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Reflection paper on poorly extractable and/or non-radiolabelled substances

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Table of contents

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 often very poorly extractable from soil. In addition, experience from registration procedures has shown that for the group of molecules used in veterinary drugs, radiolabelled analytes might be difficult to obtain as they might not be commercially available.

The aims of this paper are to:

1. Identify the issues which can arise when performing the OECD 307: Aerobic and anaerobic transformation in soil 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 that are not discussed here, such as quantification of mineralisation, determination of bound residues, identification of transformation products, elucidation of the transformation pathway, etc.

2. Discussion

Following the requirements outlined in the guideline on environmental impact assessment for veterinary medicinal products - Phase II (EMEA, 2005), applicants have to submit a study according to OECD 307: Aerobic and anaerobic[1] 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 a 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, or is even necessary to enable test requirements such as a complete mass balance at all sampling points, quantification of CO_2 and quantification of non-extractable residues. Even if radiolabelled compounds are not commercially available, often they can be synthesized in sufficient quantities for reasonable prices. Costs should thus not be an argument to not perform an experiment with radiolabeled compounds.

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 the start of the experiment (in line with the quality criteria on recovery of chemicals as per OECD 307 specifications). However, this is often not the case as some VMP active substances

[1] For regulatory purposes of VMPs, only the aerobic part is requested.

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 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, of all 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 has to be quantified.

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 degradation rate, the NER fraction should be as close to the bound-residue (BR) fraction as possible, and thus 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.

Table 1. Working definitions (based on 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. ER 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 the extraction method applied, but is extractable using harsher extraction methods such as refluxing, microwaves or PLE pressurized liquid extraction. NER are strongly associated with the matrix, but the binding with the matrix is potentially reversible. However, the partitioning is very much in favour of 'binding' to components of the matrix. Thus, the amount of NER depends not only of its physico-chemical properties, but also on the analytical procedure used.
Bound residue (BR): A residue that is tightly associated with the solid matrix, often forming covalent (or similar) bonds. Usually, BR 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 to indigenous organisms. Thus, the amount of BR depends on the physico-chemical properties and the chemical state of the compound. **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. In this working definition, NER reflects the analytical procedure and BR the chemical state of the compound. Currently, no method can distinguish between the two. This shows the importance of using the right extraction methods, so the amount of NER is as close to the actual amount of BR as possible**.

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 compound is transformed to CO_2 or volatile transformation products, bound to soil particles as NER or lost during clean-up of the samples. When the compound is sorbed 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 and irreversibly bound, this can then be assumed to be part of the degradation process. Furthermore, when the compound is not radiolabelled, often 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 ^{14}C 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 CO_2, and quantification of bound residues.

A ^{3}H 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 CO_2 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 OECD 308) studies that first, short-term extraction steps are followed by prolonged and more extensive techniques (pressurized liquid extraction (PLE), 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 limit of quantification (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 ^{14}C 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 CO_2 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 non-extractable residues or non-identified transformation products. To extract the transformation products, other extraction methods may be required than for the parent compound.

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 – using the best available extraction method apparently – the sorption of the analyte to the matrix (i.e. soil) is strong and irreversible, the non-extractable portion could be considered 'bound residue', although a clear distinction between NER and BR is technically not possible, yet. 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. This would add uncertainty and variability in a standardized test system. Moreover, in manure often high amounts of NER are built within a short timeframe, which may become available again when the manure is degraded. 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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