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COLLECTIVE EXPERT APPRAISAL: SUMMARY AND CONCLUSIONS

Related to theestablishment of a Toxicity Reference Value based on the reprotoxic effects of linuron (CAS No. 330-55-2)

AFSSET Solicited Request No. 2003/AS03

Only the French language version of this document shall prevail.

Overview of the question

AFSSET's work programme on reprotoxic TRVs included organisation of a pilot phase to ensure implementation of the recommended development method. Reprotoxic TRVs were established as part of the submissions for linuron, di-n-butyl phthalate (DnBP), benzyl butyl phthalate (BBP), nonylphenol, toluene and ethylene glycol ethyl ether (EGEE).
On 25 July 2007, the Directorate General for Health (DGS) requested that AFSSET validate these TRVs through a collective expert appraisal.

Organisation of the expert appraisal

AFSSET entrusted validation of these TRVs to the Expert Committee (CES) for "Assessment of risks linked to chemical agents". This CES mandated a *rapporteur* to conduct an expert appraisal of linuron, which was carried out in accordance with the French Standard NF X 50-110 "Quality in Expertise Activities - General Requirements of Competence for Expert Appraisals".

Description of the working method

Based on the document "*Construction/choix d'une VTR reprotoxique pour le Linuron* [Development/choice of a reprotoxic TRV for linuron]" prepared by the team of Vincent Nedellec Consultants[1], the *rapporteur* assessed the compliance of the method used compared with the recommendations of the Working Group on the following points: i) information retrieval and ii) toxicity profile, in order to select the critical effect and source study to use. He then gave his opinion about the choices made in light of available data.
The establishment of TRVs differs depending on the assumption made or data acquired on the substance's mechanism of toxic action. Currently, the default hypothesis is to consider a monotonic relationship between exposure, or dose, and effect, or response. On the basis of current knowledge and conventions, it is generally accepted that for reprotoxic effects, toxicity is expressed only above a threshold dose (with the exception of germ cell mutagenicity). Nevertheless, this assumption may be questioned if warranted by the available data.

[1] Annex 2 of the reference document for the development of a TRV based on reprotoxic effects

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Mathematically, the establishment of a TRV is therefore defined as follows[2]:

$$TRV = \frac{\text{Critical dose}}{UF}$$ where

Critical dose = NOAEL, LOAEL or BMDL
UF = globally applied uncertainty factor

In practice, establishment of the TRV involves the following four steps:

- choice of the critical effect;
- choice of a good quality scientific study usually enabling establishment of a dose-response (or dose-effect) relationship;
- choice or establishment of a critical dose from experimental doses and/or epidemiological data;
- application of uncertainty factors to the critical dose to account for uncertainties.

This method is detailed in the reference document for the establishment of a TRV based on reprotoxic effects (AFSSET, December 2006), and establishment of the TRV for linuron is based on this method.

As a result of its internal discussions, the CES has reached a decision about the choice of the critical dose and uncertainty factors. The CES emphasised the need to refer back to the supplemental studies which, while they are not directly used to identify the critical dose, are useful for choosing uncertainty factors (toxicokinetic studies, availability of other NOAELs or LOAELs, etc.)

Results of the collective expert appraisal

Summary of toxicity data

This part is based on the summary of work carried out by the submitter in 2006 as part of the pilot phase. For more information, the reader can refer to the "*Document de référence pour la construction d'une valeur toxicologique de référence fondée sur des effets reprotoxiques – annexe 2*" [Reference document for the development of a Toxic Reference Value based on reprotoxic effects – Annex 2] (AFSSET 2006). The report is based on monographs written previous to this work by national organisations and on literature published subsequent to these monographs.

Linuron is a herbicide belonging to the family of substituted ureas. Its main chemical characteristics include being highly stable, non-volatile, non-corrosive, poorly water soluble, and of low toxicity to mammals. Its use has been restricted since 2002. In Europe, annual production is 500 to 1000 tons per year (mainly in France, Italy, The Netherlands, Spain, and England). Given its uses as a herbicide, exposure can occur by respiratory or dermal routes when using products containing linuron, but also orally by ingesting foods contaminated by trace amounts of linuron. In the general population, primary exposure is by oral route.
Since 2004 (the 29th Adaptation to Technical Progress [ATP]) linuron has been classified by the European Union as a reprotoxic substance for humans, Category 2 for development (risk of harmful effects for the child during pregnancy) and Category 3 for reproduction (possible risk of impaired fertility).

[2] NOAEL: "no observed adverse effect level"; LOAEL: "lowest observed adverse effect level"; BMDL: "benchmark dose lower confidence level": lower limit of the confidence interval of the benchmark dose.

By chronic oral exposure, the haematological effects (abnormal pigmentation of the blood characterised by an absorption peak between 618 and 620 nm during transformation of haemoglobin into cyanohaemoglobin and haemolytic anaemia) are regarded as the most sensitive adverse effects in rats and dogs.
In animals, it is clearly accepted today that linuron has toxic effects on the development of the reproductive system of the male foetus during maternal exposure. In contrast, no effect has been shown in females, or in males when exposed only during adulthood. There are no data in humans.
Given the current state of knowledge, the effects on development can thus be regarded as critical effects for short-term oral exposure to linuron (during gestation).
These effects are described in rats exposed *in utero* or during early life (immature rats). Among the critical effects reported, the most common are absence of nipple regression, decreased anogenital distance, reduced weight of male genitalia, hypoplasia of the testes and epididymides, malformation of internal (epididymis, vas deferens, seminal vesicle) and external (hypospadias, cryptorchidism) sex organs.

From a review of the literature, and based on the latest data, three studies conducted in animals were selected. They are considered representative of the reprotoxic effects observed.
The first study was conducted in rats exposed for 80 days from the 20th day after birth. The adverse effect was a significant reduction in weight of the seminal vesicle and epididymides and a significant delay in the onset of puberty at the LOAEL of 40 mg/kg/day (NOAEL = 20 mg/kg/d). A significant decrease in the weights of the testes and epididymis was also shown in F1 generation males (Gray, 1999).
The second study was conducted in rats exposed *in utero* from the 12th to the 21st day of gestation. In male offspring, the incidence of hypoplasia of the testes and epididymides increased from the first dose group at 12.5 mg/kg/d (McIntyre, 2000).
The third study (Kang, 2004) was conducted in immature and castrated male rats exposed by oral gavage for 10 days. A significant decrease in the weight of seminal vesicles, ventral prostate, and Cowper glands was observed in the second dose group (LOAEL = 50 mg/kg/d; NOAEL = 25 mg/kg/d).
The three animal studies were conducted according to strict protocols, following United States Environmental Protection Agency (US EPA) good practice guidelines for the first one, a protocol similar to a standardised protocol for the second, and Organization for Economic Cooperation and Development [OECD] guidelines for the third. These studies were thus given a Klimisch rating of 1 and can be considered for the establishment of a TRV.

The toxic mechanism responsible for these effects is the anti-androgenic action (competition with testosterone at the level of the androgen receptors) responsible for anomalies in sexual differentiation during embryogenesis. Moreover, the anti-androgenic action of linuron promotes luteinising hormone proliferation causing Leydig cell tumours. It is therefore a mechanism of endocrine disruption.
In light of all the available data, it appears that the developmental effects observed in animals cannot be excluded for humans.

Analysis and assessment of the choices for establishment of the TRV

Critical effect

The most relevant developmental effects were observed in the male reproductive system, from a histological as well as functional standpoint. The primary effects thus observed were: absence of nipple regression, decreased anogenital distance, reduced weight of male

genitalia, hypoplasia of the testes and epididymides, malformation of internal (epididymis, vas deferens, seminal vesicle) and external (hypospadias, cryptorchidism) sex organs.
The critical effect chosen for the establishment of a reprotoxic TRV is consistent with published reports. The critical effect relevant for humans observed at the lowest dose is **hypoplasia of the testes and epididymides**.

Pivotal study

The three studies chosen are all of good quality. Furthermore, they were given a Klimisch rating of 1. The final choice among these three was essentially based on the ability to demonstrate a dose-response relationship (multiple dose groups were tested), publication in an international journal, and the more protective nature of the lowest LOAEL, which is consistent with current knowledge and recommendations contained in the "*Document de référence pour la construction d'une valeur toxicologique de référence fondée sur des effets reprotoxiques*" [Reference document for the development of a Toxic Reference Value based on reprotoxic effects] (AFSSET, December 2006). The lowest LOAEL was identified for hypoplasia of the testes and epididymis, observed in the study by McIntyre (2000).
Thus, the study by **McIntyre *et al.* (2000)** was chosen. This study was given a Klimisch rating of 1 (compliance with guidelines other than those of the OECD): gravid rats were exposed orally by gavage from the 12th to the 21st day to 0 – 12.5 - 25 and 50 mg/kg/d of linuron. In the male offspring (F1), the incidence of testicular and epididymal hypoplasia increased from the first dose tested: 0% in the control group and 3.6% and 1.8% respectively in the first group exposed to 12.5 mg/kg/d. These incidence rates climb respectively to 11.3% and 9% in the last group exposed to 50 mg/kg/d. There was no dose-dependent nipple regression but the difference was significant only at the highest dose (continuous variable: no incidence). A significant increase in malformations of the epididymides (6.8%) was also observed, only at the highest dose, and an absence of vas deferens (4.5%). The chosen effect is **hypoplasia of the testes and epididymides (p=0.05) in newborn males**. This effect indicates a mechanism of endocrine disruption.

Choice of the critical dose

In the study chosen, hypoplasia of the testes and epididymides were observed from the lowest dose tested, thus corresponding to an **LOAEL of 12.5 mg/kg/d**. Therefore, no NOAEL could be proposed in this study. It can simply be specified that the NOAEL is between 0 and 12.5 mg/kg/d.
To determine this LOAEL, the authors used analysis of variance (ANOVA) and analysis of covariance (ANCOVA) methods, followed by a Dunnett's test to determine the effects related to the treatment of animals with linuron. A significance threshold of 0.05 was used for interpreting results.
During the pilot phase, AFSSET proposed developing a BMDL based on the chosen effect. The obtained response was dichotomous (effect/no effect). Several models were tested (logit, probit, gamma and Weibull) and the probit model was chosen because it was the best suited to the data. A benchmark response level (BMR) of 5% in 'extra risk' was retained (extra risk corresponds to the increased risk compared to the probability of not being affected in the control group, presumably the more conservative response). A **BMD_{05} at 18 mg/kg/d** and a **$BMDL_{05}$ at 14 mg/kg/d** was obtained (the results of the entire process are detailed in the 'Reference document for the establishment of a Toxic Reference Value based on reprotoxic effects', Annex 3). There was low variability in the results of the BMDL.

Choice of uncertainty factors

- UF_A: inter-species variability: the factor chosen is the maximum factor of 10 because there is insufficient evidence in humans.
- UF_H: intra-species variability: the factor 10 is chosen by default when using studies conducted in animals, to take into account the greater variability within the human species.
- $UF_{L/B}$: use of a BMDL: AFSSET recommends a factor of 3 or 10 depending on the case. The CES proposes a factor of 3 because the 5% incidence used is low and a NOAEL at 20 mg/kg/d from another study was noted in the literature, which would favour a NOAEL close to the LOAEL.

The Expert Committee (CES) for “Assessment of risks linked to chemical agents” accepted the results of the collective expert appraisal at its meeting on 29 May 2008 and informed the Directorate General of AFSSET.

Conclusions of the collective expert appraisal

- Linuron has been the subject of several studies (dose-response relationships and mechanism of action) and risk assessment reports.
- The effects observed in animals (rats) are relevant to humans and the effect on the development of the male reproductive system (hypoplasia of the testes and epididymis) may be considered as the critical effect.
- The mechanism put forward (anti-androgenic effect by competition with testosterone) is plausible.
- There are no studies in humans.

The CES thus proposes establishing a TRV specifically for effects on development. Given the demonstrated effects on development of the reproductive system, which indicates endocrine disruption and whose critical window of exposure corresponds to the gestation period, the TRV will be applicable for sub-chronic exposure.

-- Linuron CAS No. 330-55-2

Critical effect	Critical dose*	UF	TRV
Hypoplasia of the testes and epididymis Prenatal oral toxicity study in rats (GD12-GD21) McIntyre *et al.* 2000	LOAEL = 12.5 mg/kg/d No NOAEL BMD_{05} = 18 mg/kg/d **$BMDL_{05}$ = 14 mg/kg/d**	**300** UF_A 10 UF_H 10 $UF_{L/B}$ 3	**TRV = 0.05 mg/kg/d** **Confidence level** Data collection: average (Lack of fertility study) Study: high Critical dose: high TRV: average

*Time conversion factors, allometric coefficients: NIL

Recommendations of the CES

Among the early effects, occurring at lower doses, the absence of nipple regression observed in male rats is an effect that cannot occur in humans. Nevertheless, it indicates disruption of sexual differentiation during embryogenesis, which could manifest as a different effect in humans. In addition, the decrease in anogenital distance is considered a reliable, easily identifiable and measurable marker in both humans and animals.
Current knowledge about the nature of endocrine disruption by linuron is only partial: a single animal species has been tested and impairment of fertility has not been studied. The CES thus recommends re-evaluating the TRV as new scientific data are published.

Lastly, the CES did not wish to apply an allometric adjustment[3] to the critical dose because this type of adjustment has not yet undergone extensive study in France and the reference document for establishing the TRVs based on reprotoxic effects has not yet addressed this issue. The need for a better understanding of animal-human transposition and the UF_A uncertainty factor led to the recommendation that further consideration be given to this aspect.

Maisons-Alfort, 16 June 2008.

On behalf of the Expert Committee (CES) for
"Assessment of risks linked to chemical agents",

Chairman of the CES

Mr Michel Guerbet

[3] Some agencies occasionally recommend "human equivalent" critical doses or concentrations by applying adjustments that take into account differences in body surface areas during oral exposure or other specific physiological parameters of the respiratory route. These adjustments have not yet been adequately discussed within the French Working Groups.