European Medicines Agency
Veterinary Unit and Inspections

London, 23 May 2007
Doc. Ref. EMEA/CVMP/SWP/95682/2007-CONSULTATION

COMMITTEE FOR MEDICINAL PRODUCTS FOR VETERINARY USE (CVMP)

REFLECTION PAPER ON ASSESSMENT OF BIOAVAILABILITY OF BOUND RESIDUES IN FOOD COMMODITIES OF ANIMAL ORIGIN IN THE CONTEXT OF COUNCIL REGULATION (EEC) NO 2377/90

AGREED BY SAFETY WORKING PARTY	23 February 2007
ADOPTION BY CVMP FOR RELEASE FOR CONSULTATION	15 May 2007
END OF CONSULTATION (DEADLINE FOR COMMENTS)	30 November 2007

The content of the reflection paper, once reviewed will be placed in the Volume 8 of the Rules Governing Medicinal Products in the European Union

Comments should be provided to Kornelia Grein, kornelia.grein@emea.europa.eu, Head of Sector – Safety of Veterinary Medicines

Fax +44 20 7418 8447

KEYWORDS	Bioavailability, bound, extractable, residues, consumer safety, Maximum Residue Limit, MRL

7 Westferry Circus, Canary Wharf, London, E14 4HB, UK
Tel. (44-20) 74 18 84 00 Fax (44-20)
E-mail: mail@emea.europa.eu http://www.emea.europa.eu

1. INTRODUCTION

Assessment of consumer safety from use of veterinary drugs in food producing animals requires a comprehensive set of data on the nature and quantity of residues in edible tissues and their basic pharmacokinetic behaviour. This information is required to characterize the type and level of possible dietary intake of residues. The residues may consist of a variety of drug-derived compounds: parent drug and free metabolites, conjugated residues, and residues that are bound to macromolecules or even incorporated into endogenous molecules. The relative and absolute amount of each residue fraction may vary depending on the compound, dose, tissues and time point of sampling. The relative fraction of metabolized residues or non-extractable/bound forms often increases with time after treatment.

Dietary exposure to residues is usually estimated as a function of the total drug derived residues in a model food basket, as it is assumed that all residues are equally and completely bioavailable once ingested by the consumer, and are of equal concern. This estimate of the theoretical maximum residues, though inherently safe, is likely to substantially overestimate biologically relevant exposure if the hazard is associated with a particular active residue fraction only (e.g. parent compound) or if substantial portions of the residues are non-bioavailable to the target organism/target cells due to irreversible binding to the food matrix.

While it is generally accepted to discount from the intake any components that are known to be biologically inactive, i.e. not contributing to the hazard of interest (e.g. not pharmacologically active), consideration of "non-bioavailablilty" of bound total residues in the evaluation of exposure scenarios and/or the calculation of the ADI has been an issue of controversial debate. For some types of substances, bound and thus potentially non-bioavailable residues may represent an important fraction of the daily intake and their consideration (or non consideration) can substantially influence the outcome of the risk assessment.

In practice, assessment of bound residues and their biological potential is often complicated by the fact that experimental evidence of covalent binding and bioavailability is not easily obtainable: The chemical nature or even the structural identity of bound residues and their biological significance is difficult (if not impossible) to characterise because of the complex chemical reactions leading to non-specific binding to macromolecules as well as their relatively low tissue levels. In most cases, regulatory bodies (e.g. JECFA) pursue a pragmatic approach considering (bioavailable) bound residues to be of no greater concern than parent compound, and assume that covalent binding represents a metabolically stable endpoint of biotransformation and further bioactivation into more potent forms can normally be excluded. On the other hand, there is also consensus that demonstration of covalent binding of residues to the food matrix cannot necessarily be regarded as an irreversible endpoint in relation to bioavailability. Digestive processes may alter the residues to yield cleavage or degradation products that may be bioavailable and thus contribute to biologically relevant exposure.

2. BACKGROUND

Although a provision to test for the presence of food bound residues does exist in Volume 8 of the Rules Governing Medicinal Products in the European Union, the document does not specify any criteria on how and when this information might be used in the assessment of MRLs, nor does it offer advice on the most suitable experimental approach to be followed.

Questions concerning consideration of bound residues have recently also been the subject of scientific advice to companies in cases of individual substances.

This paper is an attempt to discuss the subject on a more general level and to develop a set of criteria for a rational approach to the testing and assessment of bound residues. In this it may serve to complement and further specify the provisions of Volume 8 of the Rules Governing Medicinal Products in the European Union.

3. DEFINITIONS

It is proposed to adopt as working definitions the definitions of "bound residue", "extractable residue" and "bioavailable residues" provided by the Codex Alimentarius[1]:

Bound Residue: Residues derived from the covalent binding of the parent drug or a metabolite of the drug and a cellular biological soluble or insoluble macromolecule. These residues are not extractable from the macromolecule by exhaustive extraction, denaturation or solubilization techniques. They do not result from the incorporation of metabolized, radiolabelled fragments of the drug into endogenous compounds, or the same macromolecule by normal biosynthetic pathways.

Extractable Residue: Those residues extracted from tissues or biological fluids by means of aqueous acidic or basic media, organic solvents and/or hydrolysis with enzymes (e.g. sulfatase or glucuronidase) to hydrolyze conjugates. The extraction conditions must be such that the compounds of interest are not destroyed.

Bioavailable Residues: Those residues that can be shown, by means of an appropriate method (e.g. Gallo-Torres method), to be absorbed into systemic circulation when fed to laboratory animals

A practically equivalent definition of bound residue has been developed by the IUPAC:

"A xenobiotic bound residue is a residue which is associated with one or more classes of endogenous macromolecules. It cannot be dis-associated from the natural macromolecule using exhaustive extraction or digestion without significantly changing the nature of either the exocon or the associated endogenous macromolecule".[2]

Where residues have not been fully defined as bound they should be termed as unextractable and the extraction procedures used should be specified. In the discussion on residue binding it is important to clearly differentiate between the term "bound", which requires experimental proof or at least strong evidence of covalent binding and the term "unextractable" which could simply mean that that the methods used to extract residues were not sufficiently rigorous (to solubilize, for instance, substances that are simply absorbed).

4. GENERAL PRINCIPLES AND ASSUMPTIONS

The overall biological significance of a bound residue will depend primarily on its bioavailability and the resulting level of systemic exposure. In the assessment of bound residues the following assumptions will usually be made:

- In the absence of information to the contrary, bioavailable bound residues will be assumed to be of equal consumer concern as parent drug[3]. Consideration of a reduced contribution of the (bioavailable) bound residue to the overall toxicity of a compound would need to be supported by adequate data.

- Full chemical and structural identification (including mechanistic information) of the bound residue is normally extremely difficult to obtain and will only be requested if there is evidence

[1] *From „Glossary of terms and definitions" (Residues of Veterinary Drugs in Foods) (CAC/MISC 5-1993, Amended 2003),*
http://www.codexalimentarius.net/download/standards/348/CXA_005_2003e.pdf

[2] *Pure & Appl. Chem. Vol. 70, No. 7, pp. 1423-1447, 1998.*
http://www.iupac.org/publications/pac/1998/pdf/7007x1423.pdf

[3] *or the substance on which the ADI was based*

of formation of bound molecular species/adducts of particular concern (e.g. potentially mutagenic/carcinogenic).

- If the bound residue constitutes a (the) major fraction of the total residues and is bioavailable (partly or entirely) further chemical and toxicological characterization in comparison to parent compound or other free residues of concern could be necessary on a case by case basis.

- If bioavailable bound residues make up only a minor portion of the total residue, and in the absence of a particular concern, there may be no need to examine them further.

- If a bound residue has been demonstrated to be non-bioavailable it is possible to discount the non-bioavailable portion from the residues of concern.[4]

- Residues which have unambiguously been shown to result from incorporation of residues into naturally occurring compounds are considered innocuous and therefore of no further concern (this portion of the residues is referred to as the endogenously incorporated residue fraction).

- Apart from the hazard aspects, structural identification of the bound residues may sometimes also be required, if this information is needed to define a marker residue for residue control.

5. CALCULATIONS

Information regarding free and bound residues is considered in the assessment of potential consumer exposure and in conjunction with the estimation of MRLs. An estimate of the daily intake of residues of a drug that has a bound residue component needs to take into account the bioavailability factor of the residues and (possibly) their toxicological potency[5].

Residue of concern = $\Sigma\ TR_{ti} - (NB_{ti} + ER_{ti})$ or Σ UB + (BR x fraction bioavailable)

Bound residue = total residue - (free residues + extractable residues + endogenously incorporated residue fraction)
Bioavailable bound residue = (bound residue x bioavailablilty factor)

TR = total residue of concern
UB = unbound residue
BR = bound residue
NB = non-bioavailable bound
ER = endogenously incorporated residue fraction
Ti = edible tissue (tissues of the standard food basket)

6. EXPERIMENTAL APPROACHES

The objectives of the experiments are to quantify the bound residues remaining after exhaustive extraction, to collect information on the nature of the associated complex and to develop correlations with bioavailability of bound residues and, where necessary, their possible biological significance. The testing strategy and study design are usually developed on a case by case basis.

A combination of chemical analytical data with biological *in vitro* and/or *in vivo* methods has been shown to be the most suitable way to provide the necessary information. A variety of different

---

[4] This does not necessarily apply if the critical concern is related to non-systemic effects, i.e. local effects at sites of contact in the gastrointestinal tract. In such cases additional specific risk assessment may be required. This paper does not include guidance on testing/ assessment of bioavailability of bound residues for bacteria of the human gut flora.

[5] relative toxicological potency of bioavailable bound residues is assumed to be 1, compared to parent compound/residue of concern, when no other information is available.

techniques and methods might need to be used for quantitative determination, characterization and bioavailability testing of bound residues. This investigation can normally only be achieved by use of radiolabelled substances. The required experimental effort may only be worthwhile if significant amounts of bound residue occur (and/or if the specific radioactivity of residues is high enough for the purpose) and especially if the bound residue is of significant quantitative relevance to the risk assessment.

The sections below provide an overview of common experimental approaches that have been described in literature or mentioned in relevant international guidance documents (but do not claim to be exhaustive).

6.1. Extraction and physico-chemical characterization

Extraction with solvents
Tissue samples from radiolabelled studies are extracted with a variation of solvents to dissolve residues that are either free, encapsulated or that have weak ionic interactions with natural cell components. A wide range of solvents and extraction conditions can be applied, e.g. aqueous/organic solvents or mixtures thereof, different polarity, pH (inorganic/organic acids), temperature, single/continuous/repeat extractions, microwave assisted, pressure, solvent viscosity. The cleavage of covalent bonds during this procedure is unlikely.

Hydrolysis/Enzyme hydrolysis
A complementary strategy to release bound residues may be via (strong) acid hydrolysis or specific enzymatic hydrolysis by cleaving enzymatically degradable exocon-endocon links (e.g. by sulfatases, ß-glucuronidase, ß-glucosidase, esterases) or by solubilisation of the entire matrix via breakdown of large macromolecules into their constituent parts for releasing protein bound residues (e.g., protease/peptidase treatment).

A combination of the extraction and hydrolysis methods as described reflects the definition of "bound residue"[2].

6.2. Methods to determine the mechanisms of binding

For the characterization of the mechanism of bound residue formation, usually samples from (radiolabelled) metabolism studies in laboratory animals and target species are analyzed. Mechanistic knowledge of the chemical reactions underlying bound residue formation is not routinely required, except in the case of a specific concern (e.g. formation of potentially genotoxic adducts). Additionally, a full structural identification/chemical and mechanistic characterization of the bound residue is often a difficult technical/analytical task. However, any additional mechanistic information on the nature of binding, in particular regarding its reversibility or irreversibility, can considerably add to the understanding of the biological potential of the bound residues[6].

As the liver is the major organ involved in metabolism, *in vitro* techniques using cultured hepatocytes, liver microsomes or liver slices offer useful tools to elucidate major pathways of formation and metabolism of protein-bound residues.

Suitable experimental methods have been described in literature or relevant international guidance documents.

6.3. Testing of bioavailability

Quantitative testing of bioavailability and determination of the bioavailable residue fraction is a prerequisite in the exposure assessment of bound residues. Bioavailability after oral ingestion will vary

[6] *A very common mechanism is for instance the formation of a covalent bond from the electrophilic reaction of a residue with the nucleophilic sites on proteins or nucleic acids.*

depending on the compound involved and on the nature of binding. Studies should cover the relevant competing metabolic transformations and routes of secretion and excretion.

Bioavailability studies typically involve feeding animals (usually rat as default species) radiolabelled residues. Experimental parameters must be carefully chosen, including animal selection, dosage, quantitative collection of urine and faeces, blood, bile, saliva, respiratory products. In this context the Gallo-Torres method[7] is often recommended and has given valuable results. A possible limitation of *in vivo* bioavailability studies is that bound residues are often low and that, even when the tissues containing the bound residue are used as the only dietary source, animals are not exposed to sufficiently high concentrations/radioactivity to allow monitoring in the biological fluids/tissues with sufficient sensitivity.

In vitro/ex vivo methods using gastro-intestinal tract models to study mechanisms of absorption and first-pass metabolism can also provide valuable information. Various experimental approaches using gastrointestinal tract models including isolated epithelial cells, brush border membrane vesicles of enterocytes, isolated gut segments, and perfused gut segment models have been described.

7. SUMMARY

Chemical residues may in some cases be associated with natural macromolecules of the food matrix making them unreleasable and thus potentially unavailable for systemic absorption. Residues that are demonstrated to be bound and "non-bioavailable" may normally be assumed to be of no consumer concern and can be discounted from the dietary intake dose.

Experimental strategies accepted by international regulatory bodies are typically based on combinations of mutually complementing experiments: physical-chemical extractions, including extraction following enzymatic hydrolysis (exhaustive extraction), *in vitro* techniques to assess the mechanism leading to residue binding, and *in vivo* bioavailability studies in laboratory animals or suitable *in vitro* methods to determine the bioavailable residue fraction. Consideration of bound residues in the exposure assessment requires evidence of covalent binding and subsequent quantification of bioavailability. Mechanistic data could assist in the interpretation of the findings, but provision of such data would not be a routine requirement.

Use of physicochemical extraction methods similar to those used to characterize food-matrix binding of residues may also prove valuable for determining the fraction of a dose of antibiotic residues that are unavailable to gut bacteria due to metabolic inactivation and/or binding to contents of the colon. This information may be useful in the calculation of microbiological ADI values and the fraction of the residue dose bioavailable to gut bacteria.

---

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