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Factsheets for the (eco)toxicological risk assessment strategy of the National Institute of Public Health and the Environment (RIVM)

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Abstract

Eight fact sheets describing risk assessment methods used at the Centre of Substances and Risk assessment (CSR) of the National Institute for Public Health and the Environment (RIVM) are presented here with the main aim of promoting greater transparency in the risk assessment methods used at the Institute in general and within the Centre in particular. The fact sheets, listed below, reflect a state-of-the-art approach; they are also meant to function as a platform for discussion.

1. Methemoglobine/Heinz bodies
2. Acetylcholinesterase inhibitors
3. Pheochromocytomas
4. Assessment factors for human health risk assessment
5. Delayed Neurotoxicity/NTE inhibition
6. Residues of plant protection products on food ingested by birds and mammals
7. Degradation of veterinary drugs in manure
8. Guideline for evaluating studies to determine excretion of veterinary drugs.

The first five fact sheets are related to human risk assessment and the last three to environmental issues. Remarks, omissions or additional information sent to the editors (first name) will be appreciated.

Preface

This report was written within the framework of the project 'Risk Assessment of Substances: Science and Market'. The results as presented in this report have been discussed by members of the human and environmental peer review groups of the Centre of Substances and Risk assessment (CSR), and in some cases experts were consulted, all are acknowledged for their contribution. These members and experts are: A.J. Baars, P. van Beelen, R.B. Beems, B.J. Blaauboer, A.B.T.J. Boink, J. Janus, A.G.A.C. Knaap, A.M.A. van der Linden J.B.H.J. Linders, R. Luttik,W.C. Mennes, M.H.M.M. Montforts, M.T.M. van Raaij, A. Sips, W. Slob, G.J.A. Speijers, A. Verschoor, T.G. Vermeire, and P.W. Wester.

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Samenvatting

In dit rapport worden 8 factsheets gepresenteerd, die voor de risicoschatting van stoffen binnen het Centrum voor Stoffen en Risicobeoordeling (CSR) gehanteerd worden. De eerste 5 factsheets hebben betrekking op de humane risicoschatting en de overige 3 factsheets op het milieu.
In de factsheet '**Methemoglobine/Heinz bodies'** wordt weergeven wat methemoglobinevorming en Heinz bodies zijn, welke factoren deze effecten beïnvloeden, welke effecten als 'adverse' beschouwd dienen te worden en wat de beoordelingsstrategie voor deze effecten is.
In de factsheet '**Acetylcholinesterase inhibitors**' wordt vastgelegd welke effecten met betrekking tot AChE inhibitie als toxicologisch relevant worden beschouwd en bij welke verandering in AChE activiteit er sprake is van een 'adverse' effect.
In de factsheet '**Pheochromocytomas**' wordt vastgelegd wat de toxicologische relevantie is van een stof-geïnduceerde toename in pheochromocytomas in bijniermerg voor beoordeling en risicoschatting voor de mens.
De factsheet '**Assessment factors for human health risk assessment**' is gericht op de toepassing van probabilistische verdelingen van default assessment factoren in de risicobeoordeling van stoffen voor de mens (inter- en intraspeciesvariatie en de correctiefactor voor de duur van de studies). De voorgestelde verdelingen zullen toegepast gaan worden in de risicobeoordeling van nieuwe en bestaande stoffen en bestrijdingsmiddelen voor de interpretatie van respectievelijk de 'Margin of Safety' (MOS) en de afleiding van de ADI.
De factsheet '**Delayed Neurotoxicity/NTE-inhibition**' geeft een overzicht van wat op dit moment bekend is over OPIDPN, wat de significantie is van NTE-remming en welke rol NTE speelt bij het inschatten van mogelijk uitgestelde neurotoxische effecten van organofosfaatverbindingen.
De factsheet '**Residues of plant protection products on food items for birds and mammals**' geeft een samenvatting van recent gepubliceerd residu onderzoek, en geeft een voorstel hoe deze nieuwe gegevens in de ecotoxicologische risicobeoordeling van bestrijdingsmiddelen te gebruiken.
In de factsheet '**Degradation of veterinary drugs in manure**' wordt beschreven hoe de afbraakstudie van diergeneesmiddelen in mest beoordeeld moet worden binnen de toelatingsprocedure van diergeneesmiddelen.
In de factsheet '**Guideline for the evaluation of studies determining the excretion of veterinary drugs**' wordt beschreven hoe een excretiestudie van diergeneesmiddelen beoordeeld moet worden binnen de toelatingsprocedure van diergeneesmiddelen.

Summary

This report presents 8 factsheets for the risk assessment methods used in the Centre for Substances and Risk assessment (CSR). The first 5 of these factsheets are dealing with issues related to human risk assessment and the other 3 with environmental issues.
The factsheet **Methemoglobine/Heinz bodies** describes the mechanism of methaemoglobin and Heinz body formation, the factors influencing these effects, those effects that must be labelled 'adverse', and the assessment strategy for these effects.
The factsheet **Acetylcholinesterase inhibitors** describes which effects associated with AChE inhibition are considered as toxicologically relevant and at what change in AChE activity an effect is labelled 'adverse'.
The factsheet **Pheochromocytomas** aims to establish the toxicological significance of an agent-induced increase in adrenal medullar pheochromocytomas in experimental animals with respect to human risk assessment.
The factsheet **Assessment factors for human health risk assessment** is focussed on the Application of probabilistic distributions of default assessment factors in human health risk assessment (inter- and intraspecific variation and duration of the test). The proposed distributions will be applied in risk assessment of new and existing substances and persticides for the interpretation of the Margin of Safety (MOS) and the derivation of the ADI, respectively.
The factsheet **Delayed Neurotoxicity/NTE-inhibition** gives an overview of the current knowledge on OPIDPN, the role of NTE and the significance of NTE-inhibition data for the assessment of the delayed neurotoxic potential of organophosphorus compounds.
The factsheet **Residues of plant protection products on food items for birds and mammals** a summary of recent published research on residue levels is presented and a proposal is made how to use these new data in the ecotoxicological hazard/risk assessment for birds and mammals.
The factsheet **Degradation of veterinary drugs in manure** is a guideline for assessment of the reliability of the studies currently submitted for legislative purposes of veterinary drugs.
The factsheet **Guideline for the evaluation of studies determining the excretion of veterinary drugs** is meant as a preliminary guideline in which the requirements and criteria for an excretion study are described and that can be used to decide whether or not the results are useful for the environmental risk assessment of veterinary drugs.

Introduction

One of the main tasks of the Centre for Substances and Risk assessment (CSR) of the National Institute of Public Health and the Environment (RIVM) is to assess the risk of compounds on public health and the environment. To carry out risk assessments it is of the highest importance that adequate and up-to-date risk assessment methods are available. Some of these methods are taken over (adopted) from other organisations, but many are, for a large part, developed within the RIVM. These risk assessment methods are not rigid procedures but can be adapted based on new/developing scientific information, possible triggered by questions from policy makers or by developments in national or international organisations. For specific problems or gaps in the assessment of (eco)toxicological effects, 'factsheets' are written by employees of CSR in co-operation with experts. In these factsheets the assessment strategy of RIVM/CSR is described. After adoption of the factsheet by the advisory board and the head of the laboratory of CSR all employees of CSR have to follow the risk assessment method described in the factsheet.

In 1999 and 2000 eight factsheets were published (5 factsheets related to public health issues and 3 factheets related to environmental issues):
Factsheets concerning public health
Methemoglobine/Heinz bodies,
Acetylcholinesterase inhibitors,
Pheochromocytomas,
Safety factors,
Delayed Neurotoxicity/NTE-inhibition,
Factsheets concerning the environment
Residues of plant protection products on food items for birds and mammals,
Degradation of veterinary drugs in manure,
Guidance document for summarising/interpreting of studies describing excretion of veterinary drugs by mammals.

We hope that by publishing these factsheets, the risk asessment methods followed by RIVM/CSR will become more transparent. The authors of each factsheet have tried to describe the state of the art of their subject. Remarks, omissions or supplementary information will be appreciated and can be send to Robert.Luttik@RIVM.NL and will be passed on to the responsible authors.

1 Methemoglobine/Heinz bodies

Factsheet FSV-001/00 date 25-01-2000

Author:

W.M. Blom

1.1 Introduction and aim

The standard toxicological evaluation of a chemical substance includes the measurement a number of standard haematological and biochemical parameters in animal experiments. Certain substances, however, such as nitrite, aromatic amines, and compounds with a strong oxidating capacity, may induce additional (non-standard) haematological effects, such as methaemoglobin and Heinz bodies (*see appendix A for list with known substances*). Certain characteristics, e.g. structural analogy, blue-coloring of extrimities (skin, nails), and associated signs at organ-level in animal studies, may be indicative of methaemoglobin formation and Heinz bodies. This factsheet describes the mechanism of methaemoglobin and Heinz body formation, the factors influencing these effects, the level of effect that must be labelled 'adverse', and the assessment strategy for these effects.

1.2 Mechanism for the development of the effect, and background

Methaemoglobin
Haemoglobin (Hb) is an iron-containing, tetrameric protein, consisting of 4 protein chains forming the globin, and 4 identical haem groups. Each haem group is able to reversibly bind an O_2-molecule to its ferrous group. However, a small proportion of the oxygen becomes superoxide O_2^-, leaving one electron, so the iron is oxidized from the ferrous to ferric state, the so-called methaemoglobin (MetHb, FeMethaemoglobin, $HbFe^{3+;}$ ferrihaemoglobin; see Fig. 1).

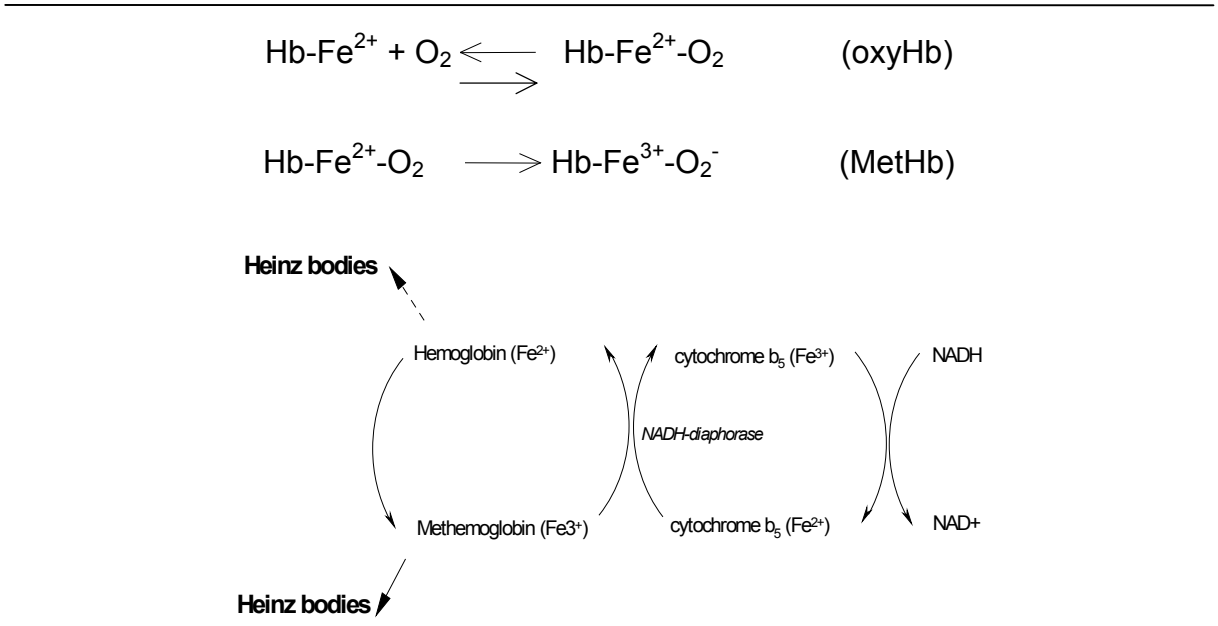

Figure 1 Schematic representation of MetHb and Heinz bodies formation. Explanation in main body of the text.

Haemoglobin consequently loses its property of combining reversibly with oxygen, and furthermore the other O_2-molecules are more strongly bound. This auto-oxidation occurs spontaneously, and normally the formed Fe^{3+} is reduced to Fe^{2+} by the NADH-dependent

cytochrome b_5 reductase (also called NADH-diaphorase). This enzyme accounts for 60-95% of the MetHb reduction.
Two other, quantitatively less important, reducing systems are cellular antioxidants, such as vitamin C, E and GSH (12-16%), and the NADPH-diaphorase (<5%) that is activated only by exogeneous electron carriers like methylene blue (Russell et al., 1982) (Calabrese, 1991).
The ultimate MetHb concentration is the resultant of the equilibrium reaction between the (slow, spontaneous) formation of MetHb and the rate of the reducing systems (notably NADH-diaphorase). In humans this reducing capacity is 250 times larger than the rate at which MetHb is formed, so that under normal conditions MetHb in humans comprises less than 1% of the total Hb percentage (Jaffé, 1981).
MetHb levels will increase with increasing formation of MetHb, or through insufficiently protective reducing capacity (caused by impairment of the NADH diaphorase itself or through depletion of NADH-generating pathways in the erythrocytes). This may occur through oxidating compounds (toxic methaemoglobinaemia) or in rare genetic defects (Calabrese, 1991).

The following broad classification for clinical methaemoglobinaemia has been widely adopted, both in the literature and in handbooks: < 15% MetHb no effects; 15-40% fatigue, weakness, dizziness, headache, tachycardia; 40-70% hypoxia, unconsciousness, coma, bradycardia, arrhythmias; > 70% fatal. This classification, however, is based on acute clinical problems (poisoning) and not on long-term exposure. Moreover, Coleman and Coleman (1996) point out that certain individuals will already show clinical signs at MetHb concentrations <10% (see section 6).

Heinz bodies
Heinz bodies are small or large precipitates in erythrocytes which develop after oxidation of Hb, or, to a lesser extent, from MetHb (through formation of haemichromes). Presumably, Heinz bodies do not necessarily develop under the influence of the same active metabolites that give rise to MetHb. But in both cases the protein in the erythrocyte cytoplasm undergoes a conformational change, denaturation, and next precipitation. This reduces the flexibility and functioning of the erythrocytes. The spleen usually removes small amounts of Heinz bodies from the blood. Heinz Bodies are demonstrable for a longer period in the blood than are increased MetHb concentrations. In general the presence of Heinz bodies nearly always points to MetHb formation, though indeed not in all cases (Smith, 1996; Russell et al., 1982).

1.3 Normal values and natural variation

Methaemoglobin
The MetHb concentration is determined by direct spectrophotometry (absorption peaks at 631 nm and 500 nm) and is expressed as percentage of the total Hb concentration. Another (somewhat older) method makes use of cyanide: added to the sample it leads to the formation of cyanmethaemoglobin. Consequently, the 631 nm absorption peak will disappear and the 500 nm peak will shift to a broad peak at 542 nm (Evelyn and Malloy, 1938).
Normal values for MetHb levels have not been reported in handbooks or relevant journals. The MetHb blood levels in various control groups presented in Table 1 provide an indication of the values usually measured in experimental animals and humans.
In animal studies under evaluation, however, the MetHb concentrations in the control groups may differ from those mentioned in Table 1. For instance when animal measurements were done with equipment specifically developed to determine human MetHb: a difference in

spectrum will than result in higher values. For the assessment of effects in exposed groups the reference values in the study under consideration are ultimately more important than any historical reference values (Boink, personal commununication).

Table 1 MetHb concentrations – reference values in man and in various animal species [a/b]

Species		MetHb (%)	Reference/source
Man			
	Volunteers study	0.22-0.56 (mean 0.44)	Kortboyer, et al. 1997ab; Kortboyer, et al. 1998
		< 1	(Jaffé, 1981; Faivre et al.., 1998)
Rat			
Wistar (Riv:Tox)	RIVM studies[c]	range 0-0.57 (mean 0.18)	Boink et al. (1996a)
		0.2-4.1 (nutrition A (RIVM))	Boink et al. (1996b)
		0.2-3.7 (nutrition B (TNO)	
		0.0-0.26 variation of the mean in the same group of rats for several weeks	Boink et al. (1997)
Sprague-Dawley	one study	range 0.4-2.1 (mean 1.1)	Aniline (EPA, 1999)
Crl:CD	one study	range 0 - 2.9	ibid
Fischer 344	4 studies	range 0.2-1.3	TNB (Choudhury, 1997)
Mouse (CD-1)[e]	one study [d]	1.56 (m) (mean) 2.19 (f) (mean)	EU-monograph Chlorpropham draft
Dog (Beagle)	one study [d] (n=4)	range 0.5-2.0 (m) (mean 1.2) range 0.4-1.1 (f) (mean 0.6)	EU-monograph Chlorpropham draft

a) Normal values derived from hematology handbooks available at RIVM/LPI, and various reference handbooks for laboratory animals (Loeb and Quimby, 1989; Lewi and Marsboom, 1981).
b) For other animal species, e.g. birds, see thesis Blaauboer (1978).
c) The values in controls did not exceed 0.5%, and values in the nitrite-exposed group ranged from 0.5-12% (Boink et al. 1996a, 1996b, 1997).
d) Heinz bodies were also determined in this study (0 in controls).
e) The mouse values in the Table are likely to be relatively high, but other reference levels are not available.

Heinz bodies
Heinz bodies are demonstrated using methyl-violet staining. In a leucocyte differential count, however, red blood cells will always be present, so that Heinz bodies may also be identified using routine Giemsa staining. Theoretically, the Heinz bodies level in blood of control animals is zero. (for more information see: (Russell et al., 1982), (Coleman and Coleman, 1996), (Chanarin, 1979), (Dabrow and Gabuzda, 1996).

1.4 Sensitivity species / groups

Methaemoglobin
Interspecies differences have been investigated only in a limited number of studies, mainly in *in-vitro* studies. Although these studies show that species differences are present, a distinct ratio from animal species to humans cannot be established, since MetHb formaation *in vivo* is highly dependent on other factors, such as the metabolism of the substance, reducing components in the blood (e.g. from food), and intraspecies differences.

Various *in vitro* studies with erythrocytes, which particularly focussed on reducing capacity, yielded a rough dichotomy for the formation of MetHb: Rat/mous/rabbit/guinea pig/monkey are less sensitive to MetHb formation and generally show a more effective reduction of induced MetHb than do man/dog/cat. The cat is most sensitive to MetHb formation, primarily because of a different type of haemoglobin.
A number of old *in vivo* studies revealed a similar division as well. Administration of acetanilid or acetophenetidin to various animal species resulted in slower MetHb formation in rat/rabbit/monkey (the latter two formed hardly any MetHb) than in man/dog/cat. However, it is not known whether for other substances a similar division exists. Following i.v. administration of 4-dimethylaminophenol the reduction of induced MetHb in mice and rabbits was very rapid, whereas in dogs and cats it was much slower.

From this pattern it cannot be concluded that e.g. the dog is the most suitable experimental animal for MetHb effects, as several other factors are involved (e.g. the substance's metabolism) in addition to the reducing property of e.g. NADH diaphorase. For more information refer to (Calabrese, 1991), (Smith, 1996), and (Blaauboer, 1978).

As to intraspecies differences, the human population may be distinguished into these subpopulations:
- **Faetuses and newborns**: characterized by low NADH diaphorase (up to 50% less) and compared with adult Hb a more fluctuating faetal Hb (HbF). Both Hb and NADH diaphorase reach adult levels about 3-6 months after birth.
- **Cytochrome b5 reductase deficiency:** occurs very rarely. The individuals concerned (hetero- and homozygotes) are extremely susceptible to toxic methaemoglobinaemia.
- **Elderly people**: *characterized by a more fluctuating Hb, which is more susceptible to oxidation.*
- **Glucose-6 phosphate dehydrogenase deficiency**: The individuals concerned show defective NADH production, which may inhibit the reducing property of the NADH diaphorase (this deficiency was observed in American black males (11-13%), Mediterranean Jews (11%), Greeks (1-2%), and Sardinians (1-8%); (Calabrese, 1991)).
- **Deficiency of enzymes involved in the RBC energy balance**.
- **Mutations in Hb** (THALASSAEMIA): owing to which it may become more susceptible to oxidation to MetHb.

References: (Dabrow and Gabuzda, 1996; Griffin, 1997; Smith, 1996; Coleman and Coleman, 1996; Jaffé, 1981).

Proposing toxic threshold values for the whole population, the US-EPA uses an additional assessment factor to account for a difference in sensitivity between adults and newborns (see, for instance, Aniline: proposed acute exposure guideline levels (AEGLs)). RIVM holds the view that the commonly used factor 10 for intraspecies variation will suffice to account for the difference in sensitivity between adults and newborns. Hence, no additional assessment factor will be applied in determining a toxicological threshold value (e.g. an ADI) on the basis of MetHb effects.

It is not known whether similar intraspecies differences occur in laboratory animals, but from appendix B it appears that two Wistar rat strains (Riv:TOX and Wu:Harlan) for example, show a difference in MetHb formation during an 8 weeks' exposure to nitrite.
Heinz bodies
Little is known about the formation of Heinz bodies, and Smith (1996) in his review points out that possible interspecies differences have not been systematically investigated. Yet he

provides a rough arrangement (omitting the rat!): the red blood cells of rabbit, monkey, chicken, and guinea pig are the least sensitive, followed by man, mouse, and dog, and finally the cat, which is most sensitive to the formation of Heinz bodies.
Strikingly, the mouse, in contrast to its sensitivity to MetHb formation, is here included in the group of sensitive species, together with man and dog. The review, however, does not provide a reason for this.
Little is known about sensitive groups within a species. But assuming that MetHb formation usually leads to formation of Heinz bodies, the above intraspecies differences are supposed to occur as well with respect to Heinz bodies formation in the human population (Smith, 1996).

1.5 Factors that may influence the MetHb concentration

A number of factors are known to influence the concentration of MetHb. These may be conditions during the experiment itself, but also after taking a blood sample.

- The timing of blood taking in a study is important, as the formation of MetHb is a relatively acute effect, and once formed MetHb is rapidly reduced. Therefore, when blood for a MetHb assay is collected at a time when the MetHb concentration in the blood is already falling or has reached baseline level, the effect is bound to be underestimated (see for instance study in EU-monograph Chlorpropham). See also the human volunteers experiment in appendix B, as well as the above studies in the literature in different animal species (see section 4). The maximum effect on MetHb strongly depends on the kinetics and metabolism of the substance.
- In addition, long-term exposure to a substance may cause adaptation of the reductase system. See the example in appendix B in which the maximum MetHb concentration in the blood of rats decreases during exposure to nitrite (total 8 weeks).
- Food intake may influence the MetHb concentration in the blood, for instance because the food itself will cause MetHb formation, or because it contains reducing components (GSH; oxidants) (Boink et al., 1996ab, and the list of substances that induce MetHb formation: Appendix A).

The conditions after blood sampling may also influence the percentage of measured MetHb (Sprokholt, 1987). For example, if red blood cells lyse, the released Hb is rapidly oxidized to MetHb. Energy depletion in the red blood cells may also result in higher MetHb level because the NADH-diaphorase system is inhibited.

1.6 Assessment and strategy in the RIVM Centre for Substances and Risk Assessment (CSR)

Until now, organisations like JMPR, WHO, or EPA have not published specific policies or guidelines for the assessment of substances that may cause methaemoglobin formation.
There are two kinds of effects to be considered when assessing MetHb-inducing substances: clinical signs and haematological parameters.

Clinical signs
The first clinical signs indicating that a substance may induce MetHb-formation, are the brown/grey colour of the blood and the blue/grey appearance of the extremities (nails, nose, fingertips, skin). These may already occur at slightly raised MetHb concentrations (<6%) in experimental animals and humans. In a long-term study these effects might well be noticed in the initial phase only, and subsequently disappear due to adaptation.

These clinical signs could point at MetHb formation, but might also be the result of other mechanisms that lead to O_2 deficiency (e.g. lung or cardiovascular effects). However, if these signs are observed, the performance of MetHb measurements are certainly indicated.

MetHb concentration in the blood and related effects

General

Evaluation of MetHb measurements should always take notice of the experimental factors, such as the time points of measurement and treatment of the samples (see section 5). The accurate points of time are determined by the kinetics and metabolism data of the substance. Blood sampling at a relative late time point in a subacute (or semichronic) study may lead to underestimation of MetHb induction. However, at the same time this sampling time could have been too early to detect some related effects (see below) that will develop over time. In view of possible adaptation (particularly in the rat) the MetHb measurements should have been performed relatively early in the study. The optimum procedure consists of repeated measurements of the MetHb concentration during the first few days or weeks after administration of the substance.

The following general considerations play a pivotal role in the assessment strategy outlined below. If an effect on MetHb concentrations is observed, it is by definition considered an adverse effect because an increase in MetHb levels is possible only when the capacity of the reducing mechanisms is exceeded (notably NADH-diaphorase). The exposure in question has then already reached such a level that considerable energy will be spent on reduction of MetHb and production of reticulocytes. This is certainly considered an adverse effect on a long term basis, as it impedes the 'biological fitness' of the organism.

Increased MetHb concentration – when is it toxicologically relevant?

The following rule of thumb applies for MetHb assessment:

> Any significant increase in MetHb concentration compared to control level is in principle considered a toxicologically relevant ('adverse') effect if a dose-response relationship is present.

This could imply that also small, though significant, increases compared to control level are considered as adverse.

An increase in MetHb concentration which is not (yet) significant, is still considered a biologically relevant effect if a dose-response relationship is observed at the consecutive dosages. This dose at which the non-significant increase in MetHb is measured, is in principle considered as NOAEL in the study in question, unless other related effects have been observed in the study in question or in other available studies at comparable dose(s) (see below). In the latter case a non-significant increase of MetHb concentrations may also be considered as toxicologically relevant.

Related effects

MetHb concentrations in exposed animals may occasionally show larger variation than those in control groups. Owing to a wide standard deviation, an increase of the MetHb concentration at a certain dose might then turn out not to be statistically significant (see e.g. appendix B and Table 1, note c). A non-significant increase in MetHb may nevertheless be associated with other, related, effects. These MetHb-related effects in blood and organs are of importance as well in the absence of a dose-response relationship for effects on MetHb concentrations (see Table 2). This is why these related effects should always be taken into consideration.

In (sub-)chronic studies the whole blood differential cell count is generally more important than just the measured amount of MetHb, as the MetHb concentration may have dropped through adaptation. Prolonged exposure to MetHb-inducing compounds may bring about several adaptation-linked related effects, such as the presence of Heinz bodies in red blood cells and changes in the differential cell count indicative of anaemia: RBC↓, Hb↓, MCV↑, and reticulocytes↑. In addition, haematopoiesis in liver and spleen, hemosiderins (insoluble

Table 2. Overall course of effects after exposure to a MetHb-forming compound.

Short-term effects mainly observable at the outset of the study	Effects observable in the course of time in (sub)chronic studies
1. MetHb-formation 2. Bluing of the extremities	1. Effects on blood parameters: RBC↓, Hb↓, MCV↑ Reticulocytes↑ Heinz bodies↑ 2.Effects on organs - liver heamatopoiesis; haemosiderins spleen haematopoiesis

iron precipitates) in the liver, and possibly (in the beginning of the study) bluing of skin and/or nose are indicative of MetHb-formation as well.

The NOAEL in a study with a MetHb-inducing substance may, therefore, also be determined by effects on the above-mentioned parameters. The following 'rule of thumb' may be applied:

The NOAEL in the study is based on a significant change in one of the above-mentioned related parameters, provided that the next, higher doses result in a) a dose-effect relationship for the parameter in question, and b) the occurrence of minimally two other related effects.

Consequently, should the MetHb-formation have been determined incorrectly or not at all, and yet the effects listed in Table 2 are present, it is not always necessary to request for a new study in which MetHb-formation is measured. The overall picture of effects may then suffice to determine a NOAEL.
If there are indeed reasons to request for a new study, a short-term study lasting maximally 2-4 weeks, with repeated measurements of blood MetHb concentrations, will suffice in most cases.

1.7 Examples

Chlorpropham: Recommendation 06120B00, Draft dd. 28-12-1998. Authors: Apeldoorn ME van, Wouters MFA, Fouw JC de, et al. MetHb formation determined the NOAEL, and because MetHb was not established until the end of the 90-day study, a safety factor of 3X was applied to the ADI. See pp. 69-70.

1.8 References

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Appendix 1 Compounds that can cause Methemoglobinemia

After Seger, 1992

Acetanilid
Acetophenetidin
Alloxans
Alpha naphtylamine
Aminophenols
Ammonium nitrate
Aniline and derivates
  Chloromethylaniline
  Paradichloraniline
  Paranitroaniline
Aniline dyes
Anilinoethanol
Antipyrine
Arsine
Benzene and derivates
  Dinitrobenzene
  Nitrobenzene
  Nitrosobenzene
Benzocaine
Chlorates
Chloranilines
Chlorobenzene
Chloronitrobenzene
Cobalt preparations
Corning extract
Crayons, wax (red or orange)
Dapsone
Diaminodiphenylsulfone
Diesel fuel additives
Dimethylamine
Dimethylaniline
Dinitrophenol
Dinitrotoluene
Hydrogen peroxide
Hydroquinone
Hydroxylacetanilid
Hydroxylamine
Inks
Kiszka
Lidocaine
Menthol
Meta-chloraniline
Methylacetanilid
Methylene blue
Monochloroaniline
Naphtylamines
Nitrites and nitrates
Amyl nitrite
  Butyl/isobutyl nitrite
  Sodium nitrite
Nitrite and nitrate preservatives in meat
Vegetables (carrots and spinach) in infants
  Contaminated well water
  Nitrite salts used in industry
  Glyceryl trinitrate (nitroglycerin)
Transdermal, sublingual, oral
  nitrite/nitrate
  Contaminants from aneasthesia
  cannisters with nitrous oxide
Nitrogen oxide
Nitrofurans
Nitroglycerin
Nitrophenol
Ozone
Parnaquine
Para-aminopropiophenone
Para-bromoaniline
Para-chloraniline
Para-toluidine
Pentaerythritol tetranitrate
Phenacetin
Phenetidin
Phenols
Phenylazopyridine
Phenylenediamine
Phenylhydrazine
Phenylhydroxylamine
Phenytoin (Dilantin)
Piperazine
Plasmoquine
Prilocaine
Primaquine
Propitocaine
Pyridium
Quinones
Resorcinol
Shoe dye or polish
Sodium nitroprusside
Spinach
Sulfonal

Sulfonamides
- Dapsone
- Prontosil
- Sulfanilamide
- Sulfapyridine
- Sulfathiazole

Sulfones
Tetranitromethane tetronal
Tetralin
Toluenediamine
Toluidine
Toluylhydroxylamine
Trichlorocarbanilide (TCC)
Trinitrotoluene
Trional

Appendix 2 Factors that influence MetHb-levels

For the measurement of MetHb levels in blood, one should consider e.g. the time point of blood sampling. (see fig. 1), the possibility of adaptation (see fig. 2), and intraspecies differences. Such effects have been investigated at the RIVM within the framework of the nitrate/nitrite research. The acute methemoglobin formation was measured in a human volunteer study (RIVM report 235802 007, Kortboyer et al., 1998). The volunteers received nitrite intraveneously, after which MetHb-levels were measured in the blood for 10 hours. In figure 1, the MetHb-levels for 2 volunteers are depicted. It is shown that, after reaching a maximum level between 1-2 hours, MetHb levels decrease rapidly reaching control levels already after several hours. This indicates that measurements of MetHb levels several hours after a single treatment are useless.

In another study, MetHb levels were determined after nitrite treatment in two different rat strains. Rats received drinking water containing nitrite for 8 weeks, and blood was sampled pre-exposure and than every two weeks. The study showed that nitrite treatment induced higher MetHb levels in Wu:Harlan Wistar rats compared to Riv:TOX Wistars.
In addition, repeated exposure of rats to nitrite resulted in a gradual adapatation in both rat strains.

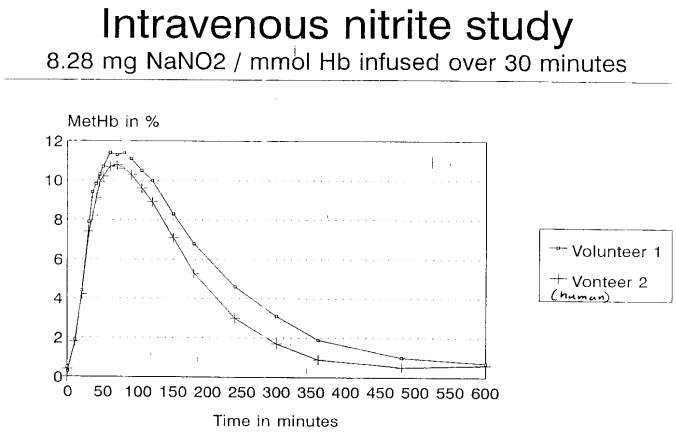
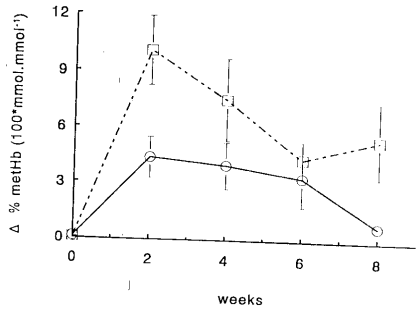


Figure 1 MetHb-levels in intrvaveneous blood of human volunteers after exposure to nitrite.
Figure derived from Dr. ABTJ Boink; published in RIVM report 235802 007 (Kortboyer et al., 1998).



Figuur 2 Adaptation of MetHb-formation in 2 different Wistar rat strains.
Net effect (difference between test group and concurrent control (=KCL, 36 mmol.l^{-1}) of exposition to nitrite (36 mmol.l^{-1}) on the methaemoglbin fraction of Riv:TOX (O) and Wu:Harlan Wistar ( ) rats during time (means ± s.d.). Uit: 'Health aspects of nitrates and its metabolites (particularly nitrate)' Proceedings International Workshop Bilthoven, The Netherlands 8-10 Nov 1994, p229-232.

2 Acetylcholinesterase inhibitors

Factsheet FSV-002/00 date 10-09-1999

Author:

M.T.M. van Raaij

2.1 Introduction and aim

Organophosporus esters (OPs) and carbamates are widely used insecticides. One of their major effects is inhibition of the enzyme acetylcholinesterase (AChE; EC 3.1.1.7). This enzyme is primarily located in the synapses of the somatic, autonomous, and central nervous systems, but also in erythrocytes and blood plasma. AChE is involved in the breakdown of the neurotransmitter acetylcholine, which diminishes or terminates the activation of postsynaptic cholinergic receptors. Inhibition of AChE leads to acetylcholine-induced overstimulation of the postsynaptic receptors, which in its turn results in so-called cholinergic toxicity or 'cholinergic crisis' [1,2,4]. Besides AChE inhibition in the central (CNS) or peripheral nervous system (PNS), OPs and carbamates can also inhibit AChE in the blood. This factsheet describes which effects associated with AChE inhibition are considered as toxicologically relevant and at what level of AChE inhibition is considered 'adverse'.

2.2 Mechanism for the development of the effect, and background

Although they both induce AChE inhibition, OPs and carbamates do not have quite the same mechanisms of action. OPs are analogues of the normal biological substrates of AChE. The various steps of the interaction between an OP and AChE are shown in Figure 1.
An OP reversibly binds to the hydroxyl group of a serine residue in the enzyme, resulting in a Michaelis-Menten complex (step 1). After separation of the residual group XH (step 2), the OP and AChE form a covalent bond (for acetylcholine the residual group is choline). The OP-enzyme complex may be reactivated (step 3). For the original substrate acetylcholine, step 3 will last a few microseconds only. However, for OPs the half-life times can be very long [2], resulting in long-term AChE inhibition. Some OPs, however, do not seem to undergo any reactivation [2]. An additional complicating factor is 'ageing'. This phenomenon refers to separation of one of the residual groups that are linked to the phosphorus through an oxygen atom (step 4). This irreversible reaction will inactivate the enzyme. The half-life times of ageing are 4 hours for dimethoxy-OPs, and about 10 hours for diethoxy-OPs. This implies that the rate of ageing (step 4) could far exceed that of reactivation (step 3), which makes the inhibition of AChE by OPs virtually irreversible [1,2,3]. Ageing is probably an essential step in the induction of OP-induced delayed neurotoxicity (OPIDN). This subject will be dealt with in a separate factsheet.
The behaviour of carbamates is different from that of OPs. They are hydrolized by AChE, but the enzyme-carbamate-complex has a long half-life time. This complex, unlike OPs, does not undergo ageing however, and AChE is thus reactivated according to step 3. Consequently, the AChE inhibition of carbamates is reversible (within a period lasting from minutes to hours). With regard to mechanisms of action refer to refs. for further information [1,2,3,4].

As mentioned, AChE is found particularly in the *CNS* and the *PNS*. The CNS is protected by the so-called 'blood-brain-barrier', preventing a number of AChE-inhibiting substances to reach their target site (the synapses) in the CNS. The PNS is surrounded by a perineurium, but this is less effective than the blood-brain-barrier, especially at the peripheral ganglia. Hence, exposure of peripheral nerves and ganglia may be occasionally higher than that of the CNS [3]. However, many organophosphorus esters pass the blood-brain-barrier relatively easily, with little or no difference in exposure between the CNS and the PNS [A. Moretto, personal communication].

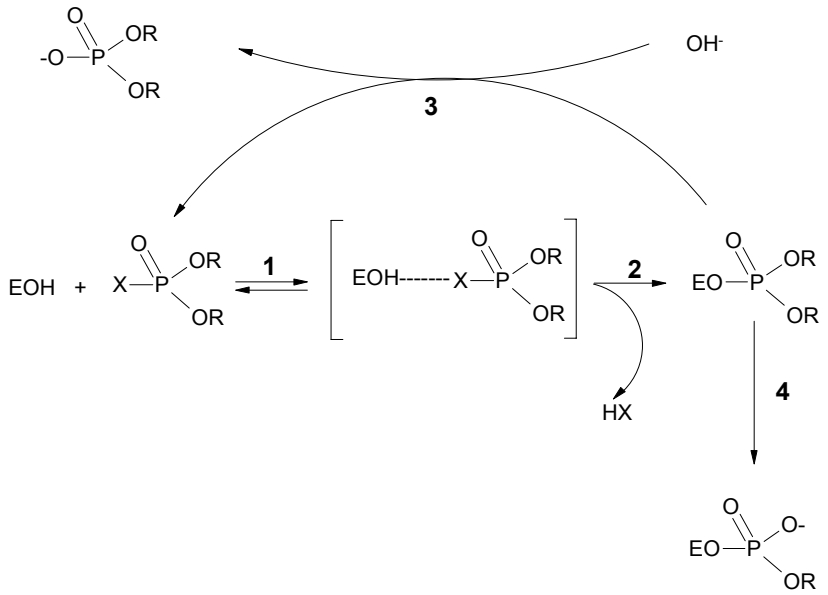


Fig. 1. Schematic representation of the interaction and enzymatic steps in the actions between an OP and AChE. (the various steps are explained in the main body of the text).

Both the erythrocytes and the blood plasma contain cholinesterases, which, in contrast to neural AChE, are not involved in cholinergic transmission. The enzyme in the *erythrocytes* is biochemically identical to that in the PNS, viz. acetylcholinesterase. The rate at which AChE is being resynthesized, is however much higher in the PNS than it is in erythrocytes [11,22]. Cholinesterase activity in *plasma* does not only comprise acetylcholinesterase but also butyrylcholinesterase (BuChE). In rat plasma the proportion of AChE and BuChE is 1:1, but in humans it is about 1:100 [2,5,11]. This means that plasma cholinesterase activity in humans almost exclusively relates to BuChE. This is a so-called 'pseudo-cholinesterase', which is also used as a biomarker. Most analytical methods determine total cholinesterase activity by measuring the conversion of acetylthiocholine in a colorimetric assay in which no actual differences between AChE and BuChE are established.

The physiological role of cholinesterase activity in erythrocytes and blood plasma is not known. From the available literature it appears that selective inhibition of erythrocyte or plasma ChE itself does not induce harmful effects [6]. Nevertheless, BuChE might be involved in the detoxification of other xenobiotics (e.g. cocaine), which suggests that inhibition of plasma ChE activity may indeed have physiological consequences [1].

2.3 Normal values and natural variation

In the toxicological literature effects on AChE activity are usually expressed as percentage inhibition relative to the concurrent control group, or to pre-exposure values in the exposed group. Absolute quantities of AChE activity are rarely reported in the public literature. Besides, as the reported data are largely influenced by the experimental methods applied (see also section 5), comparison of the various values obtained for AChE activity is less useful [see for absolute values e.g. refs. 4,5,6,7,8].

The intraspecies variation in AChE activity is dependent upon the target organ in which the activity is measured. In general, the brain shows less intraspecies variation of AChE activity than does the blood. The mean variation of AChE activity in the brain ranges from 10-15%, whereas in blood it ranges up to 20% [2,4,5,7,8,9].

The interspecies variation in total cholinesterase activity is characterized by both qualitative and quantitative differences between the various species. Blood ChE in particular shows great differences. Erythrocyte AChE activity in various species ascends in this order: cat << rat < rabbit, dog < horse < guinea pig < pig < cow < chimpanzee < human. The differences in erythrocyt AChE activity between rat and human could amount to a factor 5 to 10 [18,4,9,10]. Plasma ChE activity consists of AChE and BuChE (see section 2). These two enzymes show variable proportions in the various species [2,5,11]. As standard assays only measure total cholinesterase activity, they do not distinguish between AChE and BuChE. For a conclusion on interspecies variation in brain AChE activity, sufficient data were not available.

2.4 Sensitive Species / Groups

Although substantial species differences have been noted with respect to the sensitivity for OP-induced delayed neurotoxicity (OPIDN), we are currently not aware of specifically sensitive species and/or groups with regard to AChE inhibition that require a specific assessment procedure or should be considered a special risk group.
Nevertheless, there are some indications for differences in sensitivity between various species [21], between males and females (females more sensitive) [18], and for an increased sensitivity in neonates [11,12,13,14,15,16,21].
However, the major differences in inter-individual sensitivity are probably caused by differences in metabolism and kinetics of the AChE inhibiting substances, notably in the detoxification routes [2].

2.5 Factors that may influence the measurements

There are several factors that influence the activity of AChE, such as age, sex, stress, endocrine status, and seasonal variation [4]. The ultimately measured AChE activity, however, is largely determined by experimental factors. The major factors influencing the actually measured/reported AChE levels are listed below.

- Timing of sampling. Especially when dealing with carbamates or after single doses of OPs, the time of sampling must be accurate in order to provide an optimal measurement of AChE inhibition (at the time of peak inhibition). Kinetic data or AChE activity measurements at different time points could be helpful in this respect [22].
- AChE inhibition might be underestimated if samples are not correctly treated and/or stored. Also when stored, samples may show, for instance, spontaneous reactivation of AChE. This is particularly a problem with carbamates. Samples should preferably be stored at a temperature of –60 or –80 °C [17,18,22].
- The assay conditions may exert a great influence on the results obtained through the so-called ‘Ellman-method’ (also available as standard kits): buffer composition, pH, substrate concentration, temperature, sample dilution, and the measuring wavelength. For instance, the used wavelength can be increased from 412 to 480 nm in order to reduce the interference of haemoglobine (in the case of blood/RBC measurements) [17,18,22].

- According to Wilson et al. [17] it is essential to include tissue blanks in addition to substrate blanks, and to apply pre-incubation.
- Standard kits designed for measuring AChE activity in rat blood are not directly suitable for human samples [7].

The above indicates that the methods of measuring ChE should be carefully assessed, especially in key studies. The latter implies that particularly in studies in which there is doubt about the analytical conduct, and in the study that yields the overall NOAEL (if based on cholinesterase inhibition), the analytical performance of the study should be carefully examined. References [4,7,8,17,18] provide detailed discussions and points for attention for such an analytical assessment.

2.6 Assessment and RIVM/CSR Strategy

In the past two decades the assessment of cholinesterase inhibition by OPs and carbamates has been subject to discussion, notably on the relevance of AChE inhibition in the blood. See for a historical review of the various discussions and propositions ref [19].
Three organisations in particular have published their policies on cholinesterase inhibition: WHO/UNEP [20], U.S. EPA [19,21], and JMPR [3,7,18,22,23] (The WHO's opinion is based on the JMPR assessments). In spite of minor differences in the approach of acetylcholinesterase inhibition assessment, the WHO, U.S. EPA, and JMP approaches show no fundamental differences. This is why the RIVM/CSR strategy outlined below basically links up with these international approaches.

AChE inhibition in toxicity studies should be assessed using a multi-stage approach ('weight of evidence approach' [19]) examing the following endpoints in the order of importance:

1) Clinical signs and/or other behavourial or neurophysiological effects in laboratory animals or humans,
2) AChE inhibition measurements in the CNS and/or the PNS,
3) AChE inhibition measurements in erythrocytes or whole blood,
4) ChE inhibition measurements in plasma/serum.

NOTE: The analytical conduct of AChE measurements should be assessed as well (see section 5).

Clinical signs
The occurrence of (cholinergic) clinical signs, behavourial or neurophysiological effects provides direct indications for an adverse effect of ChE-inhibiting substances. Dependent on the distribution and kinetics of the substance (combination of PNS and/or CNS effects) a range of clinical patterns may be observed [2,19]. The most frequently observed cholinergic symptoms are: headache, dizziness, anxiety and restlessness, muscle-contractions, weakness, tremor, incoordination, vomiting, abdominal spasms, diarrhoea, miosis, sweating, salivation, and lacrimation. [2,19,24].
The incidence and/or severity of the clinical signs may decrease ('adaptation') upon repeated exposure to the substance. This suggests that in such a case the substance actually becomes less toxic. This 'adaptation', however, is merely the result of neurobiochemical adaptations at receptor level that impede the 'biological fitness' of the organism, or may be associated with other manifestations of neural dysfunction [25,26,27].

AChE inhibition in the CNS
AChE inhibition in the CNS is an indicator of an adverse effect as it directly interferes with the deactivation of acetylcholine, and thus influences the cholinergic activation of neurons. The inhibition of AChE in the brain may strongly differ between various regions of the brain (e.g. hypothalamus, striatum, hippocampus, cerebellum, cortex). Measurements at various specific brain areas could, therefore, be more conclusive than 'whole brain' measurements. For the present factsheet insufficient information was available to establish which regions of the brain are the most sensitive to AChE inhibition.

AChE inhibition in the PNS
AChE inhibition in the PNS is an indicator of an adverse effect. Although it is technically feasible to conduct PNS measurements, PNS AChE is rarely measured. The major PNS target tissues include: skeletal muscles, heart, diaphragm, salivary glands, and autonomous ganglia [21].

AChE inhibition in the erythrocytes
Inhibition of erythrocyte (and plasma) AChE itself does not lead (as far as known) to adverse effects. The enzyme in the erythrocytes is identical to that in the CNS and PNS [8,11]. Moreover, if measured correctly, a distinct correlation will be shown between blood/erythrocyte AChE activity and CNS or PNS activity, although quantitatively this relation is different for various substances [2,5,14,15,28]. This is why erythrocyte AChE may serve as an indicator for AChE inhibition in the CNS and/or PNS. It is difficult (if not impossible), however, to distinguish between the effects of AChE inhibition in the CNS and in the PNS.

During evaluation of a study three situations may arise:
1) Measurements of CNS, PNS, and erythrocyte AChE are all available. As in this situation data are available for the primary target organs (CNS and PNS), the erythrocyte measurements are not (or less) relevant.
2) Measurements of CNS and erythrocyte AChE are available, but those in the PNS are lacking. This is the prevailing situation for most substances. The CNS AChE measurements provide a direct indication for effects in the CNS, and erythrocyte measurements are, therefore, not relevant as indicator for the CNS. However, exposure of the PNS might have been higher than the CNS (see above); the erythrocyte AChE inhibition can then be used as indicator for PNS AChE inhibition. In this case erythrocyte AChE cannot be considered completely non-relevant.
   A crucial element is the substance's passage through the blood-brain-barrier[1]. If it effectively passes through the blood-brain-barrier, the use of erythrocyte AChE is relatively conservative; in that case the brain measurements are more relevant to the PNS. If the substance poorly penetrates into the blood-brain-barrier or not at all, the PNS may be higher exposed; in that case the erythrocyte measurements are indeed relevant to the PNS.

[1] Whether a substance actually passes through the blood-brain-barrier can be deduced from kinetic data (distribution of the substance among the organs/ brain) or from AChE inhibition data after acute exposure. Considerable inhibition of brain AChE (compared with blood/plasma) measured after acute exposure points at substantial passage of the blood-brain-barrier. If relatively little brain AChE inhibition (compared with blood/plasma) is observed, this could indicate poor passage of the blood-brain-barrier [A. Moretto, personal communication].

3) Measurements of CNS and PNS AChE inhibition are not available; only erythrocyte AChE measurements have been performed. In that case the erythrocyte measurements are used as indicator for AChE inhibition in both the CNS and the PNS.

Primarily in the case of short-term exposure (< 4 weeks) it is justified to use erythrocyte AChE inhibition as indicator for peripheral effects (PNS). Erythrocyte AChE measurements are probably very conservative in long-term exposure, as erythrocyte AChE is resynthesized more slowly than that in the PNS.
Exceptions to the above guidelines:
For several reasons it may be justified to use erythrocyte AChE inhibition as toxicological endpoint (also for establishing an ADI, RfD, or TDI) (see refs 19,21,23 for discussions on this issue). Examples are:

- Blood measurements are often the only available indicators for AChE inhibition. As CNS or PNS measurements are not available in humans, blood AChE actually forms the only or most critical toxicological endpoint for AChE inhibition in humans. Consequently, if human data are used to establish, for instance an ADI, erythrocyte AChE activity is used to this end.
- A substance with a steep dose-response curve, for which blood AChE is the most sensitive parameter.
- A substance for which the LOAELs and NOAELs for the various AChE inhibition endpoints prove to be more or less similar.
- A substance for which, on the basis of the available information, it may be assumed that it not or hardly passes through the blood-brain-barrier. Mainly peripheral cholinergic effects will arise in this situation, and erythrocyte AChE could then be the most relevant parameter.

ChE inhibition in plasma or serum
As mentioned above, ChE activity in plasma (or serum) in rats consists partly of BuChE, and in humans almost entirely of BuChE. Moreover, the turnover of plasma ChE differs from that in erythrocytes and neural tissue. Inhibition of plasma ChE itself has (as far as it is known) no adverse effects. For these reasons inhibition of plasma ChE is considered only as indicator for exposure to the substance and is considered not toxicologically relevant.
Nevertheless, the correlation between cholinergic symptoms and plasma ChE activity may be the only one, or may be stronger than that of, e.g. erythrocyte AChE [2,14,15,19,23]. Plasma ChE inhibition, for instance, may be an important predictor for pesticide-related diseases, and may serve as biomarker for exposure [21]. Hence, plasma AChE inhibition as well should always be assessed accurately. Plasma AChE measurements may provide additional information for determining the substance's toxicological profile (see section 2).

What is a toxicologically relevant ('adverse') effect
In line with the JMPR [22], RIVM/CSR regards a statistically significant inhibition of ≥ 20% as toxicologically relevant ('adverse'). This applies both to the CNS and the PNS, as well as to erythrocyte AChE. The inhibition of 20% may be considered with respect to the concurrent control group or with respect to the 'pre-exposure' values in the treated group. In view of the fact that age is a modulating factor for AChE activity, (sub)chronic experiments certainly should include comparison with a concurrent control group.
A statistically non-significant inhibition of ≥ 20%, or a statistically significant inhibition of < 20% requires a more detailed evaluation of the effect. The toxicological relevance of such effects should be assed on a 'case-by-case' basis.

The JMPR does not provide specific argumentation for the 20% inhibition threshold value [22]. The normal inter-individual variation for brain and erythrocyte AChE activity is roughly ≤ 20% (see above). This implies that a < 20% change may fall within the range of normal variation. From studies investigating the correlation between brain/erythrocyte AChE and behaviour/symptoms it appears that clinical symptoms and/or behavioural changes will not become manifest until a level of ≥ 20% inhibition of brain/erythrocyte AChE activity has been reached [2,6,24,28].
The EPA [19,21] in its documents considers the value of 20% inhibition as a toxicological effect, but a 1997 policy document states with regard to brain AChE inhibition that 'Statistically significant decreases in brain ChE are generally considered toxicologically significant…' [19]. The recent 'Guidelines for Neurotoxicity Risk Assessment' of the EPA [26] do not include any statement on the toxicologically relevant level of AChE inhibition. The EPA opinion expressed in citation [19], however, does not collide with JMPR and RIVM basic assumptions.

2.7 Examples

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3 Pheochromocytomas

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3.1 Introduction and aim

The adrenal gland consists of the cortex and the central part, the medulla, which both have different endocrine functions. The cortex produces (adreno)corticosteroid hormones (e.g. glucocorticoids), the medulla produces catecholamines (CA, epinephrine, and norepinephrine). Tumors (neoplasms) that are formed in the adrenal medulla are called pheochromocytomas.
The medulla of aging rats is characterized by spontaneously developing and commonly present hyperplasia en neoplasia [1,2]. Hyperplasia of the adrenal medulla has been observed in mice as well, though much less frequent than in rats [2,3]. Medullar hyperplasia and pheochromocytomas in humans are primarily (but not exclusively) associated with Sipple`s syndrome, type II Multiple Endocrine Neoplasia (MEN) [2,3,4]. This autosomal dominant inherited disease bears some resemblance to medullar lesions in de rat [2,3], but its incidence is very low.
Pheochromocytomas can be induced in experimental animals by exposing them to certain agents, for instance polyols (sugars or sugar alcohols). Polyols, except isomalt, induce hyperplasia and/or (benign and malignant) pheochromocytomas in the adrenal medulla of rats after long-term dietary administration at 10%-20% concentrations [5,6,7,8,17].
This factsheet aims to establish the toxicological significance of an agent-induced increase in adrenal medullar pheochromocytomas in experimental animals with respect to human risk assessment.

3.2 Mechanism for the development of the effect, and background

There is great terminological variety regarding cellular proliferation in the rat medulla [1,3,11]. Compression of the surrounding tissue has generally been accepted to discriminate between hyperplasia en pheochromocytoma. The universally accepted criterion for malignancy of human pheochromocytomas is the appearance of metastases [1,9,10].

Characteristics hyperplasia/pheochromocytoma (according to Strandberg, 1983 [11]):

1. *Diffuse hyperplasia*: a diffuse increase in number and occasionally also in size (hypertrophy) of the medullar cells, but no tumor formation. The cortex is not compressed or affected.
2. *Nodular (focal) hyperplasia*: one or more concentrated accumulations of medullar cells. These cells neither affect surrounding medulla or cortex, nor compress them.
3. *Benign, differentiated pheochromocytoma*: a nodular process involving proliferation of neoplastic cells, with compression of the adjoining medulla and cortex.
4. *Malignant or non-differentiated pheochromocytoma*: tumor invasion into the adjoining medulla, cortex, capsule, and blood and lymphatics. Some will spread into the adipose tissue around the adrenal glands, or invade lymph nodes, lungs or other organs (metastasis).

These criteria have not been uniformly agreed on. For instance, the ‘European Registry of Industrial Toxicology Animal-data Group’ proposes to apply the term pheochromocytoma for hyperplasia affecting more than 50% of the medulla.
In rats, hyperplasia of the adrenal medulla seems to precede the formation of tumors [2,17].

The substances which induce pheochromocytomas in rats are pharmacologically diverse (see Appendix I). It is assumed that a common characteristic for their working mechanism is the stimulation of chromaffin cell proliferation by neural signals that 'also' regulate catecholamine production and secretion [5,7,17].
A class of components influencing the rat medulla which has been studied intensively for the underlying mechanism, is that of sugars and sugar alcohols. It is proposed that their working mechanism involves altered calcium homeostasis [5,7], and several studies supply evidence for an essential role of hyperabsorption of calcium from the intestines. This has resulted in the so-called 'calcium hypothesis' proposing the following mechanism [12]: sugar and sugar alcohols increase calcium absorption in the rat small intestine. As this is associated with an increase in urine calcium excretion, the plasma calcium concentration hardly changes. Because hypercalcemia does not occur, the 'calcium hypothesis' poses that not calcium itself but mechanisms involved in maintaining normal serum calcium concentrations will lead to changes in the adrenal medulla. Findings from two studies, by Bär [12] and by Brion and Depuis [13], provide indications for polyol-induced effects on the medulla. Bär [12] demonstrated that in rats a diet containing 20% xylitol or sorbitol resulted in increased catecholamine (CA) concentrations in the medulla. In another study, Brion and Depuis [13] demonstrated decreased CA-concentrations in the adrenal glands of rats kept on a vitamin D deficient diet. Vitamin D_3 is involved in the absorption of calcium, and therefore, the observation that adding 20% lactose to the diet could prevent CA-depletion caused by vitamin D_3 deficiency is of importance.
Pheochromocytomas have not been observed in polyol-studies in mice and in dogs, which suggests that the induction is specific for rat. Human studies provide no indications that polyols induce changes in calcium homeostasis and changes in CA-concentration in the medulla, since changes in urine calcium and CA secretion has not been observed [17]. This indicates that adrenal medullar lesions resulting from oral exposure to polyols are not relevant to humans (see also section 5).

3.3 Normal values and natural variation

The table below shows the spontaneous incidence of pheochromocytomas for the rat, the mouse, and man. Rat and mouse incidences are also given separately for some widely used strains. Because in the rat the spontaneous incidence ranges so widely, historical data are considered to be of little importance (see also section 6, general).

The spontaneous incidence of pheochromocytomas and lesions in the rat medulla is strongly related to the strain. Independent of the strain, spontaneous pheochromocytomas more frequently occur in male rats than in female rats. Another factor is age: pheochromocytomas are predominantly observed in older animals [2,7,17]. This phenomenon does not only pertain to pheochromocytomas but also to other types of tumors (for instance thyroid tumors). Dietary restriction reduces the incidence of pheochromocytomas in rats [2,7].
The mouse does not show a sex-difference in the spontaneous incidence of pheochromocytomas. Neither does dietary restriction affect the spontaneous incidence of pheochromocytomas in the mouse [7].

Species	Strain	Sex	Incidence spontaneous (%)	Reference
Rat	General	m/f	0 – 69	18
			0 - 80	15
	Sprague-Dawley	m	0.12 – 45	9
		f	0.18 – 5.1	9
	F344	m	2.8 – 45	9
		f	0 – 15	9
	Wistar	m	10.6 – 69.2	9
		f	2.1 – 3.6	9
Mouse	General	m/f	0 – 5	16
	CD-1	m	0.2 – 2	16
		f	0 – 0.98	16
	B6C3F1	m	0.31 – 2.2	16
		f	0.27 – 1.4	16
Man	General	m/f	0.005 - 0.1	4,18

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The mouse does not show a sex-difference in the spontaneous incidence of pheochromocytomas. Neither does dietary restriction affect the spontaneous incidence of pheochromocytomas in the mouse [7].
In humans, pheochromocytomas are mostly observed at the ages from 40-50 years [4]. The general symptoms in patients with pheochromocytomas are: hypertension, severe headache, palpitation, sweating, and anxiety (attributable to an increased CA-production). Pheochromocytomas in humans are notably (but not exclusively) associated with MEN, and are preceded by hyperplasia of the adrenal medulla [4].

3.4 Sensitive Species / Groups

Interspecies
Rats are probably much more sensitive to the induction of pheochromocytomas than humans are, in particular male rats. It should be noted that in rats the sensitivity is highly strain-dependent.

Although the anatomy of the adrenal medulla in rats and humans shows great similarity, there are several potentially important differences:

A. In the rat and mouse adrenal medulla, the catecholamines epinephrine (E) and norepinephrine (NE) are largely stored in different chromaffin cells, with NE cells (type I cells, 0.3µm, 26%) as islets situated in a background of E cells (type II, 0.2 µm, 20%). [3,7,17]. Type I and II cells differ in their sensitivity to Ca^{2+} as exocytose mediator [17]. In humans, chromaffin cells are not specialized into E and NE cells: E and NE are stored in the same cell [1,17]. Several studies suggest that in rats the development of medullar

hyperplasia en pheochromocytomas is associated with increasing NE/E ratios. Pheochromocytomas in rats indeed usually show a specific phenotype characterized by almost exclusive production of NE, and secretory granules that are smaller and less in number than those in normal E and NE cells.

B. The rat medulla includes a third type of cell, the SGC (small-granule containing) cell, which has no equivalent in humans.
C. The protein composition of the secretory granules differs between rats and humans: chromogranine A in rats and chromogranine A and B in humans.
D. Chromaffin cells have several types of voltage-gated Ca^{2+} channels. These have not yet been characterized in human chromaffin cells, but the channel type distribution on rat chromaffin cells differs from that on cells of various other species [5,17].
E. While chromaffin cells of rats proliferate *in vitro,* cell proliferation has not been observed in *in vitro* studies with human chromaffin cells [16].

The significance of these various interspecies differences is still unclear [17].
In rats, pheochromocytomas are often not hyperfunctional. In humans however, the occurance of pheochromocytomas often is accompanied by symptoms indicating increased CA-output (hypertension, anxiety, and sweating). This probably relates to the observation that in rats pheochromocytomas synthesize little or no E, whereas pheochromocytomas in humans produce excessive E [17].

In summary it may be concluded that with respect to some important aspects pheochromocytomas in rats differ from those in other species: they commonly occur, are often bilateral and multicentric, and can be induced by many agents. In humans, pheochromocytomas are rare and usually solitary, except in patients with a hereditary MEN syndrome. At present there are no data about the inducibility of pheochromocytomas in humans. It should be noted, however, that human exposure to agents stimulating pheochromocytomas in rats (except polyols) is probably significantly limited compared to rat exposure levels that the occurrence of induction of pheochromocytomas cannot be determined in humans.

Mouse chromaffin cells take up an intermediate position with respect to tumor frequency and cell proliferation. These cells proliferate both *in vivo* and *in vitro*. Though the anatomy of the mouse medulla resembles that of the rat medulla, pheochromocytomas rarely occur in mice, and are not induced by xenobiots (except trichloroethane). The differences between rats and mice suggest that the mouse is possibly a more relevant model for humans with respect to medullapathobiology [7,15]. Studies on the sensitivity of e.g. dogs and monkeys are lacking.

Intraspecies
Apart from strain and sex differences in the rat, there are no data available pointing at groups within a species that may be specifically sensitive to the induction of pheochromocytomas.

3.5 Polyol-induced pheochromocytomas

In rats, the formation of polyol-induced tumors in the adrenal medulla probably results from at least three factors [12], i.e.:

1. Genetic sensitivity to a proliferative reaction pattern of the adrenal medulla after a stimulus.

2. Hyperabsorption of calcium from the intestines, followed by disturbance of the calcium-regulating hormonal system.
3. A functional sensitivity of the adrenal glands to changes in calcium homeostasis or calcium regulating systems, resulting in medullar cell proliferation.

As far as known, none of these factors plays a role in the human situation [16,17]. Medullar cell proliferation does not belong to the human reaction pattern [16], polyols have no effect on calcium intake and homeostasis [17], and there is no relationship between hypercalciuria en hypercalcemia and an increased risk of pheochromocytomas in humans.

Polyol-induced pheochromocytomas do not seem to have toxicological relevance for humans, because the underlying mechanism, which seems to be based on a disturbance of the calcium homeostasis ('calcium hypothesis'), which mechanism does not occur in humans [17]. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) has established a nonspecified ADI [2] for all polyols [14,17,18].

3.6 Assessment strategy in the RIVM centre for Substances and Risk Assessment (CSR)

As argued in section 5, there are sufficient (indirect) arguments indicating that polyol-induced pheochromocytomas are not relevant to humans. Therefore, in the toxicological assessment of polyols, pheochromocytomas in rats are not considered relevant for the risk assessment.

Regarding the other types of substances the following principle will be followed: although at present there are no concrete indications for the occurrence of substance-induced pheochromocytomas in humans, on the basis of the current knowledge and information induction of pheochromocytomas in humans cannot be ruled out. In case of a substance-induced increase in pheochromocytomas in rats the following strategy is proposed:

General
In view of the enormous range in the spontaneous incidence of pheochromocytomas in the rat (and in the various factors affecting this), historical control data are considered of little importance in the assessment of pheochromocytomas. It is important, though, to check the reliability of the control group and the test conditions (food intake[3] and doses used). Furthermore, pheochromocytomas are mainly observed in the aging rat, and one should be alert to pheochromocytomas seen at a young age.
When a rat-specific working mechanism for the induction of pheochromocytomas has been shown or made plausible, an increase in the number of pheochromocytomas is not considered in the risk assessment (in analogy to polyol-induced pheochromocytomas).

[2] The statement 'ADI not specified' means that, on the basis of the available data (chemical, biochemical, toxicological, and other), the total daily intake of the substance, arising from its use at the level necessary to achieve the desired effect and from its acceptable background in food, does not, in the opinion of the Committee, represent a hazard to health. For this reason, and for the reasons stated in the individual evaluations, the establishment of an acceptable daily intake (ADI) is not deemed necessary.
[3] Rat studies have shown that dietary restriction reduces the incidence of pheochromocytomas [2,7]. This might, for example, lead to a 'lower' incidence of pheochromocytomas in a high-dose group in the case of a decreased food consumption.

In the mouse, pheochromocytomas are rarely observed, and are not induced by xenobiotics (except trichloroethane). Findings from a mouse study are always considered in risk assessments.

Relevance of pheochromocytomas in the rat
The following step-by-step scheme serves to determine whether an increase in pheochromocytomas in rats is relevant to the evaluation and/or risk assessment. To this end a distinction was made between genotoxic and non-genotoxic agents.

1. Genotoxic agents.
   It is not known whether pheochromocytomas are induced by genotoxic mechanisms. In the case of an agent with genotoxic qualities it cannot be ruled out, however, that such mechanisms are involved. If in the assessment of a genotoxic agent a toxicological relevant increase in the number of pheochromocytomas is observed in the rat, this finding should be given weight in the risk assessment.
2. Non-genotoxic substances.
   a) If the increase in pheochromocytomas in the rat is not significant and/or not dose-related, the increase is not given weight in the risk assessment.
   b) In case a significant and also dose-related increase in pheochromocytomas is observed, the agent should be considered as carcinogenic to the rat. Such a significant, dose-related increase in pheochromocytomas is given weight in the risk assessment. This also applies if a significant increase in pheochromocytomas is observed in the highest-dose group only.
   c) If data of a second species are available (e.g. from a mouse study) and proving negative, the increase in pheochromocytomas in rats is as a rule not given weight in the risk assessment.

   Note: An expert must be consulted if:
   - the increase in pheochromocytomas is the 'critical effect' in the risk assessment for establishing the NOAEL in a study.
   - in addition to a positive rat study, data of a second species are available and proving negative (item 2c relevance pheochromocytomas in the rat).

Relevance of pheochromocytomas in other species
Any increase in the number of pheochromocytomas in species other than the rat is considered relevant for the risk assessment.

3.7 Examples

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4 Assessment factors for human health risk assessment

Factsheet FSV-004/00 date 21-01-2001

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4.1 Introduction and problem formulation

In the standard procedure for deriving Human Limit Values (HLVs), such as the Acceptable Daily Intake (ADI), Tolerable Daily Intake (TDI), Reference Dose (RfD), or Health-Based Occupational Reference Value (HBORV) from animal study data or human data, the NOAEL is divided by a number of assessment factors according to the following equation:

$$ADI, TDI, RfD = \frac{NOAEL}{AF_1 . AF_2 . AF_3 \ldots\ldots}$$

The NOAEL (No-Observed-Adverse-Effect Level) is defined as the highest concentration or amount of a substance, found by experiment or observation, which causes no detectable adverse alteration of morphology, functional capacity, growth, development, or life span of the target organisms under defined conditions of exposure. The assessment factors (AFs) are meant to account for uncertainties in extrapolating from experimental data with laboratory animals or epidemiological data to the sensitive human being. These uncertainties pertain to inter- and intraspecies differences, differences in exposure time scale and others. In the absence of many substance-specific data, default assessment factors are often applied in risk assessment. A scientific justification for the size of these defaults is often lacking. However, the choice of such factors should be explained as transparently as possible.

The assessment factors are assumed to be independent from each other. Because of the multiplication, the standard method for deriving HLVs is generally considered to be conservative. Indeed, when each individual assessment factor by itself is regarded to reflect a worst case situation, their product, i.e. the overall assessment factor, will tend to be overly conservative. However, the degree of conservatism in the HLV in any particular assessment is unknown.

In addition, the uncertainty in the numerator, the NOAEL as an estimate of the 'true' No-Adverse-Effect Level ($NAEL_{true}$) in the animal is completely ignored. Depending on the study design, the NOAEL might be a poor estimate for this true (but unknown) dose below which the substance does not evoke any adverse effects. The potential deviation of the NOAEL from the $NAEL_{true}$ cannot be quantified. The latter uncertainty may be substantial and ignoring it may introduce an anti-conservative element in the derivation of HLVs (Slob and Pieters, 1998).

Slob and Pieters (1998) proposed a conceptual framework in which it is acknowledged that both the effect parameter and the assessment factors are uncertain and can best be described by lognormal distributions. The effect parameter would be a Critical Effect Dose (Benchmark Dose) derived from the dose-response data by regression analysis. This Critical Effect Dose is defined as the dose at which the average animal shows the (postulated) Critical Effect Size for a particular endpoint, below which there is no reason for concern. The distribution of the Critical Effect Dose can probabilistically be combined with distributions of assessment factors.

This concept was further operationalised by RIVM and TNO (Vermeire et al., 1999). Distributions for default assessment factors for a wide range of substances can be approached by distributions of NOAEL-ratios derived from comprehensive toxicological databases.

This factsheet will concentrate on the quantification of default distributions of the following human assessment factors:

- Interspecies factor for the extrapolation from the average experimental animal to the average human being;
- Intraspecies factor for the extrapolation from the average human being to the sensitive human being. Since this factor may depend on the population concerned, we will discuss factors for 'the general population' and for 'workers'.
- Exposure duration factor for the extrapolation from an experimental study of short duration to an experimental study of longer duration (semi-chronic to chronic, subacute to chronic, subacute to semi-chronic).

Based on the distributions selected, choices will be made for their application in day-to-day risk assessment of, at first, new and existing substances, and pesticides. The individual default assessment factors will be combined to a distribution for the overall default factor. This distribution of the overall default assessment factor will be used for:

1. The interpretation of the Margin Of Safety (MOS). The MOS is the margin between the NOAEL as derived from experimental or human studies and the expected human exposure. The MOS is, for instance, derived in the EU risk assessments for new and existing substances.
2. The comparison to the overall assessment factor as derived according to currently accepted methods. An overall assessment factor is used explicitly in the derivation of an ADI for pesticides.

4.2 Probabilistic distributions of human assessment factors

4.2.1 Interspecies factor

The interspecies factor is composed of two subfactors:

1. A default factor ($Interspecies_1$) accounting for systemic differences between species caused by differences in body size and related differences in basal metabolic rate.
2. A default distribution ($Interspecies_2$) accounting for variability in specific toxicokinetics and toxicodynamics.

$Interspecies_1$

Allometric scaling based on caloric demands is recommended to account for systemic differences between species after oral and dermal exposure (Hakkert et al., 1996; Health Council, 1985; Kalberlah and Schneider, 1998; Vermeire et al., 1999). Allometric scaling based on caloric demands is performed by assuming that doses scale with body weight to the power 0.75. This means that the laboratory animal dose rate (mg dose/ kg body weight) should be divided by an interspecies factor which is equal to (70/body weight animal in kg)$^{0.25}$. In this way the dose rate for the average person (70 kg), expressed as mg dose/ kg body weight is obtained. An overview of the scaling factors for different laboratory species is presented in 2.4 (Combination of factors, Table 8).
Please note:

- In the case of inhalatory exposure (in $mg.m^{-3}$) and dietary exposure (in $mg.kg_{food}$) the scaling factor equals unity (1) since ventilation rate and food intake can be assumed to scale with the basal metabolic rate. Therefore, in the conversion from the exposure metric

of rats ($mg.m^{-3}$ or $mg._{kgfeed}^{-1}$) to the same exposure metric of humans the difference in metabolic rate is already accounted for.

- If the HLV is derived from a diet study by recalculating the concentration in feed to an animal daily dose in $mg.kg_{bw}^{-1}.d^{-1}$, the extrapolation should incorporate a correction for basal metabolic rate.
- Allometric scaling should not be applied if the effects are independent of metabolic rate, e.g. in the case of local effects.

Interspecies$_2$

To account for the variability in toxicokinetics and toxicodynamics a default distribution is used. Ideally the default distribution should be based on a comparison of toxicity data in experimental animals and toxicity data in humans. Since data in humans are not available, a surrogate distribution based on historical analyses of 63 rat-dog NOAEL-ratios, 67 mouse-rat NOAEL ratios and 40 mouse-dog NOAEL ratios is proposed (Vermeire et al., 1999). Prior to analysis the NOAELs were adjusted by allometric scaling. Each interspecies comparison of NOAELs (rat vs. dog, mouse vs. rat and mouse vs. dog) resulted in a distribution with a geometric mean (GM) around unity. This agrees with the fundamental biological assumption that species are, on average, equally sensitive. Deviations for the mean are caused by differences in sensitivity towards individual substances as a consequence of specific kinetics and dynamics. For oral exposure, a default lognormal distribution with a GM of 1 and/or a geometric standard deviation (GSD) of 6 has been proposed for interspecies$_2$ (Vermeire et al., 1999). Based on reanalysis and an extension of this database (Rennen et al., 1999) it was concluded that the GSD could be lowered to 4.5. It was also noted that the geometric mean of the available mouse-rat ratios differed statistically significantly from one. However, a GM of 1 seems at present to be most plausible. Limited inhalatory data available suggest that this distribution may also be applied in the case of inhalatory exposure (Rennen et al., 1999).

In the literature other interspecies distributions have been proposed (Table 1). Baird et al. (1996) proposed a distribution of interspecies$_2$ based on an analysis which is comparable to the one above (GM=1). Price et al. (1997), Swartout et al. (1998) and Slob and Pieters (1998) proposed theoretical distributions of the composite interspecies factor considered to be consistent with the current use of the factor 10. They assumed this factor 10 to be conservative.

Table 1: Default distribution for interspecies extrapolation

Source	Interspecies$_2$		Composite interspecies factor		Remark
	GM	GSD	GM	GSD	
Baird et al., 1996	1	4.9			database derived
Slob & Pieters, 1998			5	1.3	theoretical (10 = P99)
Swartout et al., 1998			1* + 2.1	2	theoretical
Price et al., 1997			1* + 2.1	2	theoretical
RIVM/TNO**	1	4.5			database derived

* The whole distribution is increased by one (shifted to the right) by these authors, as they believe that the interspecies factor should not be smaller than unity

** Based on Vermeire et al. (1999) and Rennen et al. (1999)

4.2.2 Intraspecies factor

General Population

Intraspecies variation between humans is due to a number of biological factors, such as age, sex, genetic composition and nutritional status. For decades a default factor of 10 for the extrapolation from the average to the sensitive human being has been used to derive human limit values (HLV). Calabrese (1985) who argued that a factor of 10 would be sufficient to protect the majority (up to 80-95%) of the human population against adverse health effects supported the default factor of 10.

A few attempts have been made to investigate the human interindividual variation by data analysis. Hattis et al. (1987) investigated the total variation in pharmacokinetic behaviour of 49 pharmaceuticals in healthy adults and concluded that a tenfold difference in the pharmacokinetic parameters would correspond to 2.5-9 standard deviations in populations of normal healthy adults. Reanalysis of the data of Hattis et al. showed that for the plasma half-life time the variation between individuals was quite small. Defining the intraspecies factor as the ratio of the P_{50} and P_{05} resulted in a factor of 1.4 (Schaddelee, 1997).
Although from the above analysis it appears that a factor of 10 will be sufficient for pharmacokinetic variation, the real median to sensitive human variability is underestimated, since one should take into account that (i) variation also exist in pharmacodynamics and (ii) that only data from healthy volunteers were available. Renwick (1993a,b) analysed interindividual differences of healthy volunteers and patients by comparing the maximum and mean values of pharmacokinetic parameters and the minimum and mean values of pharmacodynamic parameters. Based on this analysis he proposed to subdivide the factor of 10 into a factor of 4 for pharmacokinetic differences and a factor of 2.5 for pharmacodynamic differences. Re-analysis of the Renwick data by using distributions instead of ratios max/mean and min/mean gave comparable results (Schaddelee, 1997). The results of Renwick's analysis have been adopted by the IPCS (IPCS, 1994).

Based on an analysis of the available human data, Kalberlah and Schneider (1998) proposed an intraspecies factor of 25 for the general population, composed of a factor of 8 accounting for toxicokinetic variation and enzyme polymorphism's, and a factor of 3 accounting for toxicodynamic variation. For workers they considered a total factor of 5 to account for both inter and intraspecies variation (after adjustment for differences in metabolic size). However, the combined factor for workers accounting for both inter and intraspecies variation was not adequately explained.

Several probabilistic distributions have been proposed (Table 2). Baird et al. (1996) proposed a distribution on the basis of acute toxicity data on heterogeneity in rats and on the basis of assumptions on the unknown difference in heterogeneity between rats and humans (GM = 2.7 and GSD = 2.3 with rats and humans equally heterogeneous and GM = 5.3 and GSD = 2.1 with humans 1.5 more heterogeneous than rats). This approach is considered invalid: heterogeneity in inbred rat strains is considered not relevant for humans and the quantal response a poor and crude measure. Price et al. (1997), Swartout et al., (1998) and Slob and Pieters (1998) proposed distributions considered to be consistent with the current use of the default factor of 10. They assumed this factor 10 to be conservative.
It is concluded that currently no adequate proposal for a database-derived distribution of the intraspecies factor can be made. Therefore, for the time being, a distribution consistent with the default value of 10 as proposed by Slob and Pieters (1998) will be used.

Table 2: Default distribution for intraspecies extrapolation for the general population

Source	GM	GSD	Remark
Slob & Pieters, 1998	1* + 3	1.6	theoretical
Baird et al., 1996	2.7	2.3	database derived**
Swartout et al., 1998	1* + 2.1	2	theoretical
Price et al., 1997	1* + 2.1	2	theoretical
RIVM/TNO***	1* + 3	1.6	theoretical

* The whole distribution is increased by one (shifted to the right) since by definition the intraspecies factor cannot be smaller than unity
** Assumes equal heterogeneity in rats and humans
*** Based on Slob & Pieters (1998)

Workers

No adequate proposal for a database-derived distribution of the intraspecies factor can be made for workers. Therefore, for the time being, a distribution consistent with the default value for workers of 3 - considered to be conservative - is proposed in parallel with the approach of Slob andPieters (1998). This distribution is characterised by a GM of 1+1.4 and a GSD of 1.2, resulting in a P1 of 1 and a P99 of 3 (Table 3).

Table 3: Default distribution for intraspecies extrapolation for workers

Source	GM	GSD	Remark
RIVM/TNO	1* + 1.4	1.2	theoretical

* The whole distribution is increased by one (shifted to the right) since by definition the intraspecies factor cannot be smaller than unity

4.2.3 Exposure duration factor

In general, the proposed distributions of the exposure duration factors are based on historical analyses of ratios of oral NOAELs (e.g. the ratio of a semi-chronic $NOAEL_{rat}$ and the chronic $NOAEL_{rat}$). It is assumed that although the distributions are derived from oral data, they can also be applied to systemic effects caused by inhalatory or dermal exposure, after estimation of the systemic dose.

Semi-chronic to chronic exposure duration factor

Based on a review of published data sets with 9-149 pairs of NOAELs, a default lognormal distribution with a GM of 2 and a GSD of 4 has been proposed (Vermeire et al., 1999). Taking into account another, detailed study with 70 pairs of NOAELs (Groeneveld et al., 1998), the GSD is adjusted to 3.5, the GM remaining 2.

Other distributions have been proposed. Baird et al. (1996) proposed a distribution based on two pooled data sets of both oral and inhalation studies (GM = 2.1 and GSD = 2.1). Swartout et al. (1998), Price et al., (1997) and Slob and Pieters (1998) assumed distributions considered to be consistent with the current use of the default factor of 10 (Table 4). They assumed this factor 10 to be conservative.

Table 4: Default distributions for the semi-chronic to chronic exposure duration factor

Source	GM	GSD	Remark
Baird et al., 1996	2	2.1	Database derived
Slob & Pieters, 1998	1.5	2.3	Database/theoretical
Swartout et al., 1998	1* + 2.1	2	(P99)
Price et al., 1997	1* + 2.1	2	Theoretical
RIVM/TNO**	2	3.5	Theoretical Database derived

* The whole distribution is increased by one (shifted to the right) by these authors as they believe that the exposure duration factor should not be smaller than unity

** Based on Vermeire et al. (1999) and Groeneveld et al. (1998)

Subacute to chronic exposure duration factor

Vermeire et al. (1999) concluded to a default lognormal distribution with a GM of 4 and a GSD of 4 from a modest number of 3 data sets with 20-71 pairs of NOAELs each. Based on yet another, detailed study with 35 pairs of NOAELs (Groeneveld et al., 1998), it was concluded to adjust the GM to 5 and the GSD to 3.5 (Table 5).

No other distributions have been proposed in the scientific literature.

Table 5: Default distribution for the subacute to chronic exposure duration factor

Source	GM	GSD	Remark
RIVM/TNO*	5	3.5	database derived

* Based on Vermeire et al. (1999) and Groeneveld et al. (1998)

Subacute to semi-chronic exposure duration factor

This factor is applied in occupational risk assessments. Vermeire et al. (1999) concluded to a default lognormal distribution with a GM of 2 and a GSD of 4 from one study with a data set of 35 pairs of NOAELs (Groeneveld et al., 1998).

No other distributions have been proposed in the scientific literature.

Table 6: Default distribution for the subacute to semi-chronic exposure duration factor

Source	GM	GSD	Remark
RIVM/TNO*	2	4	theoretical

* Based on Vermeire et al. (1999) and Groeneveld et al. (1998)

4.2.4 Combination of factors

In the standard procedure for deriving HLVs, various assessment factors are multiplied to obtain an overall assessment factor. However, multiplication of assessment factors implies a piling up of worst case assumptions: the probability of simultaneous occurrence of worst case

situations for the same chemical will be smaller than that of a single worst case situation to occur. Therefore, the more extrapolation steps are taken into account, the higher the level of conservatism.

The piling-up of worst-case assumptions can be avoided by using probability distributions. In this method each assessment factor is considered uncertain and characterised as a random variable with a distribution. Propagation of the uncertainty can be evaluated using Monte Carlo simulation yielding a distribution of the overall assessment factor. This method requires characterisation of the distribution of each assessment factor (see previous chapters). As a first approach it is assumed that all factors are independent.

Combining the distributions as proposed for the individual assessment factors using Monte Carlo simulation yields the following lognormal overall distributions:

Table 7: Default distributions of combined factors

Population	Combination*	GM	GSD	P90	P95
General population	$inter_2$ x intra	4	4.7	30	53
	$inter_2$ x intra x subac/c	20	7.4	264	551
	$inter_2$ x intra x semic/c	8	7.5	101	206
Workers	$inter_2$ x intra	2.4	4.5	16	28
	$inter_2$ x intra x subac/c	12	7.1	150	302
	$inter_2$ x intra x semic/c	4.8	7.1	60	121
	$inter_2$ x intra x subac/semic	4.8	7.8	67	139

* $inter_2$ = $interspecies_2$; intra = intraspecies; subac = subacute; semic = semi-chronic; c = chronic

Please note that these distributions have not yet been multiplied with the allometric scaling factor $Interspecies_1$ (Table 8), which is species dependent.

Table 8: Scaling factor ($Interspecies_1$) based on caloric demands (i.e. $BW^{0.75}$):

Species	Body weight (kg)	$Interspecies_1$*
mouse	0.025	7.3
rat	0.100	5.1
rat	0.250	4.1
guinea pig	0.750	3.1
rabbit	2	2.4
monkey	5	1.9
dog	15	1.5

* Calculated according to the formula: $(70/\text{body weight animal in kg})^{0.25}$

The final combination of the assessment factors for the different species is presented in 3 (Conclusion and RIVM/TNO strategy).

4.2.5 Limitations

It should be recognised that all distributions proposed are based on analyses of historical data, i.e. NOAEL ratios. The use of these data has the following shortcomings:

1. The criteria used by constructing databases are not always transparent and NOAEL-ratios may have been assessed without knowing the quality of the underlying data.

2. The uncertainty in the NOAEL as an estimate of the NAEL is unknown. If ratios of NAELs would have been used, the distributions would have been less wide (i.e. smaller GSD).
3. Although the proposed default distributions are considered sufficiently founded to justify their application in human risk assessment, further research on the basis of larger databases is still considered necessary, especially with regard to the intraspecies distribution.
4. In the derivation of an interspecies assessment factor from NOAEL-ratios, it is assumed that variability between laboratory animals represents animal-human variability.

4.3 Conclusion and RIVM/TNO strategy

The present human risk characterisation for new and existing substances is based on a comparison between an estimated or measured human exposure value and the NOAEL or LOAEL, resulting in a Margin Of Safety. This MOS needs interpretation on the basis of assessment factors. Alternatively, for pesticides the human exposure value is compared to the HLV, in this case the ADI, derived from the NOAEL (LOAEL) using assessment factors.

Slob and Pieters (1998) proposed a conceptual framework in which it is acknowledged that both the effect parameter and the assessment factors are uncertain and can best be described by lognormal distributions. This concept was further operationalised by RIVM and TNO and it was decided to develop the use of probabilistic assessment factors as a first step towards further national and international harmonisation (Vermeire et al., 1999). RIVM and TNO decided on the nature of the distribution of several assessment factors.

To facilitate international consensus on the assessment factors and probabilistic risk assessment methodology, the proposed distributions will be applied in risk assessments produced at RIVM or TNO. The overall probabilistic assessment factor derived will be compared to the assessment factors currently used in the interpretation of the MOS and in the derivation of an HLV. This analysis will be performed in a separate Annex to the risk assessments performed in the RIVM and TNO Institutes. A format of the risk assessment to be published in an Annex is presented in Annex 2 of this fact sheet.

The default distributions for each species can be derived from the distributions in Table 7 and the allometric scaling factors in Table 8. Table 9 summarises the results of these calculations. If, besides the allometric scaling factor, additional point estimates are involved, e.g. a factor for the quality of the database, the numbers in columns 3, 4, and 5 should be multiplied accordingly and the numbers in the last column should be estimated using the formulae in Annex I.
It should be noted that for new chemical substances and existing substances the maximum default value has been set at 1000. In the calculations below this deviation has not been taken into account.

Table 9: Default distributions of the overall assessment factors for the general population and for workers[*]

		GM	P90	P95	P of default**
Mouse (20g)(allometric factor = 7)					
General population	Inter x intra	28	210	371	79 (10x10)
	Inter x intra x semic/c	56	707	1442	92 (10x10x10)
	Inter x intra x subac/c	140	1848	3857	98 (10x10x10x10)
Workers	Inter x intra	17	112	196	81 (3x7x3)
	Inter x intra x semic/c	34	420	847	93 (3x7x3x10)
	Inter x intra x subac/c	84	1050	2114	97 (3x7x3x50)
	Inter x intra x subac/semic	34	469	969	92 (3x7x3x10)
Rat (250 g) (allometric factor = 4)					
		GM	P90	P95	P of default**
General population	Inter x intra:	16	120	212	88 (10x10)
	Inter x intra x semic/c	32	404	824	99 (10x10x10)
	Inter x intra x subac/c	80	1056	2204	99 (10x10x10x10)
Workers	Inter x intra	10	64	112	80 (3x4x3)
	Inter x intra x semic/c	19	240	484	93 (3x4x3x10)
	Inter x intra x subac/c	48	600	1208	95 (3x4x3x50)
	Inter x intra x subac/semic	19	268	556	92 (3x4x3x10)
Guinea pig (750 g) (allometric factor = 3)					
		GM	P90	P95	P of default**
General population	Inter x intra:	12	90	159	92 (10x10)
	Inter x intra x semic/c	24	303	618	97 (10x10x10)
	Inter x intra x subac/c	60	792	1653	99 (10x10x10x10)
Workers	Inter x intra	7	48	84	81 (3x3x3)
	Inter x intra x semic/c	14	180	363	94 (3x3x3x10)
	Inter x intra x subac/c	36	450	906	95 (3x3x3x50)
	Inter x intra x subac/semic	14	201	417	92 (3x3x3x10)
Rabbit (2 kg) (allometric factor = 2.4)					
		GM	P90	P95	P of default**
General population	Inter x intra:	10	72	127	93 (10x10)
	Inter x intra x semic/c	19	242	494	98 (10x10x10)
	Inter x intra x subac/c	48	634	1322	99 (10x10x10x10)
Workers	Inter x intra	6	38	67	80 (3x2.4x3)
	Inter x intra x semic/c	11	144	290	94 (3x2.4x3x10)
	Inter x intra x subac/c	29	360	725	95 (3x2.4x3x50)
	Inter x intra x subac/semic	11	161	334	92 (3x2.4x3x10)
Monkey (5 kg)(allometric factor = 2)					
		GM	P90	P95	P of default**
General population	Inter x intra:	8	60	106	95 (10x10)
	Inter x intra x semic/c	16	202	412	99 (10x10x10)
	Inter x intra x subac/c	40	528	1102	99 (10x10x10x10)
Workers	Inter x intra	5	32	56	80 (3x2x3)
	Inter x intra x semic/c	10	120	242	93 (3x2x3x10)
	Inter x intra x subac/c	24	300	604	95 (3x2x3x50)
	Inter x intra x subac/semic	10	134	278	92 (3x2x3x10)

Table 9 (continued)

Dog (15 kg) (allometric factor = 1.4)					
		GM	P90	P95	P of default**
General population	Inter x intra:	5.6	42	74	97 (10x10)
	Inter x intra x semic/c	11.2	141	288	99 (10x10x10)
	Inter x intra x subac/c	28	370	771	99 (10x10x10x10)
Workers	Inter x intra	3	22	39	83 (3x1.4x3)
	Inter x intra x semic/c	7	84	169	93 (3x1.4x3x10)
	Inter x intra x subac/c	17	210	423	95 (3x1.4x3x50)
	Inter x intra x subac/semic	7	94	195	92 (3x1.4x3x10)

* inter = $inter_1$ x $inter_2$; intra = intraspecies; subac = subacute; semic = semi-chronic; c = chronic

** 'P of default' is the percentile of the defaults currently used at the RIVM for the general population and by TNO for workers; these current default values are shown between brackets. Note that these percentiles have been estimated using the formulae in Annex I, though these formulae actually only apply to lognormal distributions whereas the intraspecies distribution is a shifted lognormal.

4.4 Example

Risk assessment of Substance X (general population and workers)

Critical study

NOAEL : 4 $mg.kg_{bw}^{-1}.d^{-1}$
Species : rat
Exposure duration: : semi-chronic
Exposure route : oral

Exposure of Human target population (general population)

Estimated exposure : 20 $\mu g.kg_{bw}^{-1}.d^{-1}$ (applying EUSES)
Exposure duration : chronic
Exposure route : oral

Exposure of Human target population (workers)

Estimated exposure1 : 1400 $\mu g.kg_{bw}^{-1}.d^{-1}$ (applying EASE: based on a body weight of 70 kg, a concentration of 50 $\mu g/cm^2$, and an exposed surface area of 2000 cm^2)
Exposure duration: : chronic
Exposure route : dermal

Estimated exposure 2 : 4 $\mu g.kg_{bw}^{-1}.d^{-1}$ (applying EASE: based on a concentration of 29 $\mu g/m^3$, a ventilation rate of 10 m^3/day, and a body weight of 70 kg)
Exposure duration: : chronic
Exposure route : inhalation

Extrapolation steps

Interspecies* : rat-human (including allometric scaling factor)
Intraspecies* : to sensitive general population/workers
Exposure period* : semi-chronic to chronic
LOAEL to NOAEL : no
Route-to-route extrapolation : yes for workers (oral to dermal and inhalation)

* These extrapolation steps are incorporated in the combined default distributions. Point estimates are to be used for the other extrapolation steps.

1. Extrapolation by using current default assessment factors

In this approach the minimal MOS is equal to the overall assessment factor. The NOAEL divided by the overall assessment factor can be considered as an HLV to be used in risk assessment.
General population
Applying the current assessment factors of 10 for interspecies differences, 10 for intraspecies differences and 10 for the extrapolation from a semi-chronic NOAEL to a chronic NOAEL the minimal MOS should be 1000.

Workers
Applying the current assessment factors of 3x4 for interspecies differences, 3 for intraspecies differences, 10 for the extrapolation from a semi-chronic NOAEL to a chronic NOAEL, and 2 for route to route extrapolation (based on an oral absorption of 50% and a dermal and inhalatory absorption of 100%) the minimal MOS should be 720.

2. Extrapolation by using the combined default distribution

In this approach the minimal MOS is, by choice, equal to the 95th percentile of the combined default distribution (if applicable combined with point estimates for additional uncertainty factors). The ratio of the NOAEL (or LOAEL) and the 95th percentile can be considered as an HLV to be used in risk assessment.
General population
Based on the distribution for:
- the interspecies variability, including the allometric scaling factor of 4 (point estimate) for a 250 g rat;
- the intraspecies variability;
- the extrapolation from the semi-chronic to the chronic time scale;

the minimal MOS should be 824 (see Table 9 of factsheet).
Workers
Based on the distribution for:
- the interspecies variability, including the allometric scaling factor of 4 (point estimate) for a 250 g rat;
- the intraspecies variability;
- the extrapolation from the semi-chronic to the chronic time scale;
- a factor of 2 (point estimate) for route-to-route extrapolation,

The minimal MOS should be 968 (see Table 9 of Factsheet)

3. Risk characterisation

General population
The estimated MOS can be calculated as the ratio of the NOAEL and the estimated actual exposure, and equals to 200.
The outcome of the above mentioned three approaches are summarised in table 10.

Table 10: Comparison of a risk assessment of substance X by the default assessment factor approach and by the combined default distribution (oral exposure)

Parameter	Default factors	Combined default distribution	Risk characterisation
MOS-value	1000(minimal MOS)	824(minimal MOS)	200 (estimated MOS)
Risk level*	1 %	5 % (by choice)	19 %

.* The probability that adverse effects occur at the HLV (for the default assessment factor and the combined default distribution approach) or at the estimated actual exposure (in the risk characterisation) assuming that no adverse effects occur at the NOAEL chosen. Risk level for defaults: see Table 9 (default factor of 1000 is at P99). Risk level for the risk characterisation: use GMs of Table 9, the GSDs of Table 7, and the Formularium of Annex I.

On the basis of Table 10 a risk characterisation for substance X can be made either by an assessment factor approach or by evaluation of the estimated MOS. The outcome of the risk assessment using the current default assessment factor approach can be compared with the combined default distribution approach by comparing the risk levels in the respective columns. The probability is maximally 19% that adverse effects occur in a sensitive part of the population at the estimated actual exposure to substance X.

Workers
The estimated MOS can be calculated as the ratio of the NOAEL and the estimated actual exposure, and equals to 2.8 for dermal exposure and 1000 for inhalation exposure.

The outcome of the above mentioned three approaches are summarised in table 11 for dermal exposure and 12 for inhalation exposure.

Table 11: Comparison of a risk assessment of substance X by the default assessment factor approach and by the combined default distribution (dermal exposure)

Parameter	Default factors	Combined default distribution	Risk characterisation
MOS-value	720(minimal MOS)	968(minimal MOS)	2.8 (estimated MOS)
Risk level*	7 %	5 % (by choice)	60 %

.* The probability that adverse effects occur at the HLV (for the default assessment factor and the combined default distribution approach) or at the estimated actual exposure (in the risk characterisation) assuming that no adverse effects occur at the NOAEL chosen. Risk level for defaults: see Table 9 (the minimal MOS, i.e. the default factor of 360, combined with factor of 2 for route-to-route extrapolation, is at P93). The minimal MOS for the combined default distribution is 484 (Table 9), combined with the same factor 2. Risk level for the risk characterisation: use GMs of Table 9, the GSDs of Table 7, and the Formularium of Annex I.

On the basis of Tables 11 and 12 a risk characterisation for substance X can be made either by an assessment factor approach or by evaluation of the estimated MOS. The outcome of the risk assessment using the current default assessment factor approach can be compared with the combined default distribution approach by comparing the risk levels in the respective columns. For dermal exposure, the probability is 60% that adverse effects occur in a sensitive part of the population at the estimated actual exposure to substance. This probability is negligible for inhalation.

Table 12: Comparison of a risk assessment of substance X by the default assessment factor approach and by the combined default distribution (inhalation exposure)

Parameter	Default factors	Combined default distribution	Risk characterisation
MOS-value	720(minimal MOS)	968(minimal MOS)	1000(estimated MOS)
Risk level*	7 %	5 % (by choice)	1 %

.* The probability that adverse effects occur at the HLV (for the default assessment factor and the combined default distribution approach) or at the estimated actual exposure (in the risk characterisation) assuming that no adverse effects occur at the NOAEL chosen. Risk level for defaults: see Table 9 (the minimal MOS, i.e. the default factor of 360, combined with factor of 2 for route-to-route extrapolation, is at P93). The minimal MOS for the combined default distribution is 484 (Table 9), combined with the same factor 2. Risk level for the risk characterisation: use GMs of Table 9, the GSDs of Table 7, and the Formularium of Annex I.

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Annex I: Formulae

- Lognormal distributions are characterised by a dispersion factor (k) defined such that e.g. 95% of the values of a stochastic variable (X) is within a factor of k from the median, $M(X)$

$$p\left(\frac{M(X)}{k} > X > kM(X)\right) = 0.95$$

and

$$k = \exp(1.96 s_{\ln X})$$

- Geometric mean of the lognormal distribution

$$GM = \exp(\frac{1}{n}\sum_{i=1}^{n} \ln X_i))$$

- Sample variance of log-entities

$$s^2_{\ln X} = \frac{1}{n-1}\sum_{i=1}^{n}(\ln X_i - \ln GM)^2$$

- Geometric standard deviation

$$GSD = \exp(s_{\ln X})$$

- 95th percentile $P_{0.95}$ for a lognormal distribution

$$P_{0.95} = GM \cdot GSD^{z_{0.95}} \text{ and } z_{0.95} = \ln k / \ln(s_{lnX})$$

M: median

GM: geometric mean

k: dispersion factor

n: number of observations

X_i: lognormally distributed ith observation (e.g. NOAEL)

s_{lnX} : sample standard deviation of lognormally distributed X

$z_{0.95}$: 95th percentile of the standard normal distribution

Annex IIa: Model for the risk assessment of new and existing substances on the basis of probabilistic assessment factors

The present human risk characterisation is based on a comparison between an estimated or measured human exposure value and the NOAEL, resulting in a Margin Of Safety. This MOS needs interpretation on the basis of assessment factors.

In the application of assessment factors all variability and uncertainty involved in the extrapolation from experimental data to a limit value for the sensitive human should be considered. Two approaches prevail:
1. Application of substance-specific assessment factors,
and in the absence of sufficient substance specific data:
2. Application of default assessment factors.
The default factors currently used in the latter method have been subject to research into their validity, which has resulted in estimations of the default distributions for the interspecies factor, the intraspecies factor and the factor for the extrapolation from an experimental study of short duration to one of longer duration (Vermeire et al., 1999; Rennen et al., 1999). These distributions can be used to explore further the interpretation of the MOS or the overall assessment factor used in extrapolation procedures (Slob and Pieters, 1998).
The following risk characterisation of Substance X is based on the distributions in Table A.

Table A: Default distributions for assessment factors

Factor	GM	GSD	Remark
Interspecies	1*	4.5	database derived
Intraspecies-general population	1** + 3	1.6	theoretical based on factor 10
Intraspecies-workers	1** + 1.4	1.2	theoretical based on factor 3
Time factor: semi-chronic to chronic	2	3.5	database derived
Time factor: subacute to chronic	5	3.5	database derived
Time factor: subacute to semi-chronic	2	4	database derived

* This factor needs to be multiplied by an allometric scaling factor based on differences in caloric demand (mouse 7; rat 4; guinea pig 3; rabbit 2.4; monkey 1.9; dog 1.5)
** The whole distribution is increased by one (shifted to the right) since by definition the intraspecies factor cannot be smaller than unity

It is noted that some uncertainty factors (e.g. route-to-route extrapolation, extrapolation from a LOAEL to a NOAEL) are not incorporated in the combined default distribution. For these factors, if applicable, point estimates will be used. For all factors hold that a substance-specific point estimate is preferred to a default distribution or point estimate.

Risk assessment of Substance X (general population/workers)

Critical study
NOAEL (or LOAEL) : … $mg.kg_{bw}^{-1}.d^{-1}$ *or* mg/m^3
Species : …
Exposure duration: : …
Exposure route : oral/dermal/inhalatory

Exposure of Human target population (workers/general population)
Estimated exposure : .. $mg.kg_{bw}^{-1}.d^{-1}$ *or* mg/m^3
Exposure duration : …
Exposure route : oral/dermal/inhalation (more than one possible)

Extrapolation steps
Interspecies* : animal species-human (in/excluding allometric scaling factor)
Intraspecies* : to sensitive general population/worker
Exposure period* : subacute/semi-chronic to semi-chronic/chronic
LOAEL to NOAEL : yes/no
Route-to-route extrapolation : yes/no (correction for absorption: …)

* These extrapolation steps are incorporated in the combined default distributions. Point estimates are to be used for the other extrapolation steps.

1. Extrapolation by using current default assessment factors

In this approach the minimal MOS is equal to the overall assessment factor. The NOAEL divided by the overall assessment factor can be considered as an HLV to be used in risk assessment.
Applying the current assessment factors of .. for interspecies differences, .. for intraspecies differences, .. for the extrapolation from a subacute/semi-chronic NOAEL to a semi-chronic/chronic NOAEL, and … for other uncertainties (e.g. route-to-route extrapolation, extrapolation from a LOAEL to a NOAEL) the minimal MOS should be ..

2. Extrapolation by using the combined default distribution

In this approach the minimal MOS is, by choice, equal to the 95^{th} percentile of the combined default distribution (if applicable combined with point estimates for additional uncertainty factors). The ratio of the NOAEL and the 95^{th} percentile can be considered as an HLV to be used in risk assessment.
Based on the distribution for:
- the interspecies variability, including the allometric scaling factor of .. (point estimate) for a .. g rat/mouse/dog/monkey/guinea pig/rabbit;
- the intraspecies variability;
- the extrapolation from the subacute/semi-chronic to the semi-chronic/chronic time scale;
- a factor of . (point estimate) for … (other factors (see above) + explanation)

the minimal MOS should be .. (see Table 9 of factsheet).

3. Risk characterisation

The estimated MOS can be calculated as the ratio of the NOAEL (or LOAEL) and the estimated actual exposure, and equals to …

The outcome of the above mentioned three approaches are summarised in table B.

Table B: Comparison of a risk assessment of substance X by the default assessment factor approach and by the combined default distribution (... (route of exposure))

Parameter	Default factors	Combined default distribution	Risk characterisation
MOS-value	…(minimal MOS)	… (minimal MOS)	… (estimated MOS)
Risk level*	100 – Y %	5 % (by choice)	100 – Z %

* The probability that adverse effects occur at the given exposure (i.e. HLV for the default assessment factor and the combined default distribution approach, or the estimated actual exposure in the risk characterisation) assuming that no adverse effects occur at the NOAEL chosen. Risk level for defaults: see Table 9 (the minimal MOS i.e. the default factor of …., [combined with a factor of . for .,] is at P.). Risk level for the risk characterisation: use GMs of Table 9, the GSDs of Table 7, and the Formularium of Annex I in Factsheet 004/00 Assessment Factors.

On the basis of Table B a risk characterisation for substance X can be made either by an assessment factor approach or by evaluation of the estimated MOS. The outcome of the risk assessment by the traditional default assessment factor approach can be compared with the combined default distribution approach by comparing the risk levels in the respective columns. The probability is maximally 100-Z% that adverse effects occur in a sensitive part of the population at the estimated actual exposure to substance X.

Key References

Rennen, M.A.J., Hakkert, B.C., Stevenson, H., Bos, P.M.J. (1999). Interspecies extrapolation: A quantitative analysis of historical toxicity data. Zeist, The Netherlands, TNO Nutrition and Food Research Institute. TNO-report No. V99.210. (Accepted for publication in Comments on Toxicology, Special issue on Noncancer Health Risk Assessment in the 21st Century).

Slob, W. and Pieters, M.N. (1998) A probabilistic approach for deriving acceptable human intake limits and human risks from toxicological studies: general framework, Risk Analysis18: 787-798.

Vermeire, T.G., Stevenson, H., Pieters, M.N., Rennen, M., Slob, W. and Hakkert, B.C. (1999) Assessment factors for human health risk assessment: a discussion paper. Crit. Reviews Toxicol. 29(5): 439-490

Annex IIb: Model for the risk assessment of pesticides on the basis of probabilistic assessment factors

The present human risk characterisation is based on a comparison between the human exposure value and the HLV, in this case the ADI or AOEL (Acceptable Operator Exposure Limit), derived from the NOAEL using assessment factors.

In the application of assessment factors all variability and uncertainty involved in the extrapolation from experimental data to a limit value for the sensitive human should be considered. Two approaches prevail:
1. Application of substance-specific assessment factors,
and in the absence of sufficient substance specific data:
2. Application of default assessment factors.
The default factors currently used in the latter method have been subject to research into their validity, which has resulted in estimations of the default distributions for the interspecies factor, the intraspecies factor and the factor for the extrapolation from an experimental study of short duration to one of longer duration (Vermeire et al., 1999; Rennen et al., 1999). These distributions can be used to explore further the interpretation of the overall assessment factor used in extrapolation procedures (Slob and Pieters, 1998).
The following risk characterisation of Substance X is based on the distributions in Table A.

Table A: Default distributions for assessment factors

Factor	GM	GSD	Remark
Interspecies	1*	4.5	database derived
Intraspecies-general population	1** + 3	1.6	theoretical based on factor 10
Intraspecies-workers	1** + 1.4	1.2	theoretical based on factor 3
Time factor: semi-chronic to chronic	2	3.5	database derived
Time factor: subacute to chronic	5	3.5	database derived
Time factor: subacute to semi-chronic	2	4	database derived

* This factor needs to be multiplied by an allometric scaling factor based on differences in caloric demand (mouse 7; rat 4; guinea pig 3; rabbit 2.4; monkey 1.9; dog 1.5)
** The whole distribution is increased by one (shifted to the right) since by definition the intraspecies factor cannot be smaller than unity

It is noted that some uncertainty factors (e.g. route-to-route extrapolation, extrapolation from a LOAEL to a NOAEL) are not incorporated in the combined default distribution. For these factors, if applicable, point estimates will be used. For all factors hold that a substance-specific point estimate is preferred to a default distribution or point estimate.

Risk assessment of Substance X (general population/workers)

Critical study
NOAEL (or LOAEL) : … $mg.kg_{bw}^{-1}.d^{-1}$ *or* mg/m^3
Species : …
Exposure duration: : …
Exposure route : oral/dermal/inhalatory
Exposure of Human target population (workers/general population)
Estimated exposure : .. $mg.kg_{bw}^{-1}.d^{-1}$ *or* mg/m^3
Exposure duration : …

Exposure route : oral/dermal/inhalation (more than one possible)

Extrapolation steps

Interspecies*	: animal species-human (in/excluding allometric scaling factor)
Intraspecies*	: to sensitive general population/worker
Exposure period*	: subacute/semi-chronic to semi-chronic/chronic
LOAEL to NOAEL	: yes/no
Route-to-route extrapolation	: yes/no (correction for absorption: …)

* These extrapolation steps are incorporated in the combined default distributions. Point estimates are to be used for the other extrapolation steps.

1. Extrapolation by using current default assessment factors

The NOAEL divided by the overall assessment factor can be considered as the ADI/AOEL, to be used in risk assessment.
Applying the current assessment factors of .. for interspecies differences, .. for intraspecies differences, .. for the extrapolation from a subacute/semi-chronic NOAEL to a semi-chronic/chronic NOAEL, and … for other uncertainties (e.g. route-to-route extrapolation, extrapolation from a LOAEL to a NOAEL) the ADI/AOEL should be ..

2. Extrapolation by using the combined default distribution

The ratio of the NOAEL and, by choice, the 95th percentile can be considered as the ADI/AOEL to be used in risk assessment.
Based on the distribution for:

- the interspecies variability, including the allometric scaling factor of .. (point estimate) for a .. g rat/mouse/dog/monkey/guinea pig/rabbit;
- the intraspecies variability;
- the extrapolation from the subacute/semi-chronic to the semi-chronic/chronic time scale;
- a factor of . (point estimate) for … (other factors (see above) + explanation)

the ADI/AOEL should be .. (see Table 9 of factsheet).

3. Risk characterisation

The ratio of the NOAEL (or LOAEL) and the estimated actual exposure is …

The outcome of the above mentioned three approaches are summarised in table B.

On the basis of Table B a risk characterisation for substance X can be made. The outcome of the risk assessment by the traditional default assessment factor approach can be compared with the combined default distribution approach by comparing the risk levels in the respective columns. The probability is maximally 100-Z% that adverse effects occur in a sensitive part of the population at the estimated actual exposure to substance X.

Table B: Comparison of a risk assessment of substance X by the default assessment factor approach and by the combined default distribution (... (route of exposure))

Parameter	Default factors	Combined default distribution	Risk characterisation
Overall factor	…	…	…
Risk level*	100 – Y %	5 % (by choice)	100 – Z %

* The probability that adverse effects occur at the given exposure (i.e. ADI/AOEL for the default assessment factor and the combined default distribution approach, or the estimated actual exposure in the risk characterisation) assuming that no adverse effects occur at the NOAEL chosen. Risk level for defaults: see Table 9 (the minimal MOS i.e. the default factor of …., [combined with a factor of . for .,] is at P.). Risk level for the risk characterisation: use GMs of Table 9, the GSDs of Table 7, and the Formularium of Annex I in Factsheet 004/00 Assessment Factors.

Key References

Rennen, M.A.J., Hakkert, B.C., Stevenson, H., Bos, P.M.J. (1999). Interspecies extrapolation: A quantitative analysis of historical toxicity data. Zeist, The Netherlands, TNO Nutrition and Food Research Institute. TNO-report No. V99.210. (Accepted for publication in Comments on Toxicology, Special issue on Noncancer Health Risk Assessment in the 21st Century).

Slob, W. and Pieters, M.N. (1998) A probabilistic approach for deriving acceptable human intake limits and human risks from toxicological studies: general framework, Risk Analysis18: 787-798.

Vermeire, T.G., Stevenson, H., Pieters, M.N., Rennen, M., Slob, W. and Hakkert, B.C. (1999) Assessment factors for human health risk assessment: a discussion paper. Crit. Reviews Toxicol. 29(5): 439-490

5 Delayed Neurotoxicity/NTE-inhibition

Factsheet FSV-005/00 date 18-07-2000

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5.1 Introduction and problem definition

Organophosphorus compounds (OP's) are widespread used as pesticides, hydraulic fluids, fuel additives, plasticizers, and flame retardants [1]. OP's are often potent inhibitors of various types of esterases such as acetylcholine esterase (see factsheet FSV 002/00 [2]). Since the 'Ginger Jake' epidemic in the 1930's it has been known that a number of OP's are also capable of inducing pathological lesions in the nervous system, known as 'delayed neurotoxicity' or 'Organophosphorus ester-induced delayed (poly)neuropathy' (OPIDPN, OPIDP, or OPIDN) which is characterised by a delayed onset of flaccid paralysis and distinct neuropathological lesions in peripheral nerves, the spinal cord, and the brain [3,4,24].
Since the late 1960's it has been postulated by Johnson that the target site for OPIDPN was an esterase called 'neuropathy target esterase' (NTE) [5,6,7,8]. This enzyme, present in neural tissues, has been shown to be a suitable tool for the assessment of the neuropathic potential of OP's since inhibition of NTE was and still is considered to be necessary for the aetiology of OPIDPN (see below). Since 1995, besides the monitoring of behavioural and neuro-pathological effects, NTE-inhibition measurements have been incorporated in OECD guidelines 418 and 419 for delayed neurotoxicity [3,9,10].
Although many years have passed since the discovery of OPIDPN and its putative target enzyme NTE, still little is known about the actual mechanism responsible for the induction of OPIDPN and the precise role of NTE herein. In the present factsheet, an overview of current knowledge on OPIDPN, the role of NTE and the significance of NTE-inhibition data for the assessment of the delayed neurotoxic potential of OP's is presented.

5.2 Mechanism of OPIDPN and background information

OPIDPN is characterised by the onset of prolonged locomotor ataxia due to flaccid paralysis of the lower and, in the most severe cases, upper limbs [24]. These signs first appear about 7-14 days after (start of the) exposure explaining the expression 'delayed neuropathy'. In test animals, the signs of ataxia are monitored by regular clinical observations and through the use of specific locomotor tests in which a scoring scale for gait alterations of at least 4 levels provides a 'clinical score' [9,10].
The morphological lesions characteristic for OPIDPN have been wrongly termed 'demyelinization' or 'demyelinating disease' because of early misinterpretation of the pathological lesions as demyelination instead of primary axonal degeneration. Although subtle compound- and species-specific differences are known to [3], the general histopathological lesions are similar for all OPIDPN – inducing compounds. The primary lesion of OPIDPN is axonal degeneration of distal parts of long and large-diameter axons especially in peripheral nerves and, when severe, in the spinal cord and sometimes the brain [3,48,24,11]. The degeneration is of 'Wallerian-type' (also known as 'dying back') and is associated with demyelination, swelling, and vacuolisation [11]. The long axons of lower limbs are first affected, but in severe cases upper limbs are involved also, whereas cranial nerves and the autonomic nervous system remain unaffected [8].

Although the actual mechanisms have not been unravelled yet, it appears that the enzym NTE plays a pivotal role in the aetiology of OPIDPN.

1) The primary step in the induction of OPIDPN is the covalent binding of the OP-compound to a specific site of NTE (phosphorylation) which results in an inhibition of NTE-activity.

The biological function of NTE as well as its normal physiological substrate are still unknown and it appears that a proper NTE-activity is not essential for the health of the neuron since severe inhibition of NTE has apparently no effect on neuron function. The inhibition of NTE-activity or its biological function *per se* is therefore not the key step in OPIDPN[4].

2) The critical step in the induction of OPIDPN appears to be a second step after binding to NTE: i.e. the irreversible hydrolysis of an alkyl- or aryl-group of the OP- moiety bound to NTE. This reaction is called 'ageing' and leaves a negatively charged group on the enzyme-OP-complex similar to the 'ageing' process for AChE [see factsheet FSV002/00]. Ageing of NTE occurs very rapidly, within a few hours after binding of the OP to NTE. This process has long been considered to be the crucial step in the induction of OPIDPN since it was observed that substances which inhibit NTE-activity without ageing apparently did not cause OPIDPN.

In the public literature it has been proposed repeatedly that a 'threshold-level' of 70-80% inhibition of NTE-activity with the subsequent occurrence of ageing was correlated with the development of OPIDPN [3,5,6,24,23,27,28]. However, the actual cellular or molecular mechanisms leading from NTE-inhibition with ageing to the induction of OPIDPN have never been identified although some hypotheses have been put forward [see refs. 7,12,20].

From the information above it can be concluded that only substances which inhibit NTE with the subsequent occurrence of ageing can cause OPIDPN. In Appendix A a list of substances is provided which have been shown to induce OPIDPN: It is known that phosphates, phosphonates, phosphorothioates and phosphoroamidates can cause OPIDPN [1] while other NTE-inhibitors such as phosphinates, sulphonates, and carbamates do not induce OPIDPN [8].

Although the information presented above seems to provide a 'straight forward' explanation for the induction of OPIDPN, recent research has shown that the mechanisms involved are far more complex [5,6,8,13,14]. Various experimental observations challenge or modify the above 'straight forward' explanation on the induction of OPIDPN. Although an extensive discussion on these themes is beyond the scope of this factsheet, some of these observations are summarised below very briefly.

1) *Some OP's do cause OPIDPN without apparent ageing of NTE*. For example, methamidophos inhibits NTE at about 90% and induces only a mild OPIDPN whereas mipafox inhibits NTE at about 70% and induces a severe OPIDPN. Nevertheless, both chemicals form a type of inhibited NTE which could always be reactivated (ex vivo) by potassium fluoride indicating the absence of ageing [5].
2) *Protection and Promotion* Some compounds such as phenyl N-methyl N-benzyl carbamate (PMBC) phenylmethanesulfonyl fluoride (PMSF) inhibit NTE without ageing but are known to protect or reduce the induction of OPIDPN by other, neuropathic, OP's [5]. On the other hand, another group of OP's (not capable of inducing OPIDPN by themselves) can act as promotors when given before or after an exposure to neuropathic OP's. These substances exacerbate the severity of OPIDPN and seem to lower the 'threshold' of NTE-inhibition required for the development of OPIDPN [5,6,8,13]. Although such 'promotors' discovered so far are indeed NTE-inhibitors, various publications suggest that NTE is not the target for their promotive action. This suggests

[4] For example, some substances are capable of inhibiting NTE activity to almost 100% without inducing OPIDPN [5,6,8].

that besides the straight forward NTE-inhibition and ageing, other processes are involved in the induction of OPIDPN too.

3) *The level of NTE-inhibition determines the induction of OPIDPN.* Some OP's do induce OPIDPN only when NTE inhibition is ≥ 90% but not when it is 75-85% even though it can be expected that ageing has occurred also at the lower NTE-inhibition, based on the structure of these compounds [5]. This indicates that the 'threshold' for NTE-inhibition and induction of OPIDPN is possibly substance specific but certainly not uniform.
4) *Various subcellular forms of NTE exist*. At least two forms of NTE have been identified in nervous tissue: particulate NTE fraction (P-NTE) recovered from microsomal fractions and soluble NTE (S-NTE) recovered from the cytosol. At present it is not clear whether distribution and/or sensitivity differences between the two forms play a crucial role in the induction of OPIDPN[5].

The information presented above indicates that the straight forward hypothesis for the induction of OPIDPN (a defined 'threshold' for NTE-inhibition with subsequent ageing), is actually far more complex and other, not yet elucidated, processes do participate in the aetiology of OPIDPN.

5.3 Normal values and characteristics of NTE-activity

In a comparable fashion as described for AChE-inhibition (see Factsheet FSV-002/00), NTE-inhibition data are often presented only as a percentage of the control value limiting the number of sources for absolute NTE-activity values. In addition, several units are used for the expression of NTE-activity such as nmol/min/g tissue or nmol/min/mg (membrane) protein. In the following table, some indications for normal NTE-activity in various species and tissues is presented.
In any given species, NTE-activity is highest in brain (although regional differences within the brain do occur) followed by the spinal cord and peripheral nervous tissues [23]. Outside the nervous system, appreciable amounts of NTE-activity are present in the adrenal gland and peripheral lymphocytes amongst other sites [23]. Furthermore, the enzyme is lacking in plasma and erythrocytes but is present in platelets. The physiological function of NTE in these non-neural tissues is unknown.
Although the peripheral nerves are the most sensitive tissues to OPIDPN, mainly brain NTE activity has generally been used for screening the OPIDPN inducing potential of chemicals. The OECD guidelines 418 and 419 prescribe the brain and the lumbar spinal cord to be assayed for NTE-activity and the analysis of sciatic nerve is recommended. It can be questioned which tissue measurement is most relevant to monitor the potency of an OP for OPIDPN: brain, spinal cord or sciatic nerve. Especially since the peripheral nerves like sciatic nerve are most susceptible. Nevertheless, investigation of NTE-inhibition in the peripheral nervous tissues is becoming more common.

5.4 Susceptible Species or Groups

Interspecies differences
OPIDPN is characterised by species selectivity. Over the years susceptible species have been

[5] Most NTE in the brain consist of P-NTE with only 1% S-NTE. However, sciatic nerve has a ratio of 1:1 for P-NTE/S-NTE [14,15]. The P- and S-NTE fractions have been shown to have different sensitivities for various OP's [14,15].

Species	Tissue	NTE-activity	Units	Reference
Hen	Cerebrum	6800	nmol/min/mg membrane prot	15
	Midbrain	5200	,,	
	Cerebellum	7400	,,	
	Spinal cord (cervical, thoracic, lumbar)	4400-5500	,,	
Hen	Brain	25	nmol/min/mg prot	16
Hen	Brain	30-60	nmol/min/mg prot	17
Hen	Brain	2141-2505	nmol/min/g tissue	5 (review of various studies)
	Spinal cord	539-657	nmol/min/g tissue	
	Peripheral nervous tissue	83-102	nmol/min/g tissue	
Hen	Brain	25	nmol/min/mg prot	18
Rat	Brain	8.8	nmol/min/mg prot	
Human/Hen	Brain	> 2000	nmol/min/g tissue	20
Rat/Mouse	Brain	30-40% of human		
Mouse	Brain	4.5	nmol/min/mg prot	21
Hen	Splenic lymphocytes	6.3	nmol/min/mg prot	16
	Blood lymphocytes	5.9	nmol/min/mg prot	
Human	Blood lymphocytes	5-12	nmol/min/mg prot	
Human	Blood lymphocytes	10	nmol/min/mg prot	1

identified and include human, chicken, hen, cat, dog, horse, cow, sheep, pig, water buffalo, ferret and monkeys. Species which apparently do not develop OPIDPN or at least develop OPIDPN inconsistently are small laboratory animals such as rat, mouse, rabbit, guinea pigs, hamsters, gerbils and most birds (other than chicken) even though they exhibit severe cholinergic effects upon treatment with neuropathic OP's [3,7,21] [6].

With respect to these interspecies differences, the characteristics of NTE from various species and tissues have been investigated. Generally it can be concluded that brain NTE of various species is similar with respect to inhibitor sensitivities, pH sensitivity, and molecular weight indicating that biochemical differences of NTE are not the cause of the species selectivity [7,19].

There is a general correlation, across a range of vertebrate species, between low levels of brain NTE activity and resistance to OPIDPN showing that species with high NTE-levels are most susceptible. Humans and chickens have a brain NTE activity of > 2000 nmol/g brain/min while rats and mice have only 20-40% of this level of activity [see table above and ref. 20]. Furthermore, the turnover of NTE is higher in mice than in chickens [20]. In addition to these factors it has been argued that the relative insensitivity of small rodents for OPIDPN cannot be attributed to difference in sensitivity of the neurons themselves but to differences in pharmacokinetic factors (absorption, metabolism, deposition) and the higher regenerating capacity of rodent neurons [21].

The relative insensitivity of rodents for OPIDPN is possibly also related to age (see below) because old rats do indeed develop histopathological lesions characteristic for OPIDPN although behavioural effects are far less likely to occur in this species [8,18,19,26]. Besides kinetic or regenerating differences, the absence of behavioural effects in rats might also be due to the fact that rats are more susceptible to the acute cholinergic effects of OP's which indicates that rats will already die because of acute cholinergic effects at dosages which do not inhibit NTE severely enough to trigger OPIDPN [18].

Taken together, the hen remains probably the most appropriate test species for OPIDPN, as recommended in OECD guidelines 418 and 419 [9,19,18,22].

[6] In some species or strains, OPIDPN can be induced only when the acute cholinergic effects of the OP are tempered by treatment with atropine.

Intraspecies differences
There is a marked age-related dependency in sensitivity to OPIDPN: the young of a given species are more resistant than adults [3,23]. In chicken, the standard species-model for investigating OPIDPN, sensitivity to OPIDPN becomes apparent at about 40-60 days of age [3,23]. Interestingly, the relative insensitivity of rats has been suggested to be related to age-dependency since in most experiments only young adult animals have been used whereas it has been observed that 'older' rats are relatively more susceptible [23]. The basis for age-dependency is unknown but is not related to the level of NTE-activity in the neurons [3]. Metabolic factors as well as differences in the capacity for regeneration and repair of neuronal tissues (the capacity of neural regeneration is greater in young animals) have been suggested to be important factors associated with age-dependent sensitivity to OPIDPN [3,23].

5.5 Miscellaneous

5.5.1 Experimental Variables and Analytical Factors

NTE-activity is measured by the hydrolysation of phenyl valerate, the optimum artificial substrate in vitro. The normal physiological substrates of NTE have not been identified. NTE represents only 6% of the total phenyl valerate hydrolysing activity in hen brain [3]. Therefore, the assay is based on the difference between the hydrolysing activity in the presence of paraoxon (a non-neuropathic esterase-inhibitor which is intended to occupy all irrelevant sites) and the activity in the presence of paraoxon + mipafox (a neuropathic OP). NTE activity is thus characterised as the paraoxon resistant, mipafox non-resistant esterase activity. The liberated phenol from phenyl valerate is measured colorimetrically. It is recommended that 'no-tissue' blanks are included in the assay since phenyl valerate can also hydrolyse spontaneously. NTE-activity is sensitive to pH changes; it declines as the pH of the medium drops. At pH < 5, NTE becomes unstable. NTE remains fully stable at pH 8.0 at 37°C for 4-5h [1]. These factors should be kept in mind when evaluating NTE-activity data.

5.5.2 New Developments

During the last decade several initiatives have been undertaken to provide additional test methods to the in-vivo hen test for screening the potential of substances to induce OPIDPN. In short, three area's of development should be pointed out, although it is not the purpose of this section to provide a full review on these subjects.

1. Acetylcholinesterase (AChE) is the main target for a majority of OP's used, especially for pesticidal OP's [24]. However, the anti-cholinergic activity of OP's is not related to their ability to inhibit NTE or to induce OPIDPN. For example, some non-pesticidal OP's (e.g. tri-ortho-cresylphosphate (TOCP)) which induce OPIDPN do not inhibit AChE at all [24]. Nevertheless, the balance in potency of a substance to bind to AChE or to NTE does indicate whether in vivo an animal will experience primarily cholinergic toxicity or the risk of developing OPIDPN [18]. After all, NTE-inhibition has to reach a high level before OPIDPN will develop. It has been shown for several OP's that the in-vitro ratio between the concentration needed to inhibit AChE and that to inhibit NTE (AChE IC_{50}/NTE IC_{50} ratio) correlates with the in-vivo ratio between the dose which causes death due to acute cholinergic effects and that which may cause OPIDPN [16,25,26]. Such additional in vitro data may provide valuable information to assess the potency of a substance for inducing OPIDPN in vivo.

2. Lymphocytic NTE is highly similar to brain NTE and the IC_{50} values in vitro of both NTE's show close correlation upon exposure to OP's [16]. The relatively easy (compared to brain NTE studies) measurement of peripheral blood lymphocytic NTE inhibition may be a useful tool to determine the OPIDPN potency of substances [1,16]. However, the measurement of peripheral lymphocytic NTE has not yet been incorporated in any guideline or accepted procedure. For the present factsheet no information was available with respect to the sensitivity of the lymphocyte NTE method.
3. Based on the in-vitro binding of OP's to AChE and/or NTE and their ratio, quantitative structure activity relationships (QSAR's) have been proposed for NTE and AChE binding [17].

Although none of these methods has been incorporated in accepted guidelines or procedures, these methods (especially the in-vitro AChE / NTE ratio) may provide useful additional information about the potency of OP's to induce OPIDPN [25]. For example, a high AChE/NTE ratio indicates that an animal will experience primarily cholinergic toxicity reaching lethal levels before sufficient NTE-inhibition occurs. Such a substance is not likely to induce OPIDPN in vivo.

5.6 Assessment and CSR Strategy

In their Guidelines for Neurotoxicity Risk Assessment [27], the U.S.-EPA stated that 'the conclusion that a chemical produces OPIDPN after acute or repeated exposure should be based on at least two out of three factors: 1) Evidence of a clinical syndrome (behavioural effects), 2) Pathological lesions, and 3) Neurotoxic esterase (NTE) inhibition'. It is further stated that NTE inhibition is necessary, but not sufficient, evidence of the potential to produce OPIDPN when there is at least 55-70% inhibition after acute exposure and at least 45% inhibition following repeated exposure [27].

The JMPR has recommended that delayed neurotoxicity testing need not to be done for mono-methyl-carbamates, phosphinates, or sulfonates. Two types of studies are generally required by JMPR for chemicals suspected to produce OPIDPN: 1) the in-vivo hen tests (acute or semi-chronic) basically as described in OECD guidelines 418 and 419, and 2) determination of NTE activity in the hen brain[7] after a single maximum tolerated dose [28]. For acute exposure, the JMPR uses a threshold value of 80% NTE inhibition (at < 60% no clinical signs will develop). During (sub)chronic exposure, JMPR states that when NTE inhibition is less than 50%, OPIDPN will not occur.

To evaluate the potential of OP's for inducing OPIDPN, the following strategy will be followed by RIVM-CSR.

In line with U.S. EPA, the effects characteristic for OPIDPN are divided into 3 categories (see below). It should be noted that when studies are performed according to the present OECD guidelines 418 and 419, all three categories of effects are investigated. However, for 'old substances' the database may be incomplete.

[7] It should be noted that presently (since 1995) NTE-inhibition measurements have been incorporated into OECD guidelines 418 and 419.

When data on all 3 categories of effects are available, a specific OP-ester is considered to produce OPIDPN when positive results have been found in 2 out the following 3 categories of effects:

1. Behaviour: Evidence of a clinical syndrome of OPIDPN (gait abnormalities, flaccid paralysis of (lower) extremities) in a hen test or in humans.
2. Pathology: The presence of (histo)pathological lesions in the peripheral nerves, spinal cord or brain characteristic for OPIDPN (see section 2 for a description of pathological lesions).
3. Biochemical: The presence of an appropriate level of brain NTE-inhibition.

With respect to interpretation of NTE-inhibition data, the following aspects need to be considered.

- NTE-inhibition measurements in brain, spinal cord and/or peripheral neurons need to be performed at or around the time of peak inhibition before any re-synthesis of the enzyme has occurred. Generally, peak inhibition occurs from 3 to 48 hours post-dosing depending on the pharmacokinetics of the compound. The OECD guidelines 418 and 419 recommend to measure NTE-inhibition at 24 and 48h post dosing.
- After acute exposure to OP's, the threshold value for NTE-inhibition at or around peak inhibition is 70% inhibition. NTE-inhibition of ≥ 70% is considered to be a positive result. When 55-70% inhibition is reported (in line with EPA), a detailed evaluation of the data should be performed.
- During (sub)chronic treatment, the threshold value for NTE-inhibition at or around peak inhibition is 60% inhibition. NTE-inhibition of ≥ 60% is considered to be a positive result. When 45-60% inhibition is reported (in line with EPA), a detailed evaluation of the data should be performed.
- When NTE-inhibition is not adequately measured at or around the time of peak inhibition, and/or when NTE-inhibition is 55-70% after acute or 45-60% during subchronic treatment, the kinetic data of the compound and the analytical procedures should be closely examined on a case-by-case basis. Depending on the conclusions on the other 2 categories of effects, a request for additional data may be considered.

Additional Remark
When only one 'positive' result has been observed of the 3 categories presented above, it cannot be concluded that OPIDPN has been induced. For example, gait abnormalities can also result from other types of neurotoxic insults. In addition, it should be emphasised that NTE-inhibition above the proposed thresholds provides an indication for the potency of the substance to induce OPIDPN but provides no conclusive evidence alone. In fact, NTE-inhibition per se does not indicate OPIDPN since an additional step (i.e. 'ageing') is considered to be necessary for the induction of OPIDPN and ageing is not monitored by measuring NTE-activity.

When data on only 2 categories of effects are available, the following additional considerations apply.

When in-vivo tests have been well performed (generally according to the 'old' OECD guidelines 418 and 419 from before 1995, when NTE-inhibition was not yet included into the guidelines) and these studies show negative results for behaviour and neuropathology, no additional NTE-inhibition data are necessary.
Additional NTE-inhibition studies are needed only when 1) the study design for behavioural and neuropathological effects show limitations (e.g. dosages were not high enough) or 2)

other indications (e.g. structure analogies) are present based on which doubts remain with respect to the negative results obtained.
When NTE-inhibition data are available in association with data on either the clinical syndrome or neuropathological lesions showing both negative results, additional data on the third category of effects is needed only when doubts remain with respect to negative results obtained for NTE or when NTE-inhibition is between 55-70% after acute or 45-60% after subchronic treatment. In the latter case, a request for additional data on the third category of effects is recommended.
When data are available for only 2 categories of effects and one of these shows positive effects, additional data on the third category of effects are needed.

When data on only 1 category of effects is available, the following additional considerations apply.

When only data of one category of effects is available a conclusion on the potency of an OP to induce OPIDPN is not possible in principle, and additional data are needed. However, on a case-by-case basis a request for additional data may be unnecessary when e.g. no NTE-inhibition whatsoever has been observed in an adequately performed study and/or when additional data may provide useful information on the potency of the substance to induced OPIDPN (e.g. the in vitro AChE / NTE ratio). Argumentation for not requesting additional data in these cases should be clearly provided.

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APPENDIX 1: Binding of Organophosphorus compounds and Ageing

Illustration derived from Johnston M.K. (1990), Toxicol. Appl. Pharmacol. 102; 385-399

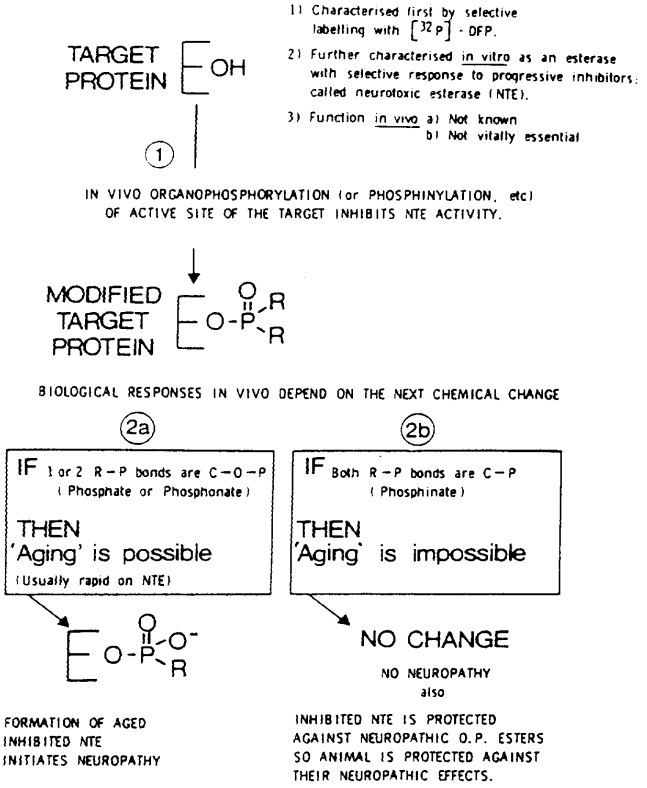

FIG. 1. The respective consequences of alternative further chemical modifications (2a and 2b) of the OPIDP target protein after it has been modified by covalent binding of certain organophosphorus esters: the pathological effects are related to the biochemical effects. (Reprinted by permission of the publisher from "The Target for Initiation of Delayed Neurotoxicity by Organophosphorus Esters: Biochemical Studies and Toxicological Applications, by M. K. Johnson; E. Hodgson, J. R. Bend, and R. M. Philpot (Eds.), *In* Reviews in Biochemical Toxicology, Vol. 4, pp. 141–212, Copyright 1982 by Elsevier Science Publishing Co., Inc.)

APPENDIX 2: Organophosphorus compounds shown to induce OPIDPN

After David Ray (1998), Organophophorus esters: An evaluation of chronic neurotoxic effects, MRC Institute for Environment and Health, University of Leicester, U.K.

Compound	**Class**
Amiprophos	Others
Chlorpyriphos	Di*ethyl* phosphorothioates (P=S) or phosphates (P=O)
Coumaphos	Di*ethyl* phosphorothioates (P=S) or phosphates (P=O)
cyanofenvos	-
Dichlorvos	Di*methyl* phosphorothioates (P=S) or phosphates (P=O)
Diisopropylfluorophosphate (DFP)	Others
Dioxabenzophos	Others
Diphenyl-2-isopropyl phosphate	-
O-ethyl-phenylphosphonothioate (EPN)	Phosphonothioates (P=S) or phosphonates (P=O)
Fenthion	Di*methyl* phosphorothioates (P=S) or phosphates (P=O)
Haloxon	-
Isofenvos	Phosphoro (mono- or di-) amidates
Leptophos	Phosphonothioates (P=S) or phosphonates (P=O)
Methamidophos	Phosphoro (mono- or di-) amidates
Merphos	Others
Mertriphonate (Trichlorophon)	Phosphonothioates (P=S) or phosphonates (P=O)
Mipafox	Phosphoro (mono- or di-) amidates
Omethoate	-
Parathion	Di*methyl* phosphorothioates (P=S) or phosphates (P=O)
Phenyl salignin phosphate (PSP)	-
Tributyl-phosphorotrithionate (DEF)	Others
Trichloronate	Phosphonothioates (P=S) or phosphonates (P=O)
Tri-ortho-cresylphosphate (TOCP)	-
Tri-ortho-tolyl phosphate (TOTP)	-
Triphenyl phosphite (TPPi)	-

6 Residues of plant protection products on food ingested by birds and mammals

Factsheet FSM-001/00 date 25-01-2001

Author:

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Résumé of factsheet

This factsheet presents a summary of recent papers dealing with pesticide residue values on plants/plants parts or insects. A comparison has been made with the so called 'nomograms of Kenaga' which are used in many of the first tier risk assessments for pesticides in the world, including the USA and the Netherlands. In Table 1 new values are presented to be used for realistic worst cases and most likely cases when assessing the risk of sprayed products for birds and mammals.

Table 1 Relationship between most likely case and realistic worst case residue concentrations on plants or parts of plants and insects (in mg/kg fresh weight) and the dosage (D) of plant protection products (in kg active ingredient per hectare) immediately after spraying.

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Food type	Most likely case 50^{th} percentile	Realistic worst case 90^{th} percentile
Short grass	62 * D	142 * D
Long grass	21 * D	69 * D
Leaves, leafy crops, forage crops and small seeds	25 * D	87 * D
Fruit, pods and large seeds	2.3 * D	11 * D
Insects (foliar application)	2.7 * D	11 * D
Insects (soil incorporation)	0.1 * D	1 * D

6.1 Introduction

In 1992 a RIVM-report (Luttik, 1992) was published in which a hazard/risk assessment method of the use of plant protection products for birds and mammals was described. This method was thereafter, beside some small changes, used in the process of placing plants protection products on the Dutch market (Handleiding voor de toelating van Bestrijdingsmiddelen van het CTB).
In the report of 1992 a method was described for estimating the residues on food items for birds and mammals due to spraying of plant protection products. The method is based on research carried out by Hoerger and Kenaga in 1972 in which they analysed data on residues of 28 plant protection products on 60 different crops. They provided maximum and 'typical' values (the typical values are the mean values of the maximum for each crop/pesticide combination) that can be expected immediately after spraying on the vegetation (see Table 2). In 1973 Kenaga proposed, for lack of measurements, to use the residue data of forage crops and cereals for small and large insects, respectively. Based on a smaller data base (27 plant protection products and 36 crops) the so called 'nomograms of Kenaga' were developed by the U.S.EPA (Urban and Cook, 1986). This nomogram is still playing an important role in the first tier hazard/risk assessment in the USA.
Premises are that the residues that one can expect are not the result of the compound but of the crop and that the initial concentration increases proportional with increasing dose.

Table 2 Relationship between 'typical' and maximum residue concentrations on plants or parts of plants (in mg/kg fresh weight) and the dosage (D) of plant protection products (in kg active ingredient per hectare) immediately after spraying (according to nomogram of Kenaga).

Plant/plant parts	Typical values	Maximum values
Short grass	112 * D	214 * D
Long grass	82 * D	98 * D
Leaves and leafy crops	31 * D	112 * D
Small seeds / forage crops[8] /small insects	29 * D	52 * D
Pods	2.7 * D	11 * D
Cereals / large insects	2.7 * D	8.9 * D
Fruit	1.3 * D	6.3 * D

Recently several studies have been carried out; in the first place to check whether nowadays the results of the research of 1972 are still valid (different compounds, low volumes, etc.) and in second place to provide better data for small and large insects:

- residues on plants by Fletcher et al. (1994) and Pfleeger et al. (1996),
- residues on invertebrates by Fischer and Bowers (1997), Brewer et al. (1997) and Joermann (1998), and
- Residues on weed seeds by Edwards et al. (1998).

[8] In the Hoerger and Kenaga paper the fourth category (29*D/52*D) is termed 'forage crops' (based exclusively on alfalfa and clover); the sixth category is termed 'grain' (mainly based on cereal grain, but also on cotton and soybeans).

In this factsheet a summary of the results of this research will be given and a proposal how to use these new data in the hazard/risk assessment for birds and mammals will be presented.

6.2 Summary of new residue literature

Fletcher et al. (1994)
This study re-examines the Kenaga nomogram using information compiled at the University of Oklahoma. The database has 42000 individual records pertaining to over 1000 different organic chemicals, 65% of which are plant protection products. There are data for more than 400 species of plants, representing 95 plant families and all major crops.
Pesticide residue levels on days 0 and 1 following application were examined for 72 plant species and 68 chemicals. Most residue data pertained to leaves and leafy crops, legume foliage, and fruit. In Table 3 the maximum and typical data of Kenaga are presented, the percentage of measurements found by Fletcher that were higher than the values of Kenaga (% of exceeding), the mean values found by Fletcher and the 95th percentile values, estimated as the mean plus 1.6 times the standard deviation.
Fletcher et al. propose to use higher maximum values for small seeds/forage crops and fruit, 121 instead of 52 and 13 instead of 6.3, respectively. They propose to combine two categories pods/large seeds and fruit to one with a maximum value of 13 and one category for leaves/leafy crops and forage crops/small seeds with a maximum value of 121.
The percentage of exceeding is low for the categories of short grass, long grass, leaves and leafy crops, but considerable for forage crops/small seeds and for the category of fruit.
The linear relationship that the Kenaga nomogram has between application rate and residue amounts is consistent with the findings of Fletcher et al.
No indications were found to treat one particular compound group differently from the others. No correlation was found for morphological differences (e.g. surface texture, leaf shape).

Pfleeger et al. (1996)
The objective of this study was to evaluate the nomogram using field data. Six plant protection products (azinphos-methyl, dimethoate, disulfoton, esfenvalerate, endosulfan en chlorobenzilate) were tested on 15 different plant species with application rates ranging between 0.06 to 2.8 kg/ha.
The percentages of measurements above the maximum values of Kenaga for the categories short grass, long grass, forage crops/small seeds, pods/large seeds and fruit are 0, 16, 3, 17, 21 and 0%, respectively. These data indicate that three of the nomogram categories need to be altered: long grass, forage and pods. In the case of wheat (large seeds), the exceeding values may be an artefact of the sampling design (entire heads and not just grain). No higher values were found for the category short grass. Probably the reason that the value of 214 in the nomogram of Kenaga was not exceeded is that the values (gallons per acre) from the original literature source were mistakenly made equivalent to pounds per acre by Kenaga and Hoerger. A statistical analysis carried out by Pfleeger et al. showed that it is not necessary to distinguish between the two categories forage crops/small seeds and leaves/leafy crops. The models developed in this experiment are quadratic, suggesting that the assumption that a linear relationship between application rate and residue level is not necessarily true.

Table 3 Residue values (normalised for an application rate of 1 kg active ingredient per ha); typical and maximum values according to Kenaga, mean and 95th percentile values according to Fletcher et al. and the percentage of values found by Fletcher et al. above the maximum values of Kenaga (% of exceeding).

Plants/plant parts	Kenaga 'typical'	Kenaga Maximum	Fletcher Mean	Fletcher 95th percentile	Fletcher % exceeding Kenaga
Short grass	112	214	76	164	0 (0)
Long Grass	82	98	32	92	4 (2)
Leaves and Leafy crops	31	112	31	98	3 (0)
Forage crops/ small seeds	29	52	40	121	22 (9)
Pods, large seeds (cereals)	2.7	11	4	13	8 (4)
Fruit	1.3	6.3	5	20	19 (7)

Fisher and Bowers (1997)

Fischer and Bowers (1997) compiled measurements made in terrestrial field studies conducted by industry in the late 1980's and early 1990's (see also ECOFRAM, 1999, chapter 3.10.6.3). This data base included measurements made within 24 h of 175 foliar applications and 56 soil applications to actual field study sites. Descriptive statistics (mean, standard deviation, etc.) of these data sets are given in Table 4 and in Table 5 for selected percent exceedence probability levels. Measurements at foliar sites were close to the Fletcher nomogram model estimates for fruits which EPA has assumed are a surrogate for large insects, but much less than the corresponding nomogram values for forage crops which EPA has assumed are a surrogate for small insects. For example, Fletcher et al. (1994) reported a mean and standard deviation residue level per 1 kg/ha applied in/on fruits of 4.8 and 8.8 mg/kg respectively. The comparative values measured by Fischer and Bowers for invertebrates were 5.1 and 8.2 mg/kg, respectively. Measured residues in invertebrates at sites where applications to the soil were made were much lower with the mean in these cases being <1 mg/kg. It is not surprising that these levels were lower since incorporation of the chemical into the soil mechanically, or via watering, 'dilutes' the amount of residue that is likely to contact invertebrates crawling on or in the soil at these sites. The invertebrates in these studies were mostly collected in pitfall traps set immediately after application and retrieved the next morning, or by sweep netting the top of the treated vegetation a few hours after application. These collection methods have potential biases. For example, a net swept against the surface of treated vegetation is likely to remove dislodgeable residues and these residues may in turn adsorb to the surface of insects caught in the net. Thus, these insect samples might have artificially inflated pesticide concentrations. On the other hand, an opposite bias may be associated with pitfall trap samples. This is because although some individuals falling into the traps 'rain down' from the vegetation upon death after an insecticide application, most animals probably fall in while walking across the ground. In the case of insecticide applications, which represent the vast majority of samples in Fischer and Bower's data set, the most highly exposed individuals are expected to become immobilised and therefore have a lower chance of encountering and falling into a pitfall trap. If this is true, the residue levels in pitfall trap samples might by biased on the low side. The following study (Brewer et al.,

1997) has been conducted that controls for these sources of bias and allows one to judge their likely significance in the Fischer and Bowers data set.

Table 4 Residue levels (mg/kg wet weight) for insects/invertebrates as a result of an application rate of 1 kg active substance per hectare (according to Fischer and Bowers, 1997).

Application type	Mean		Maximum	Minimum
	Normal	Geometric		
Foliar	5.1	1.9	48	0.036
Soil-incorporated	0.53	0.036	23	0

Table 5 Residue levels (mg/kg wet weight) for insects/invertebrates as a result of an application rate of 1 kg active substance per hectare for selected percent exceedence probability levels (according to Fischer and Bowers, 1997).

Application type	Calculation method	Exceedence probability level			
		50%	20%	10%	5%
Foliar	Observed data	1.5	7.8	14.3	20.8
	Regression model	1.9	6.8	13.3	23.0
Soil-incorporated	Observed data	0.026	0.20	0.44	1.2
	Regression model	0.036	0.20	0.54	1.3

Brewer et al. (1997)
Brewer et al. (1997) conducted small plot residue trials with several compounds specifically to obtain measurements of residues in invertebrates (see also ECOFRAM, 1999, chapter 3.10.6.3). In these trials, adult insects (crickets and/or beetles) and 'wormy' larvae (beet armyworms and/or beetle larvae) were placed just prior to application on the ground or on vegetation within a spray swath and confined there until they were collected several hours later. Mobile individuals (i.e., adults) were confined to the spray path by pinning them to vegetation or placing them in enclosures. Residue levels (see Table 6) in these samples fell well within the range of observations in the Fischer and Bowers data set. The average values as a result of an application rate of 1 kg active substance per hectare for both adult insects (3.3 mg/kg) and larvae (2.1 mg/kg) were below the average of the Fischer and Bowers data set (5.1 mg/kg). This finding is inconsistent with the potential concern that Fischer and Bowers data are biased on the low side due to the use of pitfall traps as a collection method.

Joermann (personal communication, E-mail dd. 18 February 1998)
G. Joermann of the Biologische Bundesanstalt für Land- und Forstwirtschaft (BBA) in Braunschweig (Germany) has carried out a small literature research concerning residue levels on arthropods as a result of spraying. In Table 7 the results of this research are presented. Most of the data are within the range presented by Fischer and Bowers. In a few cases higher values are found than the concentration presented for 5% percent exceedence probability level.

Table 6 Pesticides residue levels per unit dose of 1 kg/ha measured in adult and larval insects confined to the spray swap during foliar applications to experimental field plots (according to Brewer et al., 1997).

Insect type	n	Mean		Maximum	Minimum
		Normal	Geometric		
Adult crickets and beetles	5	3.3	2.4	4.8	0.34
Larval armyworms and beetle larvae	5	2.1	6.4	0.29	0.29

Edwards et al. (1998)
Little published data are available for weed seeds. An important food item for many wildlife. Edwards et al. conducted two residue trials. In study 1 the lentil crop and surrounding natural vegetation were aerially sprayed at a rate of 550 g a.i./ha and in study 2 weed plots and wheat stubble were ground sprayed at 1000 g a.i./ha. Differentiation was made between whole seeds sprayed on plants, whole seeds sprayed on ground, seeds sprayed on plant in pod and seed sprayed on plant then dehusked. Residue values were adjusted for unit dose (1 kg a.i./ha) and for low interception on artificial soil surface (x 0.6).
The results of these residue trials are presented in Table 8. According to these results there is no need to discriminate between seeds sprayed on the plant or on the ground because residues are similar. Mean residue levels on weed seeds (= small seeds) is 43 mg a.i./kg (fresh weight). There is no need to discriminate between the large seed category of Hoerger and Kenaga (1972) and seeds in pods because residue levels are similar. There is a need to discriminate between birds which do and do not dehusk seeds before consumption; about 80-95% of the spray residue is on the husk. For birds with a body weight smaller than 50 g a dehusking factor of 0.13 should be used (43 ➔ 5.6 mg a.i./kg (fresh weight).

6.3 Conclusions

The maximum residue per unit dose values found by Hoerger and Kenaga on long grass and leaves/leafy crops (98 and 112 mg/kg wet weight) are comparable with the 95th percentile data of Fletcher et al. (92 and 98 mg/kg ww).
The maximum residue per unit dose values found by Fletcher et al. for small seeds/forage crops and for fruit, respectively 121 and 20 mg/kg ww, are higher than the data provided by Hoerger and Kenaga (52 and 6 mg/kg ww).
To adjust the forage crops/ small seeds category from 52 to 121 mg/kg ww the number of exceeding values on day 0 would be reduced from 22% to 5%.
To adjust the category of fruit from 6 to 13 mg/kg ww the number of exceeding values on day 0 would be reduced from 19% to 8%.
The maximum residue per unit dose values for short grass given by Hoerger and Kenaga are probably based on a mistake.
Because the residue per unit dose value of 121 for forage crops/small seeds found by Fletcher et al. is close to the existing residue per unit dose value of 112 for leaves and leafy crops, it would be appropriate to combine the two categories. This was also affirmed by the research of Pfleeger et al.

Table 7 Measured residue levels on arthropods. Application rate in kg active ingredient/ha. Residue levels in mg /kg wet weight, standard residue levels normalised for an application rate of 1 kg active ingredient/ha (after Joermann).

Active Substance	Appl. Rate	Crop	Arthropod	Measured Residues	Standard Residue	Author
Acephate	0.61	Rangeland	Grasshopper	10.8-14*	18-23	Stromborg et al. 1984
Carbaryl	0.50	Rangeland	Grasshopper	17	34	Fair et al. 1995
Carbofuran	0.14	Pasture	Grasshopper	2.2-3.9 max 5.7	16-28 max 41	Forsyth and Westcott 1994
Carbofuran	0.13	Laboratory	Grasshopper	2.1-2.9	22	Forsyth and Westcott 1994
Carbofuran	0.13	Laboratory	Grasshopper	6.1-6.8	46-51	Martin et al. 1996
Carbofuran	0.53	Laboratory	Grasshopper	35-38	66-72	Martin et al. 1996
Chlorpyrifos	0.72	Pasture	Leatherjacket	Max 1.2	max 1.7	Clements et al. 1988
Chlorpyrifos	0.28	Laboratory	Grasshopper	16-19	57-68	Martin et al. 1996
Chlorpyrifos	1.12	Laboratory	Grasshopper	73-83	65-74	Martin et al. 1996
Diazinon	0.84	Tobacco	Hornworm	Nd-2.5	nd-3.0	Stromborg et al. 1982
Diflubenzuron	0.81	Trees	Caterpillars	78	96	deReede 1982
Diflubenzuron	0.3	Trees	Caterpillars	2	7	deReede 1982
Diflubenzuron	0.08	Trees	Caterpillars	3-11	37-137	deReede 1982
Dimethoate	0.21	Laboratory	Grasshopper	4.0-4.1	19	Martin et al. 1996
Dimethoate	0.85	Laboratory	Grasshopper	13.4-16.4	16-19	Martin et al. 1996
Fenitrothion	0.3	Forestry	Caterpillar	1.3-2.7	4.3-9.0	Hamilton et al. 1981
Fenitrothion	0.3	Forestry	Different Invertebrates	0.5-25	1.7-83	Hamilton et al. 1981
Fenitrothion	0.21	Forestry	Budworm	0.7-1.2	3.3-5.7	Forsyth and Martin 1993
Fenthion	0.052	Grassland	Different Invertebrates.	0.28	5.4	Powell 1984
Fenvalerate	0.112	Cotton	Grasshopper	0.18-0.24	2.2	Bennett et al. 1983
Fenvalerate	0.112	Cotton	Ground beetle	0.55	4.9	Bennett et al. 1983
Fenvalerate	0.112	Grassland	Grasshopper	0.03-0.33	0.3-2.9	Bennett et al. 1986
Fenvalerate	0.112	Grassland	Ground beetle	Nd-0.15	nd-1.3	Bennett et al. 1986
Fenvalerate	0.112	Grassland	Crickets	Nd-0.1	nd-0.9	Bennett et al. 1986
Isazofos	5.0	Turf	Mole cricket	0.06-1.3	0.01-0.3	Brewer et al. 1988
Malathion	0.61	Rangeland	Grasshopper	1.4-2.8	2.3-4.6	Stromborg et al. 1984

* Acephate + Methamidophos; nd = not detectable

Table 8 Measured residue levels on weed seeds

Description	Mean		Minimum value	Maximum value	Number of samples	Number of species
	Normal	Geometric				
Whole seed sprayed on plant	42	37	17	76	6	5
Whole seed sprayed on ground	45	44	31	52	4	4
Whole seed sprayed on plant and ground	43	40	17	76	10	6
Seed sprayed on plant then dehusked	6.5	5.4	2.1	14	5	4
Seed sprayed on plant in pod	1.8	1.4	0.7	3.8	3	3

In a similar fashion, fruit (new residue per unit dose value 13) and pods/large seeds (old residue per unit dose value 11) could be placed in a single category.
The research carried out by Fisher and Bowers, Brewer et al. and Joermann showed that the residue levels proposed in earlier days for small and large insects by Kenaga, and still used nowadays in the hazard/risk assessment, are in most cases too high.
Although only a small number of initial residue trials have been carried out with small seeds the results found by Edwards et al. do not give an indication for changing the values proposed by Hoerger and Kenaga and by Fletcher et al. for the categories of small seeds and large seeds or pods.

6.4 Recommendations

Because the database used in the Fletcher et al. research is much larger and more a reflection of the state of the art than the one used by Hoerger and Kenaga, preference is given to the Fletcher et al. database.
It is recommended to use four plant categories and two insect categories:
- short grass,
- long grass,
- leaves, leafy crops, forage crops and small seeds
- fruit, pods and large seeds,
- insects (foliar application), and
- insects (soil incorporation).

In Table 9 the proposed values for the ‘mean’ situation are given. Multiplying the arithmetic mean by the application rate (in kg/ha) of the compound of concern gives the residue on the food item in mg/kg food.
The ECOFRAM report (1999) suggests that the data probably are lognormally distributed. Fletcher et al. (1994) give the percentage of values that exceed the upper Kenaga limit, and indeed these percentages better match the lognormal parameters than the linear parameters (pers. comm. G. Joermann). Therefore, the lognormal transformed mean and standard

deviation are also presented in Table 9. The arithmetic mean and standard deviation are lognormal transformed using the following two formulas:

$$s_y^2 = \ln[1 + (s_x^2 / m_x^2)]$$

$$m_y = \ln m_x - 0.5 * \ln[1 + (s_x^2 / m_x^2)]$$

Table 9 Mean data and standard deviations according to Fletcher et al. (1994) and Fischer and Bowers (1997) for six types of food (normalised for an application rate of 1 kg active ingredient/ha).

	Arithmetic		lognormal transformed		
Description	Mean	Std	Mean	std	n
Short grass	75.7	53.8	4.12	0.64	18
Long grass	32.1	36.3	3.06	0.91	46
Leaves etc	40.2	50.6	3.22	0.98	96
Fruit etc.	4.8	8.8	0.84	1.21	108
Insects foliar	5.1	8.2*	0.99	1.13	175
Insects soil	0.5	3.0*	-2.37	1.87	56

* Data according to ECOFRAM (1999) page 3-88

Based on the lognormal transformed data for four percentiles (50, 90, 95 and 99) at three levels of confidence (95, 50, and 5%) values have been calculated according to the method described by Aldenberg and Jaworska (2000). This method takes the samples sizes into account. The calculated values are presented in Table 10.

Table 10 Residue values (normalised for an application rate of 1 kg/ha) for four different percentiles (50, 90, 95 and 99) at three levels of confidence (5, 50 and 95%).

Food type	50th percentile			90th percentile			Sample Size (n)
	Lower	Median	Upper	Lower	Median	Upper	
Short grass	47.2	61.6	80.2	105	142	218	18
Long grass	17.0	21.3	26.8	52	69	96	46
Leaves etc.	21.2	25.0	29.6	71	87	111	96
Fruit etc.	1.9	2.3	2.8	9	11	15	108
Insects foliar	2.3	2.7	3.1	10	11	14	175
Insects soil	0.1	0.1	0.1	1	1	2	56
	95th percentile			99th percentile			n
	Lower	Median	Upper	Lower	Median	Upper	
Short grass	129	180	296	186	281	533	18
Long grass	70	96	141	121	178	292	46
Leaves etc.	99	125	164	181	244	345	96
Fruit etc.	13	17	24	28	39	58	108
Insects foliar	14	17	22	29	38	50	175
Insects soil	1	2	4	4	7	18	56

It is recommended to use the median values of the 90th percentile for the realistic worst case assessment and the median values of the 50th percentile for the most likely case assessment. It is recommended to use a dehusking factor of 0.13 for small passerine birds (<50 gram) because they generally dehusk small seeds before consumption.

6.5 References

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7 Degradation of veterinary drugs in manure

Factsheet FSM-002/00 date 04-12-2000

Authors:

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7.1 Introduction and problem definition

In the environmental risk assessment of veterinary medicinal products, the half-life for the degradation (DT_{50}) of the active ingredient –and/or other relevant substances- during manure storage is a criterion to be assessed. To date no protocol exists describing the performance of an experiment to determine a DT_{50} in manure, nor is a CSR assessment guideline available. This factsheet is a guideline for assessment of the reliability of the studies currently submitted.

7.2 Description and background

The verification and determination of the degradation half-life in manure is performed only for products that are used with animals that are kept on stable. After medication of these animals, a proportion of the active ingredient can enter the manure storage by excretion of faeces and urine. If the registration holder of a product can demonstrate that the degradation rate in manure is above a critical level, a further environmental risk assessment is not needed. In the guideline of the European Agency for the Evaluation of Medicinal Products that is currently in force (EMEA, 1998), this is stated as follows:

> *'Substances likely to be rapidly **degraded** in manure (DT50 in manure less than 30 days) are exempted from further testing. The conclusion 'rapidly degradable' can be based on theoretical calculations or experimental studies in relevant compartments. The presence or degradation of relevant residues can also be shown in bioassays involving relevant target organisms.'*

The parameter '*degradation*' in the above-mentioned guideline is not further elaborated. Therefore, in this factsheet the following definition is given with respect to degradation of veterinary drugs, metabolites or additives:

> Degradation is dissipation of the compound under investigation due to chemical and/or biological transformation plus irreversible binding (=formation of bound residue).

The dissipation of the compound as the sum of the aforementioned processes can be monitored in time by measuring the concentration of parent compound. The formation of irreversible bound residue is regarded as dissipation because this fraction is considered not to be biologically available after spreading of the manure onto land. The extraction procedure should therefore be performed in such a way that the complete fraction of reversible bound residue is recovered (Kearny, 1982).

7.3 Other factors

In the Dutch procedure for the environmental risk assessment of veterinary drugs the following target animals are distinguished that receive medication in the stable: cattle, pigs, horses, chickens (layers and broilers), turkeys and ducks. The animal categories goats and sheep spent most of the year in the field, manure of these animals is not collected in a manure storage tank. A DT_{50} during manure storage is not incorporated in the emission route in the risk assessment of products intended for these animals. The microbiological and chemical degradation of a substance is influenced by the conditions in the manure, these vary depending on the type of manure, the type of stable en the way the manure is stored. Per

target animal category, the prevailing conditions are taken as a starting point, these condition should be applied in the test. The following conditions are distinguished:

Pigs. Manure is collected in a manure pit, where it is mixed with urine and forms a wet mass. The temperature of the pit is 20°C. The major part of the manure volume is expected to be anaerobic.
Cattle. Manure is collected in a manure pit, where it is mixed with urine and forms a wet mass. The temperature of the pit is 10°C. The major part of the manure volume is expected to be anaerobic.
Horses. Manure that is collected in quantities sufficient to be spread on small parcels (e.g. allotments) is stored until use on a dunghill. The temperature in the dunghill is 25°C. The conditions are expected to be anaerobic
Poultry. In most cases excreta are collected, aerated and dried. The temperature in the stable and of the manure is 20-25°C. Most types of chicken manure have a high dry matter content (40 - 60%) and are aerated after removal from the stable (see the schedule in appendix 1). The condition in the manure are expected to be aerobic.

Table 1. Average and standard deviations of several physical properties and pH values of animal manure types (Bouwman en Reus, 1994).

	Dry matter	Organic matter	Ash weight	pH
pigs, slurry	7.4 ± 2.5	4.9 ± 1.9	2.5 ± 0.7	8.0 ± 0.2
cattle, slurry	9.6 ±2.1	6.8 ± 1.5	2.8 ± 1.1	8.2 ± 0.2
chickens, slurry	14.4 ± 3.3	9.2 ± 1.8	5.2 ± 1.8	7.5 ± 0.3
broilers, manure	58	46	12	7.8

All values (except pH values) are percentages of total fresh weight. Presented are Dutch data, values for pigs, cattle and chickens are based on approx. 400 samples of comparable farms.

7.4 Assessment and CSR strategy

The starting point for the assessment of test reports is Table 2 (Montforts, 1999, p. 122). This table is a list of all test components (‘Items’) and limiting conditions about which reportage is desirable or compulsory. While judging a study one can check the conditions step by step, following the table. For determination of the reliability of the study as a whole the reader is referred to RIVM report 601300.001 (Montforts, 1999). At pages 118 till 120 the methodology for evaluation of studies and the assignment of reliability indices, currently in force within CSR, is explained.

Explanation of Table 2 and supplementation
The points mentioned under ‘Items’ in Table 2 in the sections ‘Description’ en ‘Results’, should be reported in a study.

7.4.1.1 Description

Ad. 1.1. Tests carried out in a controlled laboratory environment are preferred. If the test is performed in a stable, the incubation conditions should be constant. In both cases, measurement and reporting of test conditions is compulsory.
Ad. 1.2 The test is rejected when the wrong condition is employed.
Ad. 1c. Incorporating a sterile control is preferred. Not performing a sterile control alone is not consequential enough to lower the reliability of a study.
Ad. 2. The substance to be tested is the form of the molecule as it is *present in manure* after excretion by the animal. This is not necessarily the equal to the form of the substance in the (formulated) product. In products often hydrochloride- or hyclate

Table 2. Summary table 'Manure transformation studies'

	Items	Notes	Reliability lower ?
Description	**1. test type**		
	1.1. laboratory/stable		
	1.2. aerobic/anaerobic	1.2 aerobic: chickens, anaerobic: horses, cattle and pigs	1b. Y
	1.3. sterile/non-sterile	1.3 method of sterilisation should be given	
	2. test substance and position of label	2. [^{14}C] labelled material preferred	
	3. vehicle		3. E
	4. manure	4.	4. Y
	1.1 type	4.1 give type (slurry, stable manure); fresh, unaltered manure should be used of target animal species	
	1.2 microbial activity		
	4.3 pH		
	4.4 water content	4.4 determine dry weight	
	4.5 % o.m.		
	4.6 storage conditions	4.6 storage before testing should be appropriate	4.6 Y
	5. weight of sample	5. weight sample should be ≥25 g d.w.	5. E
	6. temperature	6 . temperature should be constant (±2°C)	
	7. application method and rate	7. test substance should be homogeneously distributed, rate of a.i. realistic value	
	8. light condition	8. incubation in the dark[9]	
	9. test system	9. closed with volatile traps?	10. E
	10 incubation time	10 preferred until 90% transformation or up to 100 days	11. E
	11 sampling frequency	11 ≥5 time points in degradation area are needed for adequate regression analysis	
	12 extraction/analysis method	12 This should be appropriate for the substance and the metabolites, and the recovery of the substance should be above 70% and below 110% (cold substances) or above 90% and below 110% (labelled substances)	12. Y
Results	**1. DT_{50} and %a.i. at end**	1. if 1st order kinetics: perform appropriate curve fitting	
	2. total recovery	2. (if applicable): >80% at every time-point	2. E
	3. kinetic order	3. check 1st order kinetics with Hockey-stick model if 1st order not appropiate	4. E
	4. bound residue	4. (if applicable):maximum and time and amount at end	
	5. produced CO_2	5. (if applicable): amount at end	
	6. metabolites:	6. identified and quantified separately	
	6.1 ≥ 20%:	6.1 chemical name, maximum and time, and amount at end	
	6.2 < 20%: number	6.2 number of metabolites >5%	
	6.3 ≥ 20%: DT_{50}	6.3 if reliable DT_{50} can be calculated, these can be used.	
Pay attention	1. the dissipation type	1. transformation or dissipation	
	2. the manure	2. the manure structure and components might influence the transformation rate. The manure structure depends a.o. on the feed type.	
	3. the concentration tested.	3. the substance might inhibit microbial activity. Concentrations that differ a factor X [X= 5] from the calculated are considered less reliable unless it has been proven that the substance does not inhibit microbial degradation at either the expected concentration or the highest concentration tested.	
	4. the weight of the analysis samples in relation to the distribution within the manure	4. the substance might not be homogeneously distributed; coarse material should be removed from analysis samples.	
	5. lag-phase	5. a lag-phase should be identified with at least three time-points.	

Legend

Y Y(es) indicates that solely based on not fulfilling this requirement for this item, the reliability of the entire study is expected to decrease. This can be reflected in assigning an RI of 2 to a test, or even assigning an RI of 3. It is up to expert judgement in the latter case, to decide how many 'Y'-items are required for assigning an RI of 3 to a particular test.

E E(xpert judgement), indicates that no clear guidance can be given. The reviewer can consult a specialist.

[9] Unless it has been shown that phototransformation is of no importance.

forms etc. are used. Purity and/or radiochemical purity of the substance should be reported.

Ad. 3. The substance is solved preferably in water, for addition to the test system. When it is inevitable, the use of an organic solvent is allowed when a minimum amount of a water miscible solvent is used at a maximum of 1% v/v. A too high concentration of solvent can influence microbial activity, the reliability of a test in such cases will be lower. In anaerobic tests methanol, ethanol, acetone, and dimethylsulfoxide (DMSO) can be used as solvents.

Ad. 4 The test conditions (temperature, water content, pH) should reflect the conditions of the manure storage (see § 3 and appendix 1).

Ad. 4.1a A study only complies when manure/slurry is tested of the animal for which registration is requested. Slurry or manure of the following animals are distinguished: pigs, cattle, calves, horses, poultry and ducks.

Ad. 4.1b. When a test is initiated with 'clean' manure, it should not have been exposed to the active ingredient (in accordance with other degradation tests, e.g. OECD, 1992)) or antibiotics before. When manure is used from animals medicated with the active ingredient, a *starting concentration* has to be determined. For this purpose, is important to select an extraction-, cleanup- and analytical method that determines the total amount of reversibly bound active ingredient. To that end, it should be demonstrated that the recovery of the complete procedure is sufficiently high (see ad. 12).

Ad. 4.3. The pH should be determined at the beginning and at the end of the test.

Ad. 4.4 The dry matter content or water content of the manure/slurry should be determined.

Ad. 4.6 If the manure is stored before use, pig-, cattle-, horse-, and calf manure should be stored wet (i.e. dehydration should not occur or should not have occurred), cool and dark before the start of an experiment. Poultry-, turkey- and duck manure can be stored dry, cool and dark when necessary. When one or more of the mentioned storage conditions is not met, the reliability of the test decreases.

Ad. 5 The minimum amount of manure is 25 g (dry weight). Using small batches of manure increases the possibility of dehydration and hence alteration of the microbial activity during the test.

Ad. 6 Pig manure: 20ºC, cattle and calf manure: 10ºC, horse- and poultry manure: 25ºC. The temperature during the experiment should be reported. Tests at other temperatures are excepted within a range of approx. 10°C. The resulting DT_{50} of a test is recalculated to the compartment temperature using the Arrhenius equation (Mensink et al., 1995).

Ad. 8. All tests should be performed in the dark.

Ad. 9. Anaerobic tests: see point 5, the manure in the test should have the same water content as determined in 4.3; prevent evaporation; perform test in a closed system, e.g. with water seal or CO_2-trap.

Aerobic tests: see point 5, the manure should have the same water content as determined in 4.3; prevent evaporation.

Use of tests of other types. 'Readily biodegradability' tests are relevant for the determination of DT_{50} <30 d.; when the substance is demonstrated to be 'readily' or 'inherently biodegradable'. Hydrolysis tests over a broad pH range that demonstrate rapid hydrolysis are also relevant.

Ad. 11. At a minimum of five time points in the range where degradation occurs. Each data point should consist of at least two independent, duplicate measurements. A test with only two data points (in which t=0 is included) is considered unreliable.

Ad. 12. The recovery should be between 70% and 110% for unlabeled compounds and between 90% and 110% for labelled compounds (OECD, 2000). Method of analysis and validation should be submitted en performed according to existing guidelines and reported properly. Extraction efficiency should be given (e.g. sequential extraction). The absolute recovery of the extraction method (including further steps like clean up, concentration, derivatization etc.) should preferably be determined. When validation of extraction, cleanup and analysis is not reported or out of the above-mentioned ranges, the reliability of the test decreases.

7.5 Results

Ad. 1. In order to derive a DT_{50} for degradation, the basic assumption is that the process obeys 1st order kinetics. When regression analysis shows that DT_{50} <30 d. with 95% confidence, this result is accepted and the risk assessment stops for this particular compound and target animal. When DT_{50} >30 d., regression analysis[10] provides a DT_{50} value to be used in the emission scenario of the risk assessment. Regression analysis is performed using the software program Graphpad Prism (GraphPad Software Inc, 1996). Use 'Linear regression' or 'Non-linear reg. (fit)'. When linear regression is performed, first carry out log-transformation of the y-values (% a.i. disappeared) and enter all individual values (not means). When choosing non-linear regression, next choose for 'Built-in equations', then 'One phase exponential decay', click on 'Initial Values' and fix 'PLATEAU' at 0 (zero) by clicking 'Auto'.

Ad. 2. At each time point in the experiment, irreversible disappearance (biodegradation and formation of bound residue) should be distinguished from sorption (reversible). To that end, the mass balance (total recovery) should correspond, a minimum value is 80% demanded.

Ad. 3. When a 1st order model does not describe the data properly, check the degradation curve on the presence of a hinge point using the hockey stick model (Mensink et al.., 1995).

Ad. 4. Formation of bound residue is seen as disappearance of the compound, and should be distinguished from (reversible) sorption.

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Appendix 1. Overview of manure types and treatment: poultry

The information below is obtained from the Produktschap Pluimvee at Beekbergen.

Table 1 on the next page gives an overview of different poultry manure types and various treatment procedures. The scheme applies for the target animal categories as distinguished by RIVM at present in the framework of risk assessment of veterinary medicinal products (ecotoxicity). The scheme shows the best estimates based on current knowledge. For most categories in the the table, it is possible that manure is marketed to arable farmers. This can imply that manure is transported to the farmer during the year and will be stored there temporarily (usually on the field). During this storage, heating (NL: broei) can occur, depending on the dry matter content. This applies mainly for those manure categories where heating is likely during the first part of storage.

Apart from the mentioned animal categories, poultry farming recognizes layers in aviaries, rearing of layers (in battery and ground housing), parent broilers, parent broilers in rearing, beef turkeys.

Table 1 Overview of different poultry manure types and various treatment procedures

Animal category	Manure type	Removal from stable	Storage/ (follow up) treatment	Dry matter content(%)		Temperature (°C)		Heating possible		Marketed to
				stable	Storage/ (follow up) treatment	Stable	Storage/ (follow up) treatment	Stable	Storage/ (follow up) treatment	
Layers Battery	wet	1x per year or less	basement under stable or pit/silo		±15		20-25	no	no	arable farming
	dry	1 or more times per week	discharge in container	40-60		20-25	?	no	yes	arable farming
			shed	40-60	40-80	20-25	20-60	no	yes	arable farming, export
			drying by aeration	40-60	60-80	20-25	20-25	no	no	arable farming, export, granule press
			drying by composting	40-60	60-80	20-25	50-70	no	yes, delibera-tely	arable farming, export, granule press
Free range-chicken	dry	1x per round (±15 months)		±60		20-60		ye		arable farming
			aeration in pit	60-80		20-25		no		arable farming
		1 or more times per week	aeration on conveyor belts in pit, discharge in container	40-60		20-25	?	no	yes	arable farming
Broilers	dry	1x per round (±8 weeks)		50-70		25-40		yes		arable farming, mushroom compost

8 Guideline for evaluating studies to determine excretion of veterinary drugs

Factsheet FSM-002/00 date 04-12-2000

Authors:

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8.1 Introduction and problem definition

To assess the environmental risk of veterinary drugs (VMPs) it is necessary to have information on the amount of VMPs that is excreted by the target animals. This also includes the metabolites that are excreted, since these compounds can have an adverse effect on the environment as well (for instance by contamination of groundwater). For this purpose the applicant has to perform an excretion study in which the amount of the active ingredient and its metabolites in the urine and manure is determined over a certain time period. The problem is, however, that no protocol is available that exactly prescribes which set-up and experimental criteria this kind of study has to fulfil and which results have to be obtained to be useful for the risk assessment. As a consequence, the applicants apply studies with a variety of methods. Whether or not the results are useful for the risk assessment is often open for debate. Therefore, guidance was developed in which the requirements and criteria for an excretion study are described. This guidance can also be used to decide whether or not the results are useful for the environmental risk assessment of veterinary drugs.

8.2 Description and background

According to the guideline of the European Agency for the Evaluation of Medicinal Products (EMEA, 1998) VMPs used for terrestrial animals can be divided in two groups: those that are applied to intensively reared and those that are applied to pastured animals. The difference between these groups is that pasture animals excrete the residues of VMPs directly into the environment via the urine and manure separately, whereas with intensively reared animals the urine and manure will be stored together as slurry (in a slurry storage tank) before it is spread on or injected into land. In the latter case the possibility exists that the residues degrade before they are spread onto the land.
In the EMEA guideline trigger values for the VMP concentration in manure, soil and groundwater are set. When it can be demonstrated that the predicted environmental concentration (PEC) of the active ingredient or its metabolites in those compartments does not exceed these trigger values the risk for the environment is considered to be negligible.
In the EMEA guideline it is stated as follows:
Exemption from further testing is in principle acceptable for:

- *Substances that will be present in manure or slurry, for spreading on land, in concentrations < 100 µg/kg*[1].
- *Substances used for animals kept on pasture that will be present in the fresh dung excreted in concentrations < 10 µg/ kg*[1].
- *Substances that have a predicted environmental concentration (PEC) in soil below 10 µg/kg*[2]
- *Substances that have a predicted environmental concentration (PEC) in ground water below 0.1 µg/l*

[1] *The excretion of the active substance and metabolites by the treated animals gives an indication of the extent of the environmental risk. Metabolites which represent less than 20% of the applied dose are not considered relevant in this respect and therefore Phase I can be limited to the parent compound for drugs weakly metabolised.*
[2] *This should be demonstrated by a 'worst case' calculation*

In this guideline it is also stated that substances likely to be degraded rapidly in manure (DT_{50} in manure less than 30 days) are exempted from further testing.

It has to be emphasized that the guideline does not provide scientific foundation for these trigger values. In the case the trigger values are exceeded a phase II risk assessment is necessary in which an effect assessment has to be performed.

8.3 Important aspects

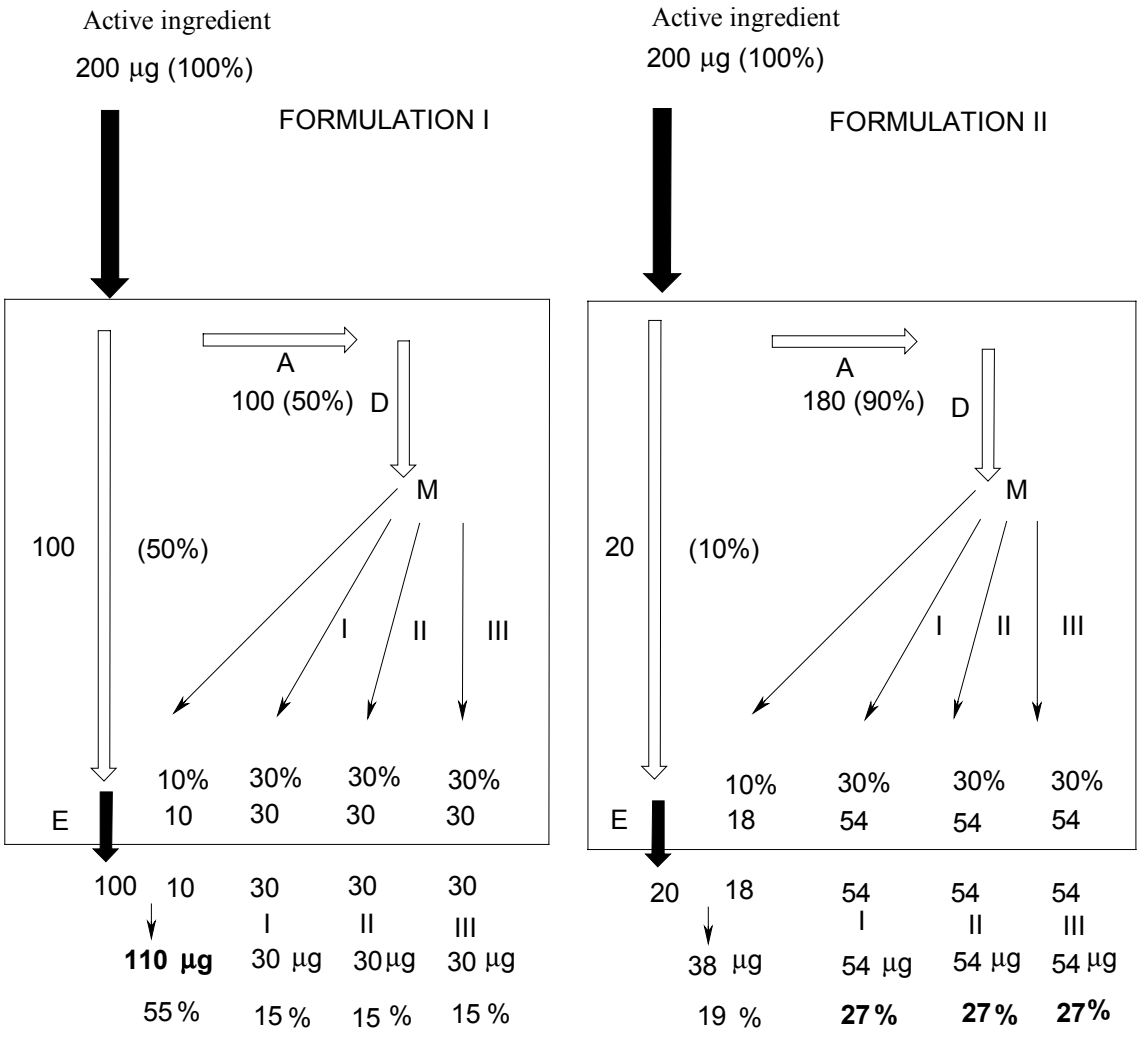
For the determination of metabolic pathway and excretion pattern the following items are considered to be of major importance.

Formulation
The adjuvants of the veterinary medical product can affect the extent to which the active ingredient is taken up by the target animal. As a consequence, products with the same active ingredient can show differences in rate and amount in which the residues (active ingredient and metabolites) are excreted by the target animal. Knowledge about these differences is not only important to determine the concentration in the excreta but it is also necessary to decide which metabolites have to be considered as relevant. In figure 1 it is illustrated that in theory it is possible that with one product no relevant metabolites will be excreted, whereas with another product with the same concentration of active ingredient but with a higher absorption of the active ingredient, relevant metabolites are excreted. A difference in absorption can also affect the rate at which the active ingredient or its metabolites are excreted and as a consequence on the maximum concentration excreted in manure (PEC_{dung}). Therefore, the excretion factor ($F_{excreted}$) and PEC_{dung} should be determined in an excretion study with the formulation in question. Only when bio-equivalence is demonstrated the result of studies performed with a formulation, different from the one for which registration is requested, mentioned in the dossier, can be used.

Dose and application frequency
In case the excretion studies are performed with a dose unequal to that prescribed in the summary of product characteristics (SPC), the applicant has to provide evidence that within an appropriate dose range the kinetic parameters are equal, *casu quo* the kinetic is linear. In other words, the applicant has to show that the ratio dose : internal exposure is not more than dose proportional. With respect to application frequency there is a possibility that due to enzyme inhibition or activation the metabolism patterns between different application frequencies are different. However, for the sake of simplicity, it is assumed that between different frequencies no differences in metabolism patterns will occur. In this the $F_{excreted}$ should be calculated by dividing the total amount of excreted substances to the total amount of active ingredient applied to the target animal.

Application frequency
Veterinary drug products can be applied via different external and internal application routes. The most important are the topical, oral and parenteral (intramuscular and intravenous) route. The way the drug is applied strongly determines to which extent the target animal absorbs the active ingredient and as a consequence strongly determines the formation of relevant metabolites. The most important difference between oral applications on the one hand and the topical and parenteral application on the other is that in the oral application the active ingredient first has to pass the liver before it enters the systemic circulation, whereas in the topical and parenteral applications the active ingredient will enter the systemic circulation directly.

Based on a conservative approach the $F_{excreted}$ has to be determined in a study in which the application route is the same as prescribed in the SPC. Only when evidence is provided that between application routes no large differences in absorption can be expected, application



A = adsorption
D = distribution
M = metabolism
E = excretion

I, II, III = metabolite I, II III

Figure 1. A theoretical example of the difference in kinetics between two products with the same concentration of active ingredient but with a difference in adsorption, caused by a difference in formulation.

routes can be considered as equal. This is probably the case within the three main groups of topical, oral and parenteral applications.
It is possible to use the $F_{excreted}$ determined in the parenteral application as a worst case for a topical application, assuming that absorption in a dermal application is always lower than in

an intramuscular and intravenous application and the proportion of metabolites formed are the same.

Species specificity
In the Dutch procedure the following groups of target animals are distinguished: cow, pig, horse, sheep, chicken, turkey and duck. Each group of target animals can again be distinguished in subgroups with regard to function, age and sex (i.e. heifer, veal calf, beef cattle, dairy cow, suckler cow). Figure 2 shows the different groups and subgroups. Based on physiological and anatomical differences it can be assumed large differences in metabolism and excretion patterns do exist between these groups. This can also depend on the physical and chemical properties of the active substance. Extrapolation to another subgroup or group is only possible on the basis of scientific knowledge on the resemblance in adsorption, metabolism and excretion. Unfortunately, due to lack of data at present a clustering of groups of target animals is not possible for any drug. The information available, as for sulfadiazines, shows large differences between the groups of mammals. As yet, the most conservative approach is used, namely that the product should be examined for every group of target animal separately, unless the applicant demonstrates otherwise.

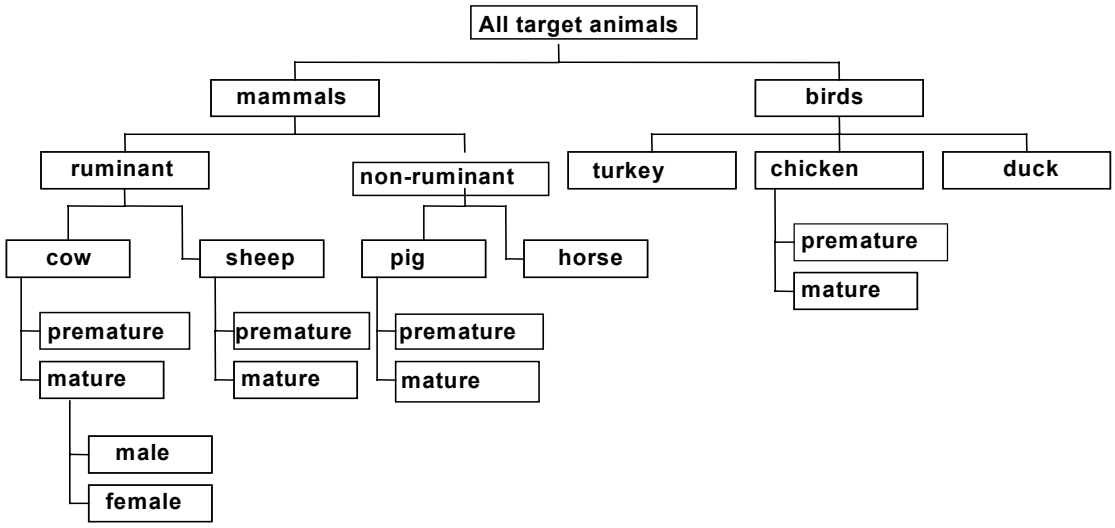


Figure 2. Clustering of target animals.

Relevant metabolites
According to the EMEA guideline all metabolites that are excreted for more than 20% of the dose are considered relevant and should be examined for their potential impact on the environment. Regarding the threshold of 20% an exception should be made for conjugated metabolites. It is known that bacteria are capable to convert the conjugated metabolites back to the original metabolite (Amdur et al., 1992). It is very likely that this process continues after the manure is excreted. Therefore, all conjugated metabolites originating from the same phase 1 metabolite should be summed and also this total amount should not be higher than 20% of the dose.

Extraction and analytical method
It is compulsory that a validated extraction and analytical method for the active ingredient and its metabolites is provided, including information on the recovery, accuracy, precision, stability, LOD and LOQ. Since substances can strongly bind to the manure matrix it is of main importance that the extraction (c.q. recovery) is efficient. For recovery, the lower

threshold criterium of 70% is used, which should be determined in spiked excreta. In table 1 the different items including the criteria are mentioned.
To correct for the influence of the excreta matrix the calibration curve should preferably be made within the same type of excreta.

Food
The presence of additives and contaminations can influence the adsorption and metabolism of the drug in the gastrointestinal tract (Timbrell, 1991). In addition, also interaction on for instance the level of the metabolism in the liver can occur. In principle it is therefore necessary to have knowledge to what extent the composition of the food can affect the metabolism and excretion of the active ingredient and its metabolites. Because knowledge is scarce it is assumed at present that differences are limited and for this reason no information on this matter is compulsory.

Table1. Criteria of validation of a routine analytical method for monitoring residues in manure

Parameter	**Definition**	**Experimental design and number of replicates considered as the minimum acceptable.**
Specificity	The extent to which the method can distinguish between the analyte and other substances under experimental conditions.	With regards to the matrix: using the analytical procedure as proposed, the absence of a response (i.e. signal, peak or ion trace) that could interfere with analyte determinations should be demonstrated by analysing a number of representative blank samples ($n \geq 20$).
		With regards to homologues analogues or metabolic products of the pharmacologically active substance (i.e. metabolites, derivatives or other veterinary drug that are related or may be co-administered): using the analytical procedure as proposed, it should be demonstrated that the accurate quantification of an analyte in fortified blank samples will not be influenced notably by the presence of relevant concentrations of these compounds.
Calibration curves	Response function: the relation between the theoretical concentrations and the response of the detector	Demonstrated by analysing standard solutions prepared as indicated in the analytical procedure at: 5 concentrations (i.e covering the expected concentrations of analyte in samples), analysed at least 3 times on the same day and/or at least on 3 different days.
	Linearity: the proportionality between the theoretical concentrations and the concentration measured	

Parameter	**Definition**	**Experimental design and number of replicates considered as the minimum acceptable.**
Limit of detection (LOD)	The lowest concentration of analyte that can be measured with statistical certainty.	The arithmetic mean of the analyte concentrations determined, using the analytical procedure as proposed, in a representative number of mutually exclusive blank samples (≥ 20) plus three times the standard deviation of the mean. Other methods of calculation providing at least equivalent statistical certainty may be used.
Limit of quantification (LOQ)	The lowest tissue analyte concentration that can be determined with a specified degree of accuracy and precision	This is the lowest concentration of analyte that has been measured in fortified blank tissue with accuracy and precision meeting the relevant criteria (see below). This value should be less than, or equal to, half the LOQ for all relevant tissues of the target species.
Precision (variation of the data around the mean)	Repeatability measured by analysis on the same day. The acceptance criteria limits for repeatability are the following values: True value — CV(%) < 1 µg/kg — 35% > 1µg/kg and < 10 µg/kg — 30% > 10 µg/kg and < 100 µg/kg — 20% > 100 µg/kg — 15%	Representative blank test samples should be spiked at 3, or more, analyte concentrations For example, fortify 3 test samples at 1 and 2 and 4 times the LOQ then take 6 test portions from each. Analyse the test portions and determine the residue concentration in each. At each fortification concentration the CV of the mean determined analyte content of all test portions may not exceed the criteria specified.
Accuracy	Closeness of agreement between the true analyte content of a test sample and the mean analyte concentration determined by applying the experimental procedure a very large number of test portions. The acceptance criteria limits for accuracy are the following values: True (or fortified) value — CV(%) < 1 µg/kg — ± 35% > 1 µg/kg — ± 20%	Accuracy (%) should be calculated from the results obtained during the precision testing phase and expressed as follows: $\frac{(\text{Analyte determined } (\mu g/kg) - \text{Analyte fortification } (\mu/kg)) \times 100}{\text{Analyte fortification } (\mu g/kg)}$ The acuracy of analyte determination must not exceed the criteria specified.
Susceptibility to interference	Non-specific influences on analyte determinations.	Conditions that may fluctuate (e.g. stability, matrix, pH and temperature) and have an effect on the outcome of analyte determinations should be identified and where appropriate operating limits should be set.
Applicability	The commodities to which the method can be applied as described or with minor modifications.	Here the minimum requirement is validation meeting the above criteria for all manure types.
Stability of analyte	The time an analyte can remain stored at a stage of analysis whilst maintaining the validation criteria.	The stability of the substance • in solvent during storage, • in matrix during storage/sample preparation, • in extract during sample

Parameter	Definition	Experimental design and number of replicates considered as the minimum acceptable.
		storage/analysis • should be tested.
Raw data	Relevant raw data, worksheets, chromatograms, calculations, statistical analyses.	All raw data (or tabulated summaries where appropriate) produced during the validation procedure should be submitted.

8.4 Assessment and CSR strategy

Table 2 shows the items and criteria for the evaluation of excretion studies.

Explanations and supplements to table 2

Legends

Y Y(es) indicates that solely based on not fulfilling this requirement for this item, the reliability of the entire study is expected to decrease. This can be reflected in assigning an RI of 2 to a test, or even assigning an RI of 3. It is up to expert judgement in the latter case, to decide how many 'Y'-items are required for assigning an RI of 3 to a particular test.

E E(xpert judgement), indicates that no clear guidance can be given. The reviewer can consult a specialist

Description

Ad 1. Preferably the tests should be performed with the formulation prescribed in the dossier. In case the study is performed with another product or with the active ingredient itself, the applicant should demonstrate that this product is bio-equivalent with the formulation in question.

Ad 2. The excretion of the products should be examined for each target animal separately.

Ad 3. Each study should be performed with a minimal number of test animals. As yet the arbitral number of 5 is chosen. Preferably the excretion should be examined in each test animal separately in order to get an insight in the variation in excretion.

Ad 4. The excretion should be examined at the maximum prescribed dose and frequency and number of treatments, as mentioned in the SPC. In all cases the actual dose should be known. This is especially important with applications in which a certain loss can be expected or with an oral application via drinking water.

Ad 5. The exposure time should correspond with the prescription in the SPC.

Ad 6. The application route should correspond with the prescription in the SPC. Other methods are only allowed when bio-equivalency is demonstrated.

Ad 7. All test animals should be healthy and not have been medicated before.

Table 2. Summary table 'Excretion studies'.

	Items	Notes	Reliability lower ?
Description	**1. test substance and vehicle**	1. test should be carried with the formulation in question	1. Y
	2. test species	2. all prescribed target species separately	2. Y
	3. number of test animal	3. min. X animals [X= 5]	3. E
	2. applied concentrations	4. prescribed max dose and max repetitions dosing regimen, dus inclusief frequentie, dosis, doseringsroute en maximale doseringsperiode	4. Y
	3. exposure time and excretion time	5. exposure: max prescribed exposure time with the prescribe interval(s) excretion : direct after the first application up to the time point that the relevant substances are no longer detectable in excreta	5. Y 6. Y
	6. application method	6. prescribed application method	7. E
	7. conditions	7. test animals should be healthy and not previously be medicated	8. E
	8. extraction/analysis method	8 A validated method should be used, which is appropriate for the parent compound, metabolites and other relevant substances, and the recovery of the substance should be >X% [X= 70] and <Y% [Y= 110]	9. E
	9. test system	9	10. E
	10 sampling frequency	10 For the determination of PEC_{dung} the intervals between sampling points should not be more than 1 day	11. E
	11 age, weight and sex of the organisms	11 one of the sexes or a specific stadium should be tested in the case the formulation is prescribe for one specific sex or age stadium.	12. E
	12 temperature and humidity	12	13. E
Results	**1. $F_{excreted}$**	1. all relevant substances (parent compound, metabolites and additives) 2.1 in manure for housed animals 2.2 in dung and urine separately for grazing animals 2.3 the highest fraction excreted in dung in one day.for grazing animals	1. Y
	2. metabolites	2. identificatied and quantified separately 1.1 chemical name, maximum and time	2. Y
Pay attention	**1. application**	**1. in the case the formulation is prescribed has to be sprayed, the actual dose has to be determined (exclusive the possible loss)**	
	2. sampling frequenty and storage	**2. F excretion can be underestimates in the case the sampling interval is higher than the DT_{50} in manure**	
	3. feeding	**3. the type of food can influence the metabolism and excretion of the ubstance**	

Ad 8. The extraction and analytical method should be validated (including the recovery, accuracy, precision and stability). The calibration should be corrected for potential interference of the manure and urine matrix.
Ad 9. In case the product in question is prescribed for pasture animals, the manure and urine should be sampled separately. For products prescribed for indoor animals only separation of the excreta is not necessary.
Ad 10. In case the product in question is prescribed for pasture animals, the excreta should be sampled in the first period, at least every 24 hours in order to determine the maximum concentration in manure (=$PEC_{max\ dung}$). In each case the sampling frequency should be

geared on the rate of biodegradation. For substances with high doses the time interval should be as short as possible to prevent an underestimation of the excretion factor.
Ad 11. Products prescribed for a target animal from a particular age category or for one of the sexes should also be examined for that particular group.
Ad 12. The test conditions, such as temperature and humidity should be in the range that can be expected in practice.

Results
Ad 1. All metabolites excreted in more than 20% of the dose should be identified.
Ad 2. For pasture animals the $F_{excreted}$ in manure and urine should be reported together with the PEC_{dung}. For indoor animals only the $F_{excreted}$ in slurry should be reported.

8.5 References

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