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Guideline on determining the fate of veterinary medicinal products in manure

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* The current revision consists of changes made in order to incorporate reference to new guidance and to correct and update the wording of the document, where appropriate. As no changes to the scientific content were made, no concept paper and no public consultation were deemed necessary.



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1. Introduction

1.1. Background

The 'Guideline on environmental impact assessment for veterinary medicinal products in support of the VICH guidelines GL6 and GL38' (EMA/CVMP/ERA/418282/2005; hereafter referred to as the CVMP 'Technical Guidance Document' [CVMP TGD]) gives further technical support pertaining to the application of VICH guideline (GL) 6 (EMA/VICH, 2000) and GL38 (EMA/VICH, 2005) on the environmental risk assessment (ERA) of veterinary medicinal products (VMPs). Besides algorithms, models, and default values for the determination of the predicted environmental concentration (PEC) of VMPs in the environment, the guideline provides a number of options to refine the exposure assessment. One of these options is the determination of the degradability of a VMP in manure of stabled target animal(s). This type of study is not required by default, but it could be used to stop the assessment in Phase I if it can be demonstrated that the active substance is mineralised or transformed into products present at less than 5% of the total applied. If the data do not allow the assessment to stop in Phase I, they could still be useful to refine the exposure assessment in Phase II.

Studies submitted should follow the present guideline and the 'Organisation for Economic Co-operation and Development' test guideline 320 (OECD TG 320 'Determining Anaerobic Transformation of Chemicals in Liquid Manure'), which provides more detailed guidance on the performance and evaluation of the test. The CVMP TGD offers only limited advice on this aspect of the risk assessment, with a focus on the use of degradation studies as part of the Phase I assessment. Since the release of the CVMP TGD it has become clear that there is a need for guidance on the design, execution, and interpretation of studies on the transformation of VMPs in manure, which can be used in the preparation of the ERA by applicants and in the evaluation of the studies by the competent authorities.

This guidance document was originally prepared with input from researchers, industry and competent authorities at a workshop organised by the European Medicines Agency (EMA) in 2009 and during a follow-up meeting in 2010.

1.2. General considerations

This guidance document attempts to standardise the methodology for degradation tests in manure from housed animals.

Animal manures contains excreta, i.e., urine and faeces, of the housed animals together with other materials of farming practice, e.g. spilled feed, straw, litter, sand, water, down and carcasses. Manure may be either liquid (slurries) or solid (farmyard manure). In Europe, and for the purpose of this guideline, (semi)-liquid manures (i.e. mixture of faeces, urine and water) are considered to be the most representative type of manure (see e.g. Weinfurter, 2011).

Manure is a highly variable mixture, and thus the parameters that influence the degradation rate of VMPs can differ to a large extent. The most striking differences are (i) the variation in dry matter content in manure due to the species of animal or the way the animals are housed; (ii) the way manure is being collected and stored; and (iii) the variation in composition due to the type of feed the animals have been fed. At present, the impact of all the different parameters on the degradation rate is not well known, which makes it difficult to select one set of conditions which mimic all conditions in which animals can be housed. In the future, it may be possible to define types of reference manure which meet certain conditions.

Considering that more research is needed to examine the various influences on test conditions, the present guideline should be considered a living document which might need revision when more

information on the variability of manure properties and the effect of this variability has on VMP degradation becomes available.

1.3. Outline

This guideline is divided into two main sections dealing with study design and the evaluation and use of the test results. These sections are subdivided into the following topics:

Study design (section 2)

- Sampling of excreta and preparation of test/reference manure (section 2.1)
- Matrix characterisation of the manure (section 2.2)
- Establishing test conditions (section 2.3)
- Test substance (section 2.4)
- Analytical methods (section 2.5)

Evaluation and use of test results (section 3)

- Treatment of results (section 3.1)
- Extrapolation within and between manure types of different target species (section 3.2)
- Use of test results to refine PECs (section 3.3)

2. Study design

2.1. Sampling of excreta and preparation of test/reference manure

Degradation studies should be performed in manure of each major species to which the VMP is administered. For the purpose of this guideline, relevant major species are those defined in Commission Regulation (EU) 2017/880, i.e. cattle, sheep for meat, pigs and chickens.

It is recommended that the degradation study is carried out in manure from animals that are reared under well-controlled conditions. Manure contaminated with other VMPs, biocides and other materials that can alter the degradation rate of the VMP under investigation should not be used. The type of animals and feed, the feeding regime and the medical history of the animals from which the manure is collected should be reported. Detailed information on the manure storage facility, the sampling procedure and storage of manure prior to the test should be given.

Freshly collected excreta from cattle and pigs should be conditioned at 20 °C until stable and strictly anaerobic conditions are reached (as measured by the redox potential) and to reduce the matrix heterogeneity by transformation of readily degradable organic substances. Subsequently, deionised water is added to prepare reference manure samples of defined dry substance contents (see section 2.3.3). Alternatively, samples taken from manure tanks under strictly anaerobic conditions can be used without conditioning, but pre-incubation at the test temperature is recommended in OECD test guideline (TG) 307 (OECD, 2002a).

As manure from chickens is stored under aerobic conditions, freshly collected manure does not need to be acclimatised. The manure samples have to be matrix-characterised (see section 2.2).

At present, it is unclear whether storage of manure at low temperature (e.g. -20 °C) before testing could influence the degradability of VMPs. For this reason, fresh manure should preferably be used.

When stored at -20 °C, it is recommended that the manure is reconditioned under ambient conditions for 3 days, and the matrix characterised again based on the parameters outlined in section 2.2.

2.2. Matrix characterisation of the manure

The degradation of a substance can be a function of different characteristics of the manure. For this reason, it is important that the following parameters are measured and reported to characterise the manure:

- pH;
- microbial activity¹;
- organic matter (% OM, total organic carbon (TOC) can additionally be determined);
- nitrogen content (total nitrogen and ammonium nitrogen);
- redox potential;
- dry matter content;
- temperature.

All the parameters should be measured at the start and termination of the study. In accordance with OECD TG 307 and OECD TG 308 (OECD, 2002b), it is recommended to monitor pH, redox potential and temperature throughout the test.

2.3. Establishing test conditions

In this section, guidance is given for a number of essential test conditions. Further guidance on the test conditions for determining the degradation of VMPs in manure from pigs and cattle is given by Kreuzig (2010) and Kreuzig *et al.* (2007; 2010a; 2010b).

2.3.1. Treatment and application of test substance

The minimum amount of manure to use in the test is 50 to 100 g (fresh weight).

Preference is given to spiked manure because it reduces the variability and makes the results easier to interpret. However, it may be possible to use manure from treated animals if a proper degradation rate and identification of the transformation products can be obtained.

The test substance should be dosed into manure at a concentration that reflects the maximum expected manure concentration. If this concentration is not high enough to identify major transformation products, incubation of separate manure samples containing higher rates may be helpful, but excessive concentrations which may influence microbial functions should be avoided.

2.3.2. Redox potential

Cattle and pig studies should be performed under anaerobic conditions. For this reason, it is recommended that the manure is incubated in a flow-through system rinsed with nitrogen (see OECD TG 307 and 308, respectively). Anaerobic conditions should be demonstrated by a negative redox potential. Weinfurter (2011) found typical redox potentials in pig and cattle manure to range from -

¹ Reduction of DMSO to DMS can be used as measurement of anaerobic microbial activity without interference as outlined by Griebler and Slezak (2001). The microbial activity can be determined by measuring the mineralisation of a readily degradable ¹⁴C-labelled compound (e.g. ¹⁴C-glucose) under anaerobic conditions.

230 mV to -400 mV. According to OECD TG 308, redox potentials (Eh) < -100 mV confirm anaerobic conditions.

For manure from chickens, the conditions should be aerobic.

2.3.3. Dry matter content

The recommended dry matter content in bovine and pig manure is 10% ± 1% and 5% ± 1%, respectively (Weinfurtner, 2011). Manure from chickens will normally have much higher dry matter content. Based on a study by Ellen (2000), a dry matter content of 60% ± 5% is recommended. If the test flasks are to be aerated, the air should be humidified to avoid loss of water from the poultry manure.

2.3.4. Sampling and measurements

It is recommended that, analogous with OECD TG 307, duplicate incubation flasks are removed at appropriate time intervals, which are chosen in such a way that pattern of decline of the test substance and patterns of formation and decline of transformation products can be established (e.g. 0, 1, 3, 7 days; 2, 3 weeks; 1, 2, 3 months). The length of the study will depend on the rate of degradation of the compound and information on the average storage time for the manure under test (see CVMP TGD, Table 6). A maximum length of 120 days could be considered in analogy with soils, provided that the test system remains sufficiently viable.

2.3.5. Temperature and light conditions

During the whole test period, the manure samples should be incubated in the dark at the relevant temperature. In the CVMP TGD, relevant temperatures for testing are considered to be 20 °C for pig manure, 10 °C for cattle manure and 25 °C for chicken manure. For the purpose of standardisation, it is recommended to test all manure types at a temperature of 20 °C. For the risk assessment, the derived half-life (DT₅₀) has to be corrected to the relevant environmental temperature (see section 3.3.5).

2.3.6. Sterile control

To obtain information on the relevance of abiotic transformation of a test substance and the nature of non-extractable residues, it is recommended to include a sterile control. Consideration should be given to the sterilisation method used in order not to change matrix properties too much. Furthermore, one should be aware that manure samples may not remain sterile when the study is conducted over a long period. For sterilisation methods, see OECD TG 307.

2.4. Test substance

The purity and/or radiochemical purity, the position of the radiolabel, the specific activity as well as the dilution ratio with non-labelled substance of the test substance should be reported.

Radiolabelled material is preferred because detailed mass balances can be determined taking into account mineralisation as well as extractable and non-extractable residues. Phenyl ring-U-¹⁴C labelling should be preferred. In all cases, the characteristics of the radiolabelled material should be reported.

When non-radiolabelled material is used, only the disappearance of the parent compound initially applied can be followed unless specific methods exist for known transformation products. The study will need to be designed in such a way that it provides equivalent data to that which would be obtained

in a radiolabelled study, i.e., it will need to identify any major metabolites and characterise the formation of non-extractable residues.

For addition to and distribution in manure, the test substance can be dissolved in water (deionised or distilled) or, when necessary, in minimum amounts of an organic solvent in which the test substance is sufficiently soluble and stable. However, the amount of solvent selected should not have a significant influence on microbial activity in the manure. The use of a positive control could be of help to determine the potential toxicity of the solvent.

2.5. Analytical methods

The study objectives include determination of the fate of the active substance in manure (i.e. degradation half-life) and the quantification of the mineralisation. Where possible, major transformation products should be identified and quantified. A major transformation product is any product representing $\geq 10\%$ of the applied dose at any time during the study.

In order to trap and analyse released $^{14}\text{CO}_2$ and ^{14}C -volatile substances under anaerobic and aerobic conditions, external traps filled with ethylene glycol, sulphuric acid and potassium hydroxide solution or solid adsorbent materials have to be linked to the incubation flask. For the determination of $^{14}\text{CH}_4$ released out of the ^{14}C -labelled test substance under anaerobic conditions, the headspace of the bioreactor flask is purged by means of nitrogen. The stripping gas stream passes different traps for $^{14}\text{CO}_2$ and volatiles. Finally, the $^{14}\text{CH}_4$ -containing gas is to be directed into a combustion apparatus where $^{14}\text{CH}_4$ is oxidised to $^{14}\text{CO}_2$, which is trapped again in an absorbing scintillation cocktail for scintillation counting (Nuck and Ferderle, 1996).

It is recommended that a sequential extraction method is followed. An exhaustive extraction is necessary with various polar and non-polar solvents (including aqueous solvent mixtures) and acid systems, the choice of which depends on the analyte (i.e. parent compound and its degradation products). First extraction steps can employ less vigorous methods (e.g. short shaking with organic solvents). However, more vigorous methods should be employed in order to destroy the manure matrix in case non-extractable residues are observed. More vigorous methods include, for example, pressurized liquid extraction, accelerated solvent extraction, reflux and Soxhlet with the appropriate solvents. The extraction of residues should not change the chemical nature of the active substance or the transformation products. The residue remaining after the last extraction step should be combusted and analysed for radiolabelled material. The possible fraction of radiolabel contained therein is termed final non-extractable residue (NER).

Validation of analytical methods (including extraction and clean-up methods) for identification and quantification of the test substance and, where applicable, its transformation products should be reported. Further guidance on the validation of analytical methods is given in the European Commission's 'Guidance Document on Pesticide Analytical Methods for Risk Assessment and Post-approval Control and Monitoring Purposes' (EC, SANTE/2020/12830, Rev.2; 2023).

3. Evaluation and use of the test results

3.1. Treatment of results

The amounts of test substance, transformation products, volatile substances (in % only) and NER should be given as % of applied initial amount and, where appropriate, as mg/kg manure (based on dry weight) for each sampling interval. A mass balance should be given in percentage of the applied initial amount for each sampling interval. Data should be reported separately for each replicate and as arithmetic mean of all replicates. A graphical presentation of the test substance concentrations against

time on a non-logarithmic scale should be included. Half-lives or DT₅₀ values and, if appropriate, DT₇₅ and DT₉₀ values should be obtained by applying appropriate kinetic model calculations (see FOCUS, 2006; 2014). The half-life and DT₅₀ values should be reported together with the description of the model used and a measure for the goodness-of-fit (see FOCUS, 2006; 2014; a free spreadsheet ['FOCUS_DEGKIN', version 2, June 2007] can be downloaded from https://esdac.jrc.ec.europa.eu/public_path/projects_data/focus/dk/software/FOCUS_DEGKIN%20v2.xls [accessed on 7 November 2023]). If appropriate, the calculations should also be applied to the major transformation products.

3.2. Extrapolation within and between manure types of different target species

In principle, extrapolation of degradation rates between manure types within the same species is possible, provided that the composition of the manure and storage conditions are comparable. At present, there is limited information available to provide more precise guidance for which species and types of manure extrapolation is feasible. For pragmatic reasons, manure from the same animal species, i.e. pigs, cattle and poultry, is considered to be comparable.

3.3. Use of test results to refine PECs

There are a number of potential outcomes from the study, namely:

- the parent compound is not degraded;
- the parent compound is completely mineralised or degraded over 30 days;
- the parent compound is partially mineralised;
- the parent compound is primarily degraded into transformation products;
- NER are formed in the manure;
- a combination of the above dissipation processes occurs.

The outcome of studies in which there is complete degradation or mineralisation can be used in Phase I. The approach for PEC refinement in Phase II is discussed in the following sections for each scenario.

3.3.1. Complete mineralisation/degradation

In instances where degradation results in complete mineralisation or complete degradation, demonstrated either by total mineralisation or by the presence of degradation products each representing 5% or less of the administered dose within 30 days, the exposure to the environment is considered negligible and the risk assessment can stop in Phase I.

3.3.2. Partial mineralisation

When partial mineralisation is observed in the study, the PEC_{soil} in Phase I can be revised using the percentage mineralisation observed after 30 days in Phase I or using an appropriate kinetic model (selected based on the degradation pattern) and average storage times recommended in the CVMP TGD in Phase II. It is recommended to use the mean of the replicate CO₂ percentages at day 30 in Phase I. Instead of mineralisation, the production of CH₄ could also be measured.

3.3.3. Degradation into transformation products

Preferably, the data obtained allow the determination of the half-life of the parent compound and the transformation products formed $\geq 10\%$ of the applied dose, based on an appropriate kinetic model. The DT_{50} values can then be used to calculate the PEC_{soil} for the parent compound and the individual degradation products, according to equation 9–11 of the CVMP TGD. If toxicity data for the parent compounds and degradation products are available, the PECs can be used to assess the risk of the individual compounds. In the absence of toxicity data for the degradation products, the total residue approach should be followed by adding the PECs and comparing them with the Predicted No Effect Concentration (PNEC) for the parent compound.

When the data do not provide individual half-lives, the concentration of the parent compound and all transformation products (including NER) $\geq 10\%$ of the applied dose which do not form part of biochemical pathways could be added at the time point equal to the half-maximal average storage time recommended in the CVMP TGD. The PEC_{soil} can be calculated by comparing the total concentration with the initial concentration of the parent compound to calculate the fraction remaining, using the following equations:

$$PEC_{soil \text{ refined}} = PEC_{soil \text{ initial}} \times \% \text{ remaining}$$

$$\% \text{ remaining} = (\% \text{ parent}) + (\% \text{ transformation product } \geq 10\%) + (\% \text{ NER})$$

If the parent compound is completely degraded into a single transformation product, the risk assessment should be focussed on this compound.

3.3.4. Non-extractable residues

The mechanisms for binding within the manure matrix can be numerous and complex, though, to date, they are not well understood. For a better understanding of the binding capacity of organic matter in various types of manure, further research is needed. It might also be necessary to highlight that, in contrast to soil and sediment, manure will be degraded to a large extent after application to soil. In that respect, it is important to rule out the possibility that the NER represents parent compound and transformation products that become available when the manure matrix degrades. In this context, the general rule that the extraction method must not substantially change the structure of the sample matrix under study applied in OECD TG 307 and TG 308 is not appropriate for manure.

At present, it is difficult to give guidance on which vigorous extraction method is sufficient to ensure that the NER will not cause any toxicity when manure degrades after having been spread on land. However, when it can be proven that NER consists of fragments incorporated into organic matter (e.g. in bacteria), then these residues should not be considered in the risk assessment. Additional information could be obtained with a sterile control in case NER are not formed in these samples. In any other case, NER should be considered to represent the parent compound, unless it can be shown that, through appropriate experiments, the toxicity of the NER is significantly lower than that of the parent compound.

3.3.5. Temperature correction

The CVMP TGD gives further guidance on how the test results can be used to refine the PEC based on the realistic storage times and environmental temperatures. As cattle and pig manure can be stored outdoors, 10 °C is considered to be a realistic environmental temperature for both manure types for Northern European countries, whereas the recommended 20 °C in CVMP TGD is more realistic for Southern European countries (Weinfurtner, 2011). By default, the PEC refinement should be based on

an outdoor temperature of 10 °C. For chicken manure, 25 °C is considered to be a realistic environmental temperature.

DT₅₀ values can be normalised to the environmental temperature using the Arrhenius equation. The European Food Safety Authority (EFSA) Scientific Panel on Plant Protection Products and their Residues (PPR Panel) recently recommended that the median E_a value of 65.4 kJ/mol corresponding to a Q₁₀ of 2.58 should be used instead of the previous recommended default E_a value of 68.9 kJ/mol mentioned in the CVMP TGD (EFSA, 2008). Based on a Q₁₀ of 2.58, the following formula can be used to converting DT₅₀ values determined at 20 °C to an environmental temperature T.

$$DT_{50, T} = DT_{50, 20\text{ °C}} \times e^{0.095 \times (20-T)}$$

in which:

DT_{50, 20 °C} = half-life at 20 °C

DT_{50, T} = half-life at the relevant temperature T [°C]

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