METABOLISM AND KINETICS IN THE REGULATION OF ANIMAL DRUGS

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Summary

"Criteria and Procedures for Evaluating Assays for Carcinogenic Residues," popularly known as the "Sensitivity of the Method (SOM)" proposal for the regulation of animal drugs and feed additives, employs an initial analysis to determine whether a compound must meet the requirements as a suspect carcinogen or the requirements for general food safety (GFS). Metabolism and kinetics of drug depletion play an important role in the six-step procedure that is used for either set of regulatory criteria. The only significant difference that exists between the two sets of criteria is the degree of testing required. However, the difference will usually not be manifest until positive findings of carcinogenicity in the chronic studies in laboratory animals trigger a further evaluation of the metabolism of the drug or feed additive. For both SOM and GFS, metabolic information will be used to determine the appropriateness of the test species as well as to help select a compound for residue analysis. Residue depletion kinetics in the food animals also play an integral role in the selection of species-strains for toxicity testing selection as well as in the selection of the compound for residue analysis in food. Furthermore, depletion kinetics are used to set a safe withdrawal period. The development of guidelines is discussed.

(Key Words: Animal Drugs, Feed Additives, Drug Depletion, Carcinogenic Residues, Metabolism, Kinetics.)

Introduction

The Food and Drug Administration (1979) published a proposal in the Federal Register that outlined a scientific and regulatory approach that the Agency proposed to pursue in the regulation of potentially carcinogenic animal drugs. This latest proposal grew out of two earlier documents on the same subject published in the Federal Register (FDA, 1973, 1977). The latest document, entitled "Chemical Compounds in Food-Producing Animals: Criteria and Procedures for Evaluating Assays for Carcinogenic Residues," is often referred to as the "Sensitivity of Method" or the "SOM" proposal. This document is still under review because of the numerous comments received. The document outlines a six-step procedure by which the carcinogenic potential of a sponsored product is evaluated. The six-step procedure is prefaced by a threshold assessment (TA) in which a preliminary evaluation of the carcinogenic potential of a compound is made by means of structure-activity relationships, in vitro genetic toxicology and 90-d subchronic toxicity tests. Also considered are chronic toxicity studies and other relevant information. A use rating that predicts potential human exposure and residue levels is also an important part of the TA. The result of the TA is a decision as to whether the compound will continue in the approval process under the criteria of the SOM proposal or under the criteria of the Agency's general food safety (GFS) requirements. Assessment under either set of criteria, however, will include a six-step evaluation procedure. The differences lie in the degrees to which the metabolic fate of a compound is pursued, the concentration at which total residues are examined, the types of toxicological studies employed and the method by which an acceptable level of drug residue is calculated. The purpose of the present paper is to outline an approach under consideration in terms of metabolism and kinetics. It should be recognized, however, that the present discussion is based on developing guideline material in response to the many comments on the SOM Proposal.
Safety Evaluation

All residues present (added directly or transformed) in food as the result of a drug or feed additive given to the animal for whatever purpose must be evaluated for safety according to the New Animal Drug sections 512(b)(7), 512(b)(8), 512(d)(1)(H) and 512(d)(2) of the Food, Drug and Cosmetic Act. The following six steps are used in this safety evaluation:

1. Metabolism studies in the target species.
2. Comparative metabolism studies in the toxicity test species-strains.
3. Toxicity-carcinogenicity testing and assignment of a safe level or acceptable level of risk for residues.
4. Determination of the marker residue and target tissue and assigning a tentative value for the marker residue (Rm) when the total residues have depleted to tolerance.
5. Development of an analytical assay for the marker residue at Rm.
6. Residue depletion studies to determine a withdrawal period.

An examination of these steps indicates that metabolic information (information on the number and nature of residues) is needed in steps 1, 2 and 4, whereas kinetic (depletion) information is needed in steps 1, 4 and 6.

Metabolic studies are necessary for a number of reasons. It is important to determine the nature and number of metabolic products present in food so that the adequacy of the species-strains used for toxicity testing may be evaluated through comparative metabolism studies. Of equal importance is the need to know the metabolic disposition of the administered compound so that the presence of a residue of possibly greater potency than the parent compound can be determined or predicted. Although many metabolites are no doubt less toxic than the parent compound, some metabolites have been demonstrated to be more toxic (Miller, 1970; Gillette, 1976). Finally, it is usually necessary to develop an analytical assay for regulatory monitoring of drug residues. In doing so, some metabolites of the administered compound are candidates for the assay rather than the administered compound because metabolites often persist longer or are found in greater concentrations than the administered compound in edible tissues after the treatment is stopped.

Radiolabeled drug or feed additive is most often employed in the metabolic evaluation. Carbon-14 is the usual choice because of its stability, lack of exchange, long half-life and availability of radiolabeled precursors for synthetic purposes. The specific activity of the tagged material may vary with the nature of the study being undertaken. It is recommended that the radiolabeled drug should have a radio-purity of at least 98% so that a minimum of nondrug-related components become labeled residues. The sponsor should determine the purity of the material upon receipt and just before its experimental use in at least two chromatographic systems. The use of high specific activity material should be reserved for special studies in which residues in the low ppb range are to be investigated, as in the case of demonstrated carcinogens.

The drug or feed additive should be radiolabeled so that portions of the compound that are of toxicologic or carcinogenic concern are labeled. The label site should also be chosen so that it is not separated by metabolic or chemical reactions from the portions of the molecule that are of interest. If metabolism cleaves the molecule into two fragments, both should be radiolabeled (usually in separate studies) if both are of toxicologic concern. The sponsor may be requested to furnish evidence of the stability of the radiolabeled site. Whenever tritium is employed, information on tritium exchange will be required. The amount of exchange may be determined by the amount of tritiated water present in excreta and tissues. Tritium exchange in excess of 10% of the administered dose is generally considered unacceptable because of the ease of tritium labeling of endogenous substances.

Antibiotic drugs should be synthesized from precursors that will incorporate the label into sites not readily removed by drug metabolism. If a multicomponent drug is produced, the major components should be adjusted to about the same specific activities in the absence of overriding information on the toxicity of the compounds. In addition, the radiolabeled components should be adjusted to a reasonable proportion of the final marketed product.

Radiotracer analytical data should include total radioactivity in the edible tissues of ex-
experimental animals and is usually determined following combustion. The following information is required.

1. Specific activity of parent drug administered.
2. Radiopurity of parent drug with supporting chromatograms from at least two different chromatographic systems.
3. Full description of tests and methods of assay.
4. Practical demonstration of the limit of detection in tissues will be assumed to be twice the background count unless demonstrated by the sponsor through fortification studies in each of the edible tissues. Parent drug should be used in these fortification experiments. Our experience indicates that 8 to 10 cpm above background is usually achievable.
5. Results of triplicate analyses of each tissue derived from medicated animals. The samples should be obtained from an ice-cold homogenate of a whole organ from chickens, for example, or a homogenate of at least .25 kg of a representative tissue (e.g., muscle, fat or representative portions of organs) from large animals.

For each experimentally derived data point, results for cpm should be calculated in dpm and then presented in terms of parent drug equivalents such as ppm or ppb. Sample weights as well as the sampling procedure and sample size should also be specified. The report should be accompanied by information on counting efficiency, quench corrections if applicable, internal standards if employed, sample calculations, conversion factors used and all appropriate raw data.

Dosing of target animals is usually accomplished by using enough male and female animals to permit sacrifice of three animals/time point. Ordinarily four time points are needed to adequately demonstrate the depletion curve. An initial sacrifice time (8 to 12 h post-treatment; 6 h for fowl) followed by three additional slaughter times is recommended. In the case of fowl, a larger number is often required so that sufficient tissue is available for residue profiling, characterization and identification, if the tissue is to be used for such purposes.

The dose given is ordinarily at the highest intended treatment level and should reflect the treatment regimen recommended for the compound. Two general exceptions are made: (1) when the sponsor is seeking a zero withdrawal time and waiver of the requirement for an analytical method and (2) when overdosed or “landmark” animals are used in preliminary metabolism studies. For the former case, 1.5 to two times the normal treatment level is administered to at least two groups of three animals for two different lengths of time and the animals are then sacrificed at zero withdrawal. Three animals are slaughtered at each of two points in time separated by at least 96 h. This is to determine whether a steady state level of residue has been achieved. Additional animals dosed for still other lengths of times may be required if steady state levels have not been achieved in the two-point study. For the second of the above-mentioned studies two or more animals are ordinarily given a 5 to 20x overdose for residue characterization and identification. The residue once identified in overdosed animals would be examined in animals dosed at use levels to verify the correspondence of the metabolic pattern at the lower level. If a drug is given only once to an animal for a specific therapeutic effect, then a single dose of radiolabeled drug is the appropriate exposure for the total residue depletion study. Where prolonged medication is intended, as for growth promotants, a limited number of daily treatments (e.g., seven) may be used. It is recognized that residues with long depletion half-lives will not achieve steady state after 7 d dosing; however, it is often impractical to administer the compound for significantly longer periods of time. Judgments involving the more persistent residues will have to consider the fact that steady state was not achieved as in the case of those involving investigational new animal drug (INAD) withdrawal times and marker residue considerations. However, for some INADs with time constraints on a withdrawal period, for certain threshold assessments and new animal drug applications (NADAs) where neither withdrawal nor a method is required, steady state levels of drug residues in tissues must be attained or estimated by acceptable means in order to approximate residue levels at the time of slaughter.

INAD Studies. Depletion data from four edible tissues (muscle, liver, kidney and fat; skin with adhering fat for poultry) will be used to assign a withdrawal period for INAD investigational animals, based on the total residue levels in the tissue that last reaches a level of residues deemed acceptable. Ordinarily this is
about 100 ppb for most compounds, unless the compound is a highly suspect or demonstrated carcinogen or has otherwise been demonstrated to be extremely toxic. Tissue consumption factors will be used to adjust the tolerance or safe level for residues in the various tissues according to available guidelines.

Residue data from the initial INAD depletion studies may be used in a TA to determine whether a sponsor's compound enters into the six-step sequence as a carcinogenic compound or under the GFS requirements. In its present form, the TA employs residue considerations only in one decision where low-use compounds are being considered. The level of 100 ppb (with permitted adjustments for consumption of various tissues) should not be exceeded in the last depleting tissue at the longest withdrawal period considered practical for the compound from animal husbandry considerations. For laying hens, however, residues in eggs for human consumption must not exceed the projected or required safe level at zero withdrawal.

At this point, the firm has learned much about the appropriateness of the radiolabel position of the drug as well as the amount of radiolabeled drug residues in tissues. If the firm suspects that a significant amount of tissue radioactivity is due to endogenous incorporation, and seeks a shorter withdrawal period, it will be necessary to demonstrate that this is a result of the incorporation of the drug label into endogenous tissue components through normal metabolic processes. Such a demonstration leads to dismissing that portion of the total residue from toxicological concern. The total drug residue level would be reduced by an amount demonstrated to be endogenous or of no toxicological concern.

**Metabolism Study in the Target Animal.**

In one approach being considered, the metabolism study is intended to provide information on the identity, amounts and persistence of the parent drug and its metabolites in the edible tissues of the target animal. The radiotracer aspects for these studies are similar to those outlined under the section dealing with radiotracer technology and INAD depletion studies. If the depletion studies were adequately performed, the tissues from those studies may be used for these metabolism studies. The stability of radioactive residues in the frozen state as a function of time should be demonstrated if these tissues are to be used for the purpose of metabolite identification and if several weeks have elapsed since the animals were slaughtered. In addition, it may be necessary to initiate a new study if a higher specific activity is necessary to determine drug residues in the low ppb range. The initial sacrifice time and the number of animals/point in time are the same as suggested previously. Additional sacrifice times may be added as needed to determine the depletion trends of metabolites that should be pursued to total residue concentrations of 100 ppb for GFS. Demonstrated and highly suspect carcinogens (those belonging to families of known carcinogens) may require metabolite confirmation until total residue reaches the low ppb range. It should be noted that identification of metabolites is usually pursued when residues are considerably above the ppb level.

Before beginning the studies on residue identification, sponsors may find it useful to begin metabolite identification studies with preconditioned animals overdosed with cold drug. These so-called "landmark" animals will provide drug residues in tissues and excreta at levels where purification and identification are easier than for animals treated at use level. However, because of overdosing, the metabolite profile in tissues may not correspond to that seen at normal use levels. In addition, the compound at exaggerated levels may significantly alter the metabolic activity of the bacterial flora of the gut. Although the overdosing approach is not required, we have observed significant success with its use and urge sponsors to consider this approach if metabolite identification is a problem at use level.

Studies employing such an approach have begun to ease the problems associated with low level residue identification (Paulson and Struble, 1980). New techniques involving the use of purification schemes in which thin layer and high pressure liquid chromatography are used before identification by mass spectrometry, infrared spectrophotometry and nuclear magnetic resonance spectrometry (Bakke et al., 1976) may alleviate some identification problems that have occurred in the past.

Individual metabolite identification should ordinarily be pursued for metabolites comprising 10% or more of the total residue or more than 100 ppb (whichever is lower) at zero withdrawal, under the GFS. For demonstrated carcinogens and compounds that come from a family of substances in which carcinogenicity has also been demonstrated, metabolite identification may be required below 50 ppb. There-
fore, the amount of metabolite identification required for suspect, but not demonstrated, carcinogens and the amount of metabolite identification required under GFS will be considered essentially the same before toxicity testing.

Because of the limitations in the area of metabolite identification, requirements will generally be limited to methods available in the field as best effort at state-of-the-art. If a compound has been demonstrated to be a carcinogen, the metabolism data developed earlier may have to be reevaluated. For demonstrated carcinogens or other special cases this may not actually go much beyond that outlined above for GFS, but a sponsor may be requested to pursue already identified metabolites to the low ppb level in depletion studies for a highly suspect or demonstrated carcinogen. Furthermore, after partial purification and before identification of metabolites of demonstrated or highly suspect carcinogens, in vitro testing of metabolites is recommended as a screen for compounds that may require identification. It is recognized, however, that de novo identification cannot begin at the low ppb level. Identification may involve some synthesis of predicted metabolites for use in reverse isotope dilution identifications for certain low level metabolites especially if certain partially purified metabolites give positive results in the in vitro tests.

The following studies provide an important part of the information in the toxicological assessment of drug residues. This information in conjunction with somewhat similar studies in laboratory animals provides a basis for acceptance of the toxicity test species-strain used in the safety evaluation studies in step three of the criteria.

1. Total residues at each time point for the four edible tissues (muscle, liver, kidney and skin-fat), milk and eggs (separate determinations for yolk and white) where applicable.

2. The percentage of the extractable vs the nonextractable total residues in appropriate tissues at each time point. Extractability should be examined with a number of solvents, at various pH levels and in the presence of denaturing agents (e.g., urea). Chemical and(or) enzymatic hydrolyses should be used after the initial extraction studies.

3. Metabolite chromatographic profiles of excreta, preferably reported early in depletion so that the full spectrum of metabolites present may be observed. Additionally, characterization and identification of metabolites in the excreta may form a useful initial step pursuant to similar studies in tissues.

4. Metabolite chromatographic profiles of the edible tissues, milk and eggs. Pooling of samples from one withdrawal time is acceptable. Profiles of metabolites at each withdrawal time should be provided.

5. Metabolite identification to construct metabolic pathways and to determine metabolite persistence as in No. 4 above, beginning in excreta, liver and other tissues as necessary. An attempt should be made to identify metabolites comprising 10% or more of the total residue or more than 100 ppb (whichever is lower) at zero withdrawal.

Both polar and nonpolar extractable metabolites from tissues should be chromatographed by procedures such as thin layer or high performance liquid chromatography. The radioactivity associated with each spot or peak is to be expressed as dpm, parent drug equivalents (ppm) and percentage of total residues for each tissue. The polar fraction should also be exposed to conjugate hydrolyzing enzymes in a second experiment and rechromatographed. Preliminary studies should be conducted to determine the best methods of extraction and separation of the metabolites in each edible tissue. The effect of varying solvent polarity, pH, dialysis and chromatography characteristics should be examined.

Metabolism Studies in Toxicity Test Animals. The comparative metabolism studies must be performed in the laboratory species and strains used for toxicity-carcinogenicity testing. In selecting the species and strain of laboratory animal for chronic or subchronic testing, it is important to choose either one that has a profile very similar to that seen in the food animal or a strain that has good proportions of all metabolites. Strains that are deficient in producing a major food animal metabolite or that do not produce several minor metabolites (perhaps in an outbranching of a metabolic pathway) are not considered to be the best test candidates. However, it is also recognized that certain species and strains are often chosen for a specific toxicological end point. In these instances, the selection of test animals for toxicological reasons may preempt their selection on a strictly metabolic basis.

The primary objective of the comparative metabolism evaluation (step two of the criteria)
is to determine the acceptability of the toxicity test species to produce an array of metabolite residues similar to those found in the edible tissues of food animals. This information is needed because a judgment must be reached that the residues to which humans will be exposed have been tested in acceptable animal models. The emphasis in these studies will be on the comparison of metabolite profiles between drug residues in the tissues of food animals and those produced in the laboratory animal. However, quantitation of the profiled metabolites may be used in making judgments on the need to test additional compounds in the toxicity test animals. Quantitation is most easily accomplished by radiotracer analysis of the chromatographed compounds or fractions. An additional and perhaps the most important aspect of these studies is to identify those compounds not present in the laboratory test species but present in the edible tissues of food animals. These latter types of metabolites are of concern because they have not been subjected to adequate toxicological testing. It is this type of drug residue that may lead to additional toxicity testing, especially if it is a major metabolite in the food animal, but is not detected in the laboratory test animal.

In doing these comparative metabolism studies it is essential to have a sufficient amount of tissue and excreta from the animals. In this regard, pooling of excreta or tissue from the toxicity test animals of like sex is recommended. Separate pools from both males and females are needed. The procedure employed here should be the same or of similar resolving power as that employed for the target animal so that a valid comparison of residue profiles may be made. It is emphasized here that it is essential to examine the profile of isolated compounds or fractions, whether they have or have not been identified, and the quantitation by means of radiotracer techniques of these residues produced in laboratory test species. Urinary profiling in some cases will be insufficient because test animal profiles will be compared with edible tissues (usually liver) from target food animals. It is well known that many drug metabolites especially those of high molecular weight are often excreted via the bile and feces and found in only minor amounts or not at all in urine. Therefore, urine may not accurately reflect the complete profile and relative proportions of metabolites produced in a given species and strain. In the comparative metabolism studies the animals would be dosed for a sufficient number of days to ensure that tissue storage sites and metabolism would approach steady state with the possible exception of the bound residues. Seven-day dosing should be sufficient for this to be achieved except for bound residues as in the case of the target food animal. Excreta from the last 2 or 3 d should also be retained for profiling. The dose should be at the highest level to be tested in the 90-d subchronic studies.

**Marker Residue and Target Tissue.** The FDA's approach to the use of kinetic data for selection of a marker compound and target tissue have been adequately discussed in a recent article (Weber, 1981). The outline of this approach will be briefly reviewed here to complete the concept of how metabolic information is integrated into the regulation of animal drugs. The marker residue and target tissue are selected for carcinogens as well as noncarcinogens by the same procedure. In essence, the identical procedures are used in steps 4, 5 and 6 of the criteria for both the regulation of carcinogens and GFS. Most often the target tissue is selected as being the tissue that is last to achieve its tolerance or acceptable level (including consumption factors) in depletion profiles of total residues. This is graphically demonstrated in figure 1. After the target tissue has been selected, a marker residue in the target tissue is chosen and its concentration (Rm) is determined when the total residue achieves its safe level (Sm). An example of this approach is shown for a hypothetical situation as seen in
The specific percentages, however, are under review. The statistical theory upon which the proposed method is based may be found in an article by Owen (1968).

Conclusion. The review of our current approach to the regulation of animal drugs and feed additives demonstrates the relevant points at which metabolism and kinetic data on residues play a key role in developing information on the safe use of chemicals used in food-producing animals. This includes the development of metabolic information used to determine the adequacy of the animal models employed in the toxicity and carcinogenicity testing of the sponsored compound as well as selection of a marker residue, target tissue Rm value and withdrawal period for food animals. An attempt has also been made to share some of the developing guideline material. It cannot be assured that the final form of the guidelines will have the exact dimensions given here, but it is reasonable to assume that in many instances what has been discussed will be a good approximation of the final product.

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