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COLLECTIVE EXPERT APPRAISAL: SUMMARY AND CONCLUSIONS

Related to the establishment of a Toxicity Reference Value based on the reprotoxic effects of benzyl butyl phthalate (CAS No. 85-68-7)

AFSSET Solicited Request No. 2003/AS03

Only the French language version of this document shall prevail.

Overview of the question

Toxicity Reference Values (TRVs) are indices enabling both a qualitative and quantitative relationship to be established between a health effect in humans and exposure to a chemical substance. They are fundamental to the process of health risk assessment.

In 2003, the Agency decided to establish a national programme on TRVs based on collective and scientifically sound French expertise on the matter. The field of reprotoxic chemical substances was proposed as a priority. For this purpose, in 2004, the Agency set up a Working Group to develop a method for establishing TRVs based on reprotoxic effects. As part of this work, the Agency published a limited call for papers for the development of reprotoxic TRVs for six substances, namely linuron, di-n-butyl phthalate (DnBP), benzyl butyl phthalate (BBP), nonylphenol, toluene and ethylene glycol monoethyl ether (EGEE), the purpose being to:

- ensure that in its current state the method proposed is sufficient to enable establishment of a reprotoxic TRV ;
- enable improvements to be made to the method proposed through practical feedback.

The development and proposal of TRVs for the six substances studied during the pilot phase are dealt with in Annexes 2 and 3 of the "*Document de référence pour la construction d'une VTR fondée sur des effets reprotoxiques*" [Reference document for the development of a TRV based on reprotoxic effects]. AFSSET, December 2006" available online at http://www.afsset.fr

This expert summary refers back to the main data collected by the submitter and takes them into account for the development of the TRV for benzyl butyl phtalate, along with the conclusions of the CES for "Assessment of risks linked to chemical agents".

Organisation of the expert appraisal

The Agency entrusted the validation of these TRVs, produced as part of the pilot phase, to the Expert Committee (CES) for "Assessment of risks linked to chemical agents". This Committee mandated a *rapporteur* to conduct an expert appraisal of benzyl butyl phthalate (BBP).

The *rapporteur*'s work was submitted to the CES on 29 May and 10 July 2008. This expert appraisal was therefore done by a group of experts with complementary skills. It was carried out in accordance with the French Standard NF X 50-110 "Quality in Expertise Activities -

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General Requirements of Competence for Expert Appraisals" to ensure compliance with the following points: competence, independence, transparency and traceability.

Description of the working method

Based on the document "*Construction/choix d'une VTR reprotoxique pour le benzylbutylphtalate* [Establishment/choice of a reprotoxic TRV for benzyl butyl phthalate]" 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.
The development 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.
Mathematically, the development 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 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 BBP 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 for the identification of the critical dose, are useful for choosing uncertainty factors (toxicokinetic studies, availability of other NOAELs or LOAELs, etc.).

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

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

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]. Given the abundance of scientific literature on the toxic effects of BBP, the report is primarily based on the monograph of the US National Toxicology Program – Center for the Evaluation of Risks to Human Reproduction [NTP-CERHR] (2003), the risk assessment report of the European Union (2007), and literature published subsequent to this monograph.

BBP is a plasticiser belonging to the family of phthalate esters. In Europe, over 90% of the BBP produced is used for the manufacture of polyvinyl chloride (PVC) and other polymers found in floor coverings, gaskets, etc. It is also used in food packaging. Because it is chemically loosely bound to the polymer matrix of the final product, it can easily migrate into food or be released into the environment. Although inhalation or dermal exposure is possible, exposure of the general population is primarily oral. In addition, the available data have mainly been obtained on the oral route. It was therefore considered appropriate, in the context of this expert appraisal, to establish a TRV for the oral route.
Since 2004 (the 29th Adaptation to Technical Progress [ATP]) , BBP has been classified by the European Union as a reprotoxic substance for humans, Category 2 for development (possible risk of harm to the unborn child) and Category 3 for reproduction (possible risk of impaired fertility).

Toxicokinetics

In rats, BBP is metabolised by esterases in monobutyl phthalate (MBuP) and in monobenzyl phthalate (MBeP) with preferential formation in MBuP. The metabolites are mainly found in in the urine. In humans, unlike rats, the primary metabolite is MBeP, which is mainly metabolised as conjugates in the urine (glucuronide conjugates). However, the impact of these differences on the toxicity of BBP between animals and humans remains to be determined (Saillenfait and Laudet, 2005).

General toxicity

The acute toxicity of BBP is low by oral and dermal routes. In sub-chronic and chronic oral studies (2 to 26 weeks) in rats, increases in liver and kidney weights were primarily observed (≥120-151 mg/kg/d). They were accompanied by specific histological changes at higher doses (liver ≥ 960 mg/kg/d; kidneys ≥ 500 mg/kg/d in males and ≥ 1200 mg/kg/d in females).

In vivo and *in vitro* genotoxicity assays were predominantly negative. Concerning carcinogenicity, a two year study in rats by the NTP (1995) confirmed the observation of carcinogenicity in males based on increased incidence of pancreatic tumours. There is equivocal evidence in females (marginal increase in pancreatic adenomas and papillomas of the bladder) at a high dose (1200 mg/kg/d). However, no evidence of carcinogenicity was found in mice (NTP, 1995; Saillenfait and Laudet, 2005).

Reprotoxic effects

It has been clearly established that BBP has toxic effects on male reproduction, as indicated by lesions of the reproductive organs and reduced sperm counts noted in rats exposed orally. Furthermore, oral administration of BBP to rats and mice during gestation causes an increase in embryo mortality, growth retardation, and malformations in young. Development

of the reproductive system and male sexual differentiation are particularly affected after exposure during a sensitive period. Alterations observed in male offspring are malformations (cryptorchidism, hypospadias, etc.), decreased anogenital distance, absence of nipple regression and/or alterations in sperm production.
From a recent review of the literature on the reprotoxicity of BBP or its main metabolites (monoesters), several studies conducted in animals were selected. They are considered representative of the reprotoxic effects observed and are summarised below.

Prenatal toxicity studies

The first study (NTP, 1990) was conducted in (CD)-1 mice chronically exposed *in utero* via feed from the 6th to the 15th gestation day. Fœtal mortality and some malformations were observed for exposures of 910 mg/kg/d, accompanied by maternal toxicity. The NOAEL was identified at 182 mg/kg/d. In another study in Wistar rats exposed *in utero* from the 1st to the 20th gestation day (Ema, 1990), a reduction in body weight gain was demonstrated with a LOAEL of 375 mg/kg/d. The NOAEL was identified at 185 mg/kg/d.
A third study was conducted in Harlan cpb WU rats exposed *in utero* by oral gavage of the dam from the 6th to the 15th or 20th gestation day (Piersma, 2000). The authors observed skeletal variations (presence of a 13th vertebra), decreased testicular weight, and increased incidence of undescended testes in the offspring.
Benchmark doses (BMDL) were calculated for different effects. A BMD of 5% was calculated at 171 mg/kg/d (95% confidence interval [CI95] at 145 to 206 mg/kg/d) or 211 mg/kg/d (CI95 at 182 to 254 mg/kg/d) depending on the duration of exposure (6-20 or 6-15 gestation days [GD]) and for skeletal variations but the authors consider a surnumeral vertebra as a harmless minor variation. A BMD of 1% was proposed for undescended testes at 163 mg/kg/d (CI95 = 95 to 280 mg/kg/d) or 251 mg/kg/d (CI95 = 153 to 433 mg/kg/d) depending on exposure duration The NOAEL was 350 mg/kg/d for most of the effects (LOAEL 450 mg/kg/d), except for the appearance of a 13th vertebra, observed from the first dose tested (LOAEL = 270 mg/kg/d).
The effects of MBeP, the primary metabolite of BBP in humans, were tested in female Wistar rats exposed by oral gavage from the 15th to the 17th gestation day. A significant increase in the incidence of undescended testes and decreased anogenital distance was observed in male rats from 250 mg/kg/d (NOAEL = 167 mg/kg/d), but maternal toxicity was observed at 167 mg/kg/d (weight gain and significantly reduced food intake) (Ema, 2003). In a study by the same team, a decrease in anogenital distance had been observed in male pups of female Wistar rats exposed from the 5th to the 17th gestation day at 500 mg/kg/d, also in the presence of maternal toxicity (Ema, 2002).

Fertility studies

In F344 male rats exposed for 10 weeks before mating with unexposed females, a decrease in sperm count, without altering fertility, was shown at 200 mg/kg/d (NTP, 1997).
In WU rats exposed by oral gavage for two weeks before mating, no decline in fertility was observed at 500 mg/kg/d but a significant decline in fertility, and testicular lesions were observed at 1000 mg/kg/d (Piersma, 1995).
In Wistar rats, no decline in fertility was observed in a single-generation study for exposure over 10 weeks before mating, until birth, at the dose of 418 mg/kg/d (no LOAEL) (TNO, 1993).

Multi-generational studies

In Sprague-Dawley rats exposed before mating and during gestation, a NOAEL of 100 mg/kg/d was identified. A decrease in ovarian weights in female F0 rats was shown at 500 mg/kg/d but reproductive performance (mating and fertility rates, gestation length, and number of live births) remained identical to the control group. The F1 offspring exposed *in utero*, during lactation and then by diet (10 weeks) to the dose of 500 mg/kg/d, also

maintained normal reproductive performance although decreased anogenital distance and weight of the testes and epididymis were observed. A transient decrease in birth weight in the F1 generation was observed from 100 mg/kg/d (NOAEL = 20 mg/kg/d). No systemic, developmental or reproductive toxic effects were observed in the F2 generation (Nagao *et al.* 2000).
In a more recent study conducted according to US EPA guidelines on two generations of Charles River rats (Tyl *et al.* 2004), there was a decline in fertility indices (mating and fertility rates) in F1 males exposed to 750 mg/kg/d *in utero*, during lactation. This was followed by dietary exposure up to mating for the next generation. The authors did not observe an absence of nipple regression and decrease in absolute but not relative weight of the testes and epididymides. Fertility in the F2 generation was not studied. Decreased anogenital distance was observed at 250 mg/kg/d.

Mechanism of action

Skeletal malformations appear to be due to a change in Py1a osteoblasts in rats. The morphology of cells exposed to BBP is modified in the microfilaments. This modification of the cytoskeleton interferes with good subsequent adhesion of osteocytes. BBP also acts as an anti-androgen by reducing fœtal testosterone levels, conferring effects on anogenital distance or testicular descent. BBP thus has an endocrine disruption mechanism.
On the basis of current knowledge, in spite of the lack of data on reproduction in humans and the differences in metabolism observed between humans and animals, the CES considers that the reprotoxic and developmental effects observed in animals could also occur in humans.

Analysis and assessment of the choices for establishment of the TRV

Critical effect

The various studies reviewed show that BBP is able to induce developmental effects during exposure *in utero*, as well as effects on fertility during exposure over several generations. Moreover, recent studies tend to confirm the endocrine disrupting properties of BBP, with absence of nipple regression, decreased weight of the testes and epididymides, or decreased anogenital distance among male offspring when exposed *in utero* or over several generations. It should be noted that the decrease in anogenital distance is the most sensitive marker, since it was observed at the lowest dose levels (LOAEL = 250 mg/kg/d in a multi-generational study). **The CES thus chose decreased anogenital distance as the critical effect. This effect indicates endocrine disruption.**

Pivotal Source study

The studies reviewed were of good quality and all were given a Klimish 1 rating (protocols following OECD or other guidelines). In multi-generational studies, early markers of anti-androgenic activity have been demonstrated (decreased anogenital distance or nipple retention) at low doses in the absence of maternal toxicity. This evidence is consistent with data on BBP that suggest endocrine disruption potential. The European Union has assessed the health risks and classified BBP as toxic to reproduction, Category 2, based on the decreased anogenital distance observed in the study by Tyl (2004) (European Union, 2007). The CES thus considers it appropriate to choose the study by **Tyl *et al.* (2004)** as the source study.
The final choice of the study by Tyl *et al.* (2004) for developing the reprotoxic TRV for BBP was based on:

- the quality of the study (Klimisch 1);

- the observation of effects indicating endocrine disruption in the absence of maternal toxicity;
- the most sensitive toxic effect observed (a NOAEL of 50 mg/kg/d was shown in the Tyl *et al.* study in 2004 for decreased anogenital distance, and a NOAEL of 100 mg/kg/d was demonstrated in the fertility study by Nagao *et al.* in 2000 for decreased weight of the ovaries).

Moreover, the choice of the study by Tyl *et al.* (2004) was supported by the fact that this study was used by the European Union to establish the NOAEL for BBP.

In this study, groups of 30 male and female CD rats were exposed to doses of BBP of 0 – 50 – 250 and 750 mg/kg/d, via diet, for 70 days prior to mating (generation F0). The next generation (F1) was then exposed for up to 70 days after birth and finally, the second generation (F2) was exposed for 28 days. At 750 mg/kg/d, the authors noted a decline in fertility indices (mating and fertility rates) in the F1 males, an increased nipple retention number and a decrease in absolute but not relative weight of the testes and epididymides. A decrease in anogenital distance was observed at 250 mg/kg/d ($p < 0.001$). The NOAEL for this effect is therefore 50 mg/kg/d.

Choice of the critical dose

In the study chosen, (Tyl *et al*., 2004), a significant decrease in anogenital distance was observed at **250 mg/kg/d**, corresponding to the **LOAEL**. The **NOAEL** was thus identified at **50 mg/kg/d**.

To determine this LOAEL, the authors used analysis of variance (ANOVA) and analysis of covariance (ANCOVA) methods. The homogeneity of variance hypothesis was examined using the Levene test. No benchmark dose (BMD) had been proposed from this study due to the methodological difficulties anticipated for the choice of response level. In fact, anogenital distance is a continual biological indicator. The development of a BMD would require defining the variation in anogenital distance considered as harmful in terms of toxicity data.

The CES considers, however, that the pair of LOAEL/NOAEL values can be proposed for establishment of the TRV. The minor difference between the two values, compared with other studies in the literature, supports the accuracy of the TRV.

Choice of uncertainty factors

- UF_A: inter-species variability: significant differences in metabolism were observed between humans and rodents, consequently the factor used is the maximum factor of 10 by default because there are no data on the specific health impact of MBuP or of MBeP, the main metabolites in rats and humans, respectively.
- 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.

The Expert Committee (CES) for “Assessment of risks linked to chemical agents” accepted the results of the collective expert appraisal at its meeting on 10 July 2008 and informed the Directorate General of AFSSET.

The CES emphasises the differences in the metabolism of BBP between rats and humans and the lack of knowledge about the impact of these differences on the health effects of BBP.

Conclusions of the collective expert appraisal

- No human studies specifically investigating the effects on reproduction or development are available for benzyl butyl phthalate (BBP), but numerous studies have been conducted in animals, to assess developmental effects on rodents in particular.
- The mechanism put forward (endocrine disruption by anti-androgenic activity) is plausible.
- The effects observed in animals (rats) seem relevant for humans, although differences of metabolites were noted.
- The decrease in anogenital distance can be used as the critical effect.

The CES thus proposes establishinga TRV specifically for effects on development. Given the demonstrated effects on the development of the reproductive system, which indicate endocrine disruption and whose window of critical exposure corresponds to the gestation period, the TRV will be applicable for sub-chronic exposure (for the duration of gestation). This TRV also protects against teratogenic effects demonstrated in animals, for which NOAELs were much higher.

-- Benzyl butyl phthalate (BBP) CAS No. 85-68-7 --			
Critical effect	**Critical dose***	**UF**	**TRV**
Decreased anogenital distance in male offspring Study of two generations of Sprague Dawley rats exposed by oral gavage Tyl *et al*. 2004	**NOAEL = 50 mg/kg/d** LOAEL = 250 mg/kg/d	**100** UF_A 10 UF_H 10	**TRV = 0.5 mg/kg/d** **Confidence level** Data collection: high Study: high Critical dose: high TRV: high

*Allometric coefficients: NIL. 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 TRV based on reprotoxic effects has not yet addressed this issue. The need for a better understanding of animal-human transposition and of the UF_A uncertainty factor led to the recommendation that further consideration be given to this aspect.

Recommendations of the CES

Given the differences in metabolism between animals and humans, the CES recommends conducting scientific monitoring of the metabolism of BBP to gain a better understanding of the metabolites responsible for the reproductive toxicity of BBP. Checking the literature will enable TRVs to be re-evaluated as new data are published.

[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.

The CES also recommends that scientific monitoring be conducted of studies of developmental toxicity using the same protocol as the study by Lee *et al.* (2004), which investigated the effects of di-n-butyl phthalate (DnBP) on development. Indeed, the CES noted that in the study by Lee *et al.*, a decrease in spermatocytes and nipple dysplasia was shown for doses as low as 2 mg/kg/d, while it is likely that BBP and DnBP have similar toxicity. Given the differences in the protocols of the toxicity studies chosen, the existence of a lower LOAEL for DnBP does not imply that it is more toxic than BBP.

Maisons-Alfort, 25 September 2008.

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

Chairman of the CES

Mr Michel Guerbet