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4 **Reflection paper on poorly extractable and/or non-**
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1. Introduction

Some of the active substances used in veterinary medicines have the propensity to strongly adsorb to environmental matrices. This is most often observed for the soil matrix, as it is the primary receiving compartment for most environmental emissions of veterinary medicinal products (VMPs), and hence the matrix which is most investigated. Examples of groups of compounds which strongly adsorb to soil include abamectins, aminoglycosides, fluoroquinolones and tetracyclines. As a result, these compounds are very poorly extractable from soil. In addition, experience within registration procedures has shown that for the group of molecules used in veterinary drugs, ^{14}C labelled analytes are often not available.

The aims of this paper are to:

1. Identify the issues which can arise when performing the OECD 307 study with poorly extractable and/or non-radiolabelled substances.
2. Propose a practical strategy on how to deal with substances which are difficult to extract from soil.
3. Propose solutions for the problems which can arise when performing the OECD 307 study.

Note:

(1) Although the text of this document focuses on the active substance, it is noted that it is also valid for relevant metabolites.

(2) Although focussing on extractability, there are other relevant outcomes of an OECD 307 study, such as quantification of mineralisation, determination of bound residues, identification of metabolites, elucidation of the metabolic pathway, etc. that are not discussed here.

2. Discussion

Following the requirements outlined in the guideline on environmental impact assessment for veterinary medicinal products - Phase II (EMA, 2005), applicants have to submit a study according to OECD 307: Aerobic and anaerobic¹ transformation in soil (OECD, 2002). Such experiments are performed to determine (i) the rate of transformation of the test substance, and (ii) the nature and rates of formation and decline of transformation products to which plants and soil organisms may be exposed.

The OECD 307 test guideline states that either labelled or non-labelled test substance can be used (see sections 5 and 6 of the test guideline). However, a radiolabelled analyte is strongly recommended as it makes several requirements easier to accomplish, such as a complete mass balance at all sampling moments, quantification of CO_2 and quantification of bound residues.

Applicants regularly submit studies on aerobic degradation in soil performed with a non-labelled analyte. This approach is not considered to be problematic if 70-110% of the added substance can be accounted for at each sampling point (quality criteria on recovery of chemicals as per OECD 307 specifications). However, this is often not the case as some VMP active substances bind strongly to soil resulting in extraction efficiencies below levels that enable a mass balance within the acceptable 70-110% range. Where a low extraction efficiency is seen at the start of an experiment ($t=0$), it is likely that this low extraction will continue for the duration of the experiment or more likely, become even

¹ In regulatory practice of VMPs, only the aerobic part is requested.

less efficient. In such instances, it becomes difficult interpreting the findings from the study. In particular, as part of an OECD 307 study, a rate of degradation of the active substance, and if applicable, for transformation products representing 10% or more of the parent compound and which do not form part of biochemical pathways, is determined over the course of the experiment.

However, a reliable estimate of the degradation rate of a substance is only possible when the total amount of non-degraded test substance is quantified at all sampling points. Therefore, the fraction of substance that is adsorbed but not degraded should be quantified.

Definitions (ECETOC 2013a)

Extractable residue (ER): A residue that is extractable using 'mild' extraction methods. This may include aqueous and cold solvent extraction using methods without excessive added energy. These residues are either freely available, or only weakly adsorbed to the matrix, are considered to be bioavailable and must be considered in any impact / risk assessment.

Non-extractable residue (NER): A residue that is not extractable using 'mild' extraction methods, but extractable under harsher conditions. These conditions may include solvent extraction using methods such as refluxing, microwaves or accelerated solvent extraction (ASE). These residues are strongly associated with the matrix, however they may be potentially reversible, but the partitioning is very much in favour of 'binding' to components of the matrix.

Bound residue (BR): A residue that is tightly associated with the solid matrix, often forming covalent (or similar) bonds. These residues usually cannot be released from the matrix or can only be released under extreme conditions where the integrity of the substance and/or matrix is likely to be affected. Such residues are often indistinguishable from the natural organic material e.g. humus in soil. These residues are not available for either degradation or available for indigenous organisms.

Often the definitions of BR and NER are used interchangeably, and confusion arises when the definition of BR is meant while the term NER is used. Care should be taken to avoid such confusion.

It is important to bear in mind that extractable residue (ER), and non-extractable residue (NER) fractions are not to be considered the result of degradation processes. To accurately determine the bound residue (BR) fraction, the best available extraction techniques should be used. This means that determination of the extractable fraction may have to be pursued by various extraction methods with increasing strength, e.g. according to the methodology as proposed by ECETOC (2013b). The evaluation of the feasibility of various extraction techniques should be reported in the final study report. The remaining substance concentration in the final residue can only be directly quantified when radiolabelled substances are used, otherwise the mass balance is incomplete. Alternatively, it would have to be assumed that the total mass fraction not accounted for in studies using non radiolabelled analyte is BR.

3. Issues to consider when conducting the study

Q. Why should I use a radiolabelled compound?

A. An OECD 307 test evaluates aerobic and anaerobic transformation of chemicals in soil. For this evaluation the fate of the chemical should be monitored. Without the use of a radiolabelled compound, it is unknown if the transformed compound is reduced to CO₂, bound to soil particles as NER or BR or lost during clean-up of the samples. When the compound is bound to the soil as NER it is potentially still available as the parent compound, indicating a potentially high persistency. If it is assumed that the BR fraction has been transformed, the persistency of the compound will be (highly) underestimated. Furthermore, when the compound is not radiolabelled, no information on the formation of transformation products (i.e. amount and identification) is available.

Q. Is there a preference for a type of radiolabel?

A. It is strongly recommended to conduct the study with a ¹⁴C labelled analyte. Arguments can be found in the OECD 307 guideline, and are i.e., the requirement of a mass balance (at all sampling times) serving full interpretation of the fate process, quantification of CO₂, and quantification of bound residues.

A ³H labelling is not a suitable method, because exchange of H atoms between analyte and water can occur, depending on the location of the label in the molecule, and mineralisation cannot be quantified using CO₂ formation.

Q. What should be done to demonstrate that the extraction method has been optimised for an active substance that is difficult to extract from the matrix?

A. The following recommendations apply:

- Use an extraction method that shows an extraction efficiency at t=0 of $\geq 70\%$. Recent scientific literature shows extraction methods with relatively high efficiencies for several groups of active ingredients (of VMPs). Example of efficient extraction methods can be found in e.g. Awad et al. (2014) and Bourdat-Deschamps et al. (2014). A more thorough exploration of techniques, other than the regularly encountered short term (5-15 minutes) soil:solvent shaking, may be necessary. It is regularly encountered in OECD 307 (and OECD308) studies that first, short-term extraction steps are followed by prolonged and more extensive techniques (pressurized liquid extraction (PSE), accelerated solvent extraction (ASE), reflux, Soxhlet, use of elevated temperatures, specific salt solutions). The introduction of a clean-up and/or concentration step may also be necessary. Different combinations of solvents or solutions may have to be tested. All tested methods and their results should be reported.
- The method of analysis has to be optimised. As a rule of thumb, the LOQ should be the lowest of either 10 µg/kg or $\leq 1\%$ of the starting concentration. Recent literature shows that the most used analytical method for several groups of veterinary drugs is LC-MS/MS, where the second MS step consists of quadrupole, TOF (time-of-flight) or Orbitrap.
- When the above conditions are met and the required minimum extraction efficiency of 70% is not reached, there is no other option than to conduct the study with the method that produces the best results. In such a situation, the applicant is asked to report the outcomes of all methods, also the unsuccessful ones, both extraction/clean up steps, the recoveries and analytical method(s).

- When ¹⁴C labelled material is used, the optimisation of extraction/analysis should be given as much care as with unlabelled material and the extraction method should be developed such that the extraction efficiency is optimal.

Q. Can a mass balance also be demonstrated without using radiolabel?

A. This is very difficult. For example, if CO₂ is formed this has to be quantified using other methods (e.g. gravimetry), which may be impracticable at low test concentrations. In addition, it has to be demonstrated that the extraction method is able to extract the test substance with high recovery (extraction efficiency). However, when recovery decreases after prolonged incubation and a portion of the analyte is not extracted, it is missing from the mass balance and it can only be assumed to be 'bound' but not extracted.

Q. When it is not possible to obtain a radiolabelled form of the active ingredient, can performance of the OECD 307 study be waived?

A. The option to waive the OECD 307 when it has been demonstrated that the requirement of mass balance cannot be met is not favoured. This also holds when it has been demonstrated that considerable effort has been put into optimisation. First, this would, potentially, create unequal treatment of applicants, which is undesirable. Second, when it has been demonstrated that – apparently- the sorption of the analyte to the matrix (i.e. soil) is strong and/or irreversible, the non-extractable portion could be considered 'bound residue'. The degradation could then be followed in the fraction that is extractable. For this reason it is important that the LOQ of the analytical method is sufficiently low, as explained above.

Q. The emission of veterinary drugs onto soil occurs mainly via manure. Should OECD 307 not be performed with spiked manure or with a soil amended with manure?

A. Although such a scenario resembles field conditions, the results that such a 'mixed' study would provide cannot be used for regulatory purposes. The starting point in such a study cannot be standardised or defined, as in manure often high amounts of NER or BR are built within a short timeframe. Consequently, no rate constant for transformation in soil can be determined from the data obtained from such a 'mixed' study, as fitting of the observed complex behaviour with the available standardised kinetic models (e.g., SFO) is not feasible. The use of manure introduces an extra variable in the degradation studies that would make a standardised performance unrealistic.

4. Conclusion

With this reflection paper the CVMP presents a pragmatic approach on how to deal with substances which are difficult to extract from soil. The CVMP proposes a number of solutions for problems which can arise when performing the OECD 307 study.

When all proposed solutions have been tried and it is not possible to reach the required minimum extraction efficiency of 70%, there is no other option than to use the results from the study with the method that produces the most reliable highest extraction efficiency. In such a situation, the applicant is asked to report the outcomes of all methods, also the unsuccessful ones, both extraction/clean up steps, the recoveries and analytical method(s).

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