculate the ADI using the residues of the drug as the marker for the other toxic substance and using the 100 ppm dose. The ADI for the total residue of the drug would be 0.1 ppm if 90-day studies were conducted (100 ppm divided by the 1000-

fold safety factor), or 1 ppm if chronic studies were conducted (100 ppm divided by the 100-fold safety factor). When this approach is used, FDA will implement the statutory requirement that the sponsor develop a regulatory assay for residues of the drug when necessary to ensure compliance with any restrictions on the product's use.

The above approach will not be acceptable for a carcinogen. In such a case, the sponsor has the following options for developing a safe product:

* Purify the drug and discard the remainder of the fermentation, or

* Reformulate the product to reduce 'the concentration of the toxicant(s) relative to the drug and demonstrate that the reformulated product is safe according to criteria in this guidance, or

* Identify the toxicant(s), and demonstrate by adequate tests on the toxicant(s) that safe conditions of use can be established for the product containing the toxicant. FDA may require the sponsor to develop a regulatory assay for the toxicant(s) to satisfy the statutory requirement of a showing of safety.

C. Quality Control

The sponsor must submit a description of the product, and a description of the methods and controls used in its manufacture to determine and preserve identity, strength, and quality (21 CFR 514.1(b)(4) and (5)). FDA will review these data as part of the human food safety evaluation to determine the adequacy of these procedures to prevent the formation of toxic components that were not subjected to the pre-clearance testing. The sponsor must follow the procedures and controls described unless a supplemental new animal drug application has been approved (21 CFR 514.8). A sponsor seeking approval under the terms of this guidance must also provides

* A description of the microbiological culture techniques used from stock culture through fermentation so that an assessment can be made of the potential for contamination, and a description of the tests used to

* A description-of the procedures used to control the genetic integrity of the inoculum, and a description of the tests used to assure that the integrity is maintained.

* A description of the substrate used for the fermentation, including a listing of the maximum allowable variations in substrate composition.

* A description of the tests used to determine drug yield in a batch, and the criteria used for accepting batches that deviate from the expected yield.

If the results of the tests and controls do not meet the specifications in the NADA, no product from the batch can be marketed for new animal drug or food additive purposes. Production records must be maintained (21 CFR 226.102).

As a condition of approval, FDA may require that the sponsor use a test for gene mutations in bacteria in the quality control procedures if other tests and controls are judged inadequate to preserve the identity, strength, and quality of the sponsored product. This decision will be made on a case-by-case basis after review of the submitted data.

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VII. GUIDANCE FOR THE HUMAN FOOD SAFETY EVALUATION OF BOUND RESIDUES DERIVED FROM CARCINOGENIC NEW ANIMAL DRUGS

A. INTRODUCTION

In I. GUIDANCE FOR METABOLISM STUDIES AND FOR SELECTION OF RESIDUES FOR TOXICOLOGICAL TESTING, FDA discussed in general terms some considerations relevant to bound residues. FDA stated that the covalently bound residue, whether derived from a carcinogenic or noncarcinogenic drug, would be considered no more toxic than the parent drug; that full structural identification of the bound residue would normally not be required; and that if the total residue is below the safe concentration at the requested withdrawal period no additional safety data would be required on the bound residue. In addition, FDA specified an acceptable procedure for discounting from the total residue a portion of the bound residue derived from a non-carcinogenic new animal drug (i.e., the Gallo-Torres technique for determining bioavailability, Ref. 1).

The human food safety assessment of bound residues derived from carcinogenic veterinary drugs has long presented FDA with both scientific and policy challenges. The complex nature of bound residues, which result primarily from the reaction of a drug metabolite with cellular macromolecules, makes the usual approaches to a safety evaluation unsuitable. In this guidance FDA describes the kinds of data it intends to consider in evaluating the toxicological significance of bound residues derived from carcinogenic animal drugs.

B. NATURE OF THE PROBLEM

The use of veterinary drugs in food-producing animals can result in residues that are not readily extractable from tissues using mild aqueous or organic extraction conditions. These residues usually represent (a) endogenous compounds derived from a portion of the drug or (b) covalently bound residues derived by reaction between a metabolite of the drug and cellular macromolecules. Those nonextractable residues shown to result from incorporation of fragments of a drug into naturally occurring molecules (for example, amino acids, fatty acids, nucleic acids) will be subtracted from the total residue because they are not of toxicological significance. Bound residues formed from pathway (b) above will be considered to be of equal toxicological concern as parent drug until sufficient information is made available that permits an assessment of the contribution of the bound residue to the overall toxicity of the drug.

The most direct route to examining the carcinogenic potential of a bound residue derived from a carcinogenic drug would be to feed test animals tissues containing the bound residue. The major drawback to this approach is the limited concentrations of bound residue in the tissues. Even when the tissues containing the bound residue are used as the only dietary source in order to reach the highest concentration possible, the total concentration of the bound residue given to rats or mice is still orders of magnitude lower than the lowest concentration of the parent compound that would give a positive carcinogenic response. Thus, it could be argued that negative results are inconclusive since the animals are not exposed to high enough concentrations of bound residue.

As noted above, if a veterinary drug is not carcinogenic, FDA discounts from toxicological concern that portion of the covalently bound residue that is demonstrated not to be bioavailable, relative to the parent, in the bile-cannulated rat (Ref. 1). However, FDA has not permitted a similar discount for bound residue derived from carcinogenic drugs solely on the basis of bioavailability data because of its concerns for gastrointestinal binding and gastrointestinal carcinogenesis.

Contributing to the difficulties in making a safety assessment on bound residues is the fact that the residue chemistry of bound residues is extremely complex. Reactive metabolic intermediates can form adducts with numerous naturally occurring compounds, yielding a myriad of products, most of which are likely to be present in extremely low concentrations. Consequently, the structural determination of the bound residue is difficult, if not impossible in most cases. In addition, although it has allowed that that portion of the bound residue shown to be owing to endogenous incorporation can be discounted from the total residue of toxicological concern, FDA is aware that such incorporation is likely to be so widespread as to make an accurate quantitation virtually impossible.

C. APPROACH TO THE ASSESSMENT OF THE BOUND RESIDUE

In proposing an approach to the safety assessment of bound residues that is thought to be both scientifically valid and reasonably capable of being accomplished, FDA is making a number of assumptions. First, FDA intends to consider only the key toxicity target of the parent, i.e., carcinogenicity. Second, FDA intends to ask that sponsors pursue a toxicological evaluation of the major adduct(s) only. Third, FDA intends to rely on data from a suitable combination of *in vitro* and *in vivo* studies, rather than from long term feeding studies, to establish the carcinogenic potential of the bound residue.

FDA recommends that sponsors wishing to obtain a safety evaluation of the bound residue derived from a carcinogenic veterinary drug develop data in four areas. The data should specifically provide information on the:

* Bioavailability of the Bound Residue,
* Toxicological Potential of the Bound Residue,
* Reversibility of Adduct Formation,
* Mechanism of Bound Residue Formation.

The above information may be collected from a combination of *in vitro* and *in vivo* testing methods. Below, FDA briefly describes how these data, taken together, can be useful in the evaluation process.

Bioavailability of the Bound Residue

Generally speaking, if the bound residue is not appreciably absorbed, then its toxicological significance is diminished. Accordingly, FDA's policy has been to use data from bioavailability studies in the bile-cannulated rat to reduce the total residue of toxicological concern when the parent drug is not carcinogenic. However, because of concerns about gastrointestinal binding

and gastrointestinal carcinogenesis, FDA has not accepted the results of bioavailability studies to reduce the total residue of carcinogenic concern. FDA has modified that policy and now intends to rely primarily on the results of chronic studies to determine the likelihood of a drug or its metabolites, whether actually identified or only proposed, to produce gastrointestinal carcinogenesis. Consequently, FDA accepts data from bioavailability experiments, together with the information in the areas described below, in assessing the safety of bound residues derived from carcinogenic drugs. In the event chronic feeding studies show that the gastrointestinal tract is a target for a drug's carcinogenicity, then FDA may require specific studies to evaluate the bound residue(s) of the drug.

Toxicological Potential of the Bound Residue

Clearly, the goal of the entire testing scheme is to permit a reasonably reliable assessment of the bound residue's toxicological potential for carcinogenesis. The information obtained on the reversibility of adduct formation and mechanism of bound residue formation will contribute to that evaluation. However, the intrinsic toxicity of the bound residue must still be assessed. FDA intends to accept *in vitro* or short-term *in vivo* tests to support that assessment.

The specific *in vitro* or short-term *in vivo* experiments that a sponsor chooses to conduct should be based on the toxicity profile of the parent and the nature of the bound residue. If the parent is carcinogenic and mutagenic, an assessment of the bound residue may be made using a suitable mutagenicity assay and appropriate material for testing. Types of material that could be tested include: bound residue, if isolable: material, either purified or as a mixture derived from the acid and enzymic treatment to stimulate GI digestion of bound residue; purified major adducts; beta-lyase treated adducts; or model compounds, such as cysteine-metabolite or glutathione-metabolite adducts.

Reversibility of Adduct Formation

Because bound residues are derived from reactive metabolites, the reversibility of adduct formation to yield reactive compounds is a key factor in the safety assessment of bound residues. A demonstration that the reactive metabolite cannot be regenerated under various conditions, such as mild acid or enzymic digestion, would lessen the concern for the toxicological significance of the bound residue.

While the reactive metabolite could be responsible for toxicity, it must be realized that bound residues may express toxicity in other ways. For example, the macromolecule to which the metabolite is bound may undergo digestion to peptide or amino acid adducts (cysteine adducts, for example) which themselves may be toxic or which may be chemically or enzymatically transformed into an active species thereby eliciting toxicity. Appropriate safety testing should follow from the knowledge gained in the mechanistic studies suggested below.

Mechanism of Bound Residue Formation

Studies on the mechanism of bound residue formation provide valuable information on the probable metabolic pathways involved in drug activation and on the nature of the bound

residue. The data from these studies, which ordinarily use rat liver microsomes but which could use food-animal liver microsomes as well, can permit a determination of the binding requirements. Moreover, the information obtained may allow a prediction as to what adducts are likely to represent major and minor contributors to the total bound residue. For example, from *in vitro* competitive binding studies it may be possible to determine whether primary binding of a drug metabolite is with proteins or nucleic acids. Additionally, similar studies with model compounds including amino acids, nucleosides or small peptides such as glutathione, may afford important structural information on the bound residue, e.g., to which amino acid on a protein the drug metabolite is likely to become attached and which functional groups on the drug metabolite and amino acid are involved in the binding.

As part of this work, FDA may request that the sponsor attempt to demonstrate that bound residue/adducts observed in the *in vitro* experiments are present as well in the target food-animal. It is likely that such work would entail no more than a qualitative comparative characterization. The need for this type of work will depend upon the particular drug and decisions will therefore be made on a case-by-case basis.

D. Conclusions/Comments

FDA has outlined an approach to the safety assessment of bound residues derived from carcinogenic animal drugs. FDA has not enumerated specific studies, because it anticipates that the particular testing package will vary depending on the drug and the nature of the bound residue. Moreover, the extent of the data collection will depend on a number of factors such as the degree of bioavailability, the amount of bound residue that must be discounted, the nature of the bound residue, and the carcinogenic and mutagenic potency of the parent. For example, if a bound residue is poorly absorbed, it is likely that the quantity of work needed to address the carcinogenic potency of the bound residue will be reduced. Thus, drugs will be evaluated on a case-by-case basis.

FDA suggests that a sponsor wishing to evaluate the safety of a bound residue derived from a carcinogenic drug develop its testing scheme using the general approach outlined herein. FDA will then meet with the sponsor to discuss the proposed scheme and the likelihood that the work will meet the sponsor's needs.

FDA has written this guidance after considering the views of numerous scientists that were expressed at the International Symposium on Biological Models to Determine the Safety of Bound Residues in the Tissues of Food-producing Animals, held in Washington, D.C., October 1988. In particular, FDA has relied upon the paper entitled "Development of a Unified Approach to Evaluate the Toxicological Potential of Bound Residues", which was presented by Dr. A. Y. H. Lu (Ref. 2). Sponsors are directed to this paper for an excellent overview of the bound residue issue. In addition, FDA suggests that sponsors considering addressing the safety of the bound residue of their own drugs refer to the paper entitled "Toxicological Significance of Covalently Bound Drug Residues" (Ref. 3). This latter paper and references therein describe the extensive work that was conducted in attempting to elucidate the toxicological potential of the bound residue of ronidazole.

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