Collective expert appraisal: summary and conclusions

Regarding the expert appraisal on setting occupational exposure limits for chemical agents

Evaluation of biomarkers of exposure and recommendations for biological reference values for

Di-n-butylphthalate (n° CAS 84-74-2)

This document summarises and presents the work of the Expert Committee on expert appraisal for recommending occupational exposure limits for chemical agents (OEL Committee) and the Working Group on biomarkers of exposure.

Presentation of the issue

On 12 June 2007, AFSSET, which became ANSES in July 2010, was requested by the Directorate General for Labour to conduct the scientific expert appraisal work required for setting occupational exposure limit values (OELVs) for di-n-butylphthalate (DnBP).

France currently has a mean eight-hour exposure value for di-n-butylphthalate of 5 $mg.m^{-3}$. This value was set in the Circular of 13 May 1987[1] of the Ministry of Labour (not published in the OJ).

The Directorate General for Labour asked ANSES to reassess this value and, if necessary, propose new occupational exposure limit values based on health considerations for di-n-butylphthalate.

ANSES OEL Committee decided to conduct the assessment of the biological monitoring data in occupational environment, in order to establish the relevance of recommending monitoring of one or more biomarkers in addition to the OEL and the establishment of biological limit values for the selected biomarker(s).

Scientific background

Biological monitoring of exposure in workplaces has emerged as a complementary method to atmospheric exposure measurement for assessing exposure to chemical agents. Biological monitoring assesses a worker's exposure by including all the routes by which a chemical

1 Supplementing and amending the Circular of 19 July 1982 on the acceptable values for concentrations of certain hazardous substances in workplace atmospheres.

penetrates the body (lung, skin, digestive tract). It is particularly worthwhile when a substance has a systemic effect, and:

- when routes other than inhalation contribute significantly to absorption,
- and/or when the pollutant has a cumulative effect,
- and/or when the working conditions (personal protection equipment, inter-individual differences in respiratory ventilation, etc.) determine large differences in internal dose between individuals that are not taken into account by atmospheric metrology.

With regard to prevention of chemical risk in the workplace, the French Labour Code authorises the use of biological monitoring of exposure and biological limit values.

OEL Committee definitions

Biomarker of exposure: parent substance, or one of its metabolites, determined in a biological matrix, whose variation is associated with exposure to the agent targeted. Biomarkers of early and reversible effects are included in this definition when they can be specifically correlated to occupational exposure.

Biological limit value (BLV): This is the limit value for the relevant biomarkers.

Depending on the available data, the recommended biological limit values do not all have the same meaning:

- if the body of scientific evidence is sufficient to quantify a dose/response relationship with certainty, the biological limit values (BLVs) are established on the basis of health data (no effect for threshold substances or risk levels for non-threshold carcinogens);
- in the absence of such data for substances with threshold effects, BLVs are calculated on the basis of the expected concentration of the biomarker of exposure (BME) when the worker is exposed to the 8-hour OEL. For carcinogens, in the absence of sufficient quantitative data, the biological limit value is calculated on the basis of another effect (pragmatic BLV). These last values do not guarantee the absence of health effects, but aim to limit exposure to these substances in the workplace.

Whenever possible, the OEL Committee also recommends biological reference values (BRVs). These correspond to concentrations found in a general population whose characteristics are similar to those of the French population (preferentially for biomarkers of exposure) or failing that, a control population not occupationally exposed to the substance under study (preferentially for biomarkers of effects).

These BRVs cannot be considered to offer protection from the onset of health effects, but do allow a comparison with the BME levels measured in exposed workers. These values are particularly useful in cases where it is not possible to establish a BLV.

Organisation of the expert appraisal

ANSES entrusted examination of this request to the Expert Committee on expert appraisal for recommending occupational exposure limits for chemical agents (OEL Committee). The Agency also mandated the Working Group on biomarkers for this expert appraisal.

The methodological and scientific aspects of the Working Group's work were regularly submitted to the OEL Committee. The report produced by the Working Group takes account of observations and additional information provided by the Committee members.

This expert appraisal was therefore conducted 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".

Preventing risks of conflicts of interest

ANSES analyses interests declared by the experts before they are appointed and throughout their work in order to prevent potential conflicts of interest in relation to the points addressed in expert appraisals.

The experts' declarations of interests are made public on ANSES's website (www.anses.fr).

Description of the method

Two rapporteurs from this working group were mandated by the Agency to produce a summary report on biomarkers of exposure (BMEs) and the recommendation of biological limit values (BLVs) and biological reference values for the BME(s) considered relevant. An ANSES employee also contributed to this report.

The summary report on the BMEs for di-n-butylphthalate (DnBP) was based on bibliographical information taking into account the scientific literature published on this substance until March 2013.

The bibliographical research was conducted in the following databases: Medline, Toxline, HSDB, ToxNet (CCRIS, GENE-TOX, IRIS) and ScienceDirect. The rapporteur reassessed the source articles or reports cited as references whenever he considered it necessary, or whenever the Committee requested it.

The collective expert appraisal work and its conclusions and recommendations were adopted on 13 December 2013 by the OEL Committee (term of office 2010-2013).

The collective expert appraisal work and the summary report were submitted to public consultation from 01/10/2014 to 01/12/2014. The people or organizations who contributed to the public consultation are listed in appendix of the report (only available in French). The comments received were reviewed by the OEL Committee (term of office 2014-2017) who adopted this version on 12 May 2015.

Result of the collective expert appraisal

Introduction

The scientific articles selected for evaluating biomonitoring data on di-n-butylphthalate were identified using the following keywords: "di-n-butylphthalate", "biomarker", "biomonitoring", "biological monitoring", "urine", "blood" and "occupational", while limiting the search to human data.

Toxicokinetics data

Skin contact does not appear to be a major route of absorption in humans (permeation flow of 0.07 µg.cm^{-2}.h^{-1} from Scott et al., 1987 and 0.59 µg.cm^{-2}.h^{-1} from Beydon et al., 2010). Absorption by inhalation has not been described in the literature for animals. For humans, oral absorption of DnBP has been observed but the absorption rate is not known. DnBP is absorbed in the gastro-intestinal tract as mono-n-butylphthalate (MnBP) due to high esterase activity (Silva et al., 2007).

There are no quantified data on distribution in human organs.

The side chain of MnBP may then be oxidised, causing 2 secondary metabolites to form: mono-3-hydroxy-n-butylphthalate (MHBP) and mono-3-carboxypropylphthalate (MCPP).

Anderson et al. (2001) showed that 64% and 73% (molar fraction, depending on the administered dose: 255 and 510 µg respectively) or 50 and 58% (mass fraction) of ingested DnBP was eliminated in the urine as MnBP (free and conjugated) within 24 hours. MnBP was no longer detectable in the urine 24 hours after the end of exposure. One study showed that conjugated (mainly glucuroconjugated) MnBP accounted for 94% of total urinary MnBP in humans (Seckin et al., 2009).

Choice of biomarkers of exposure and effect

Three metabolites of DnBP can be detected in human urine: MCPP, MnBP and MHBP.

Due to a lack of data on urinary concentrations of MHBP in the general population and in the workplace, this BME was not chosen.

MCPP was not chosen as a BME either since it is not specific to DnBP exposure: it is also a metabolite of di-n-octylphthalate.

The chosen BME is urinary MnBP since it is the main metabolite of DnBP and is associated, in some studies in humans, with sperm parameter abnormalities and decreased anogenital distance in male newborns whose mothers had higher urinary concentrations of MnBP. This BME is not specific to DnBP exposure since it is also a metabolite of BBzP, but in cases of exposure to BBzP, MnBP is seldom detected or not detected at all, even with high exposure (Anderson et al., 2001).

Therefore, the OEL Committee proposes only using urinary MnBP as a biomarker of occupational exposure to DnBP.

The literature reports certain effects of exposure to DnBP and more broadly to phthalates, and particularly effects on reproduction (changes in hormone levels, impaired sperm quality) and development (decreased anogenital distance at birth in boys born to mothers exposed to certain phthalates). In the absence of additional data, there are not enough studies to clearly identify a relevant target in humans or biomarkers to be monitored in the exposure-effect continuum (sperm counts, hormonal assays).

Information on biomarkers of exposure identified as relevant for the biological monitoring of exposed workers

Name	**Urinary mono-n-butylphthalate (MnBP)**
Other substances giving rise to this biomarker	Butylbenzyl phthalate
Concentrations found in exposed workers or volunteers	Field studies: Corresponding atmospheric exposure levels not specified – various industry sectors Urinary [MnBP] at end of shift: from 2 µg.g^{-1} creatinine (cr) to 1800 µg.g^{-1} cr Studies in volunteers: Anderson et al. (2001) - Oral exposure - 255 µg of DnBP: 129 µg of MnBP.24h^{-1} - 510 µg of DnBP: 298 µg of MnBP.24h^{-1}
Conversion factor	molecular weight (MW): 222.24 1 µg.L^{-1} = 0.0045 µmol.L^{-1} 1 µmol.L^{-1} = 222.24 µg.L^{-1} 1 µg.g^{-1} cr = 0.51 µmol.mol^{-1} cr 1 µmol.mol^{-1} = 1.96 µg.g^{-1} cr
Concentrations in the general population	USA-NHANES (2009-2010) - 95th percentile (20 years and over, 1914 samples): 68.9 µg.L^{-1} and 50.9 µg.g^{-1} creat (CDC, 2013)
Recommended limit values for exposed workers	None

Study of relationships between concentrations of BMEs for di-n-butylphthalate and certain health effects

Studies in human adults

The epidemiological studies in which BMEs for DnBP have been assessed in relation to health effects have primarily involved the general population. It should be noted that four publications (team of Hauser et al.: Duty et al., 2003; Duty et al., 2004; Duty et al., 2005 and Hauser et al., 2006) refer to a cross-sectional study (consultation for infertility in the same clinic) that appears to have been reproduced several times using the results of the previous study each time, thus increasing the population size with each publication but not showing independent results.

In 2003, Duty et al. found a relationship between increased urinary MnBP concentrations and lower sperm motility. In the second tertile of the concentrations that were studied (12.2 to 20.1 µg.L^{-1}), decreases in sperm motility were twice as common but this result was not statistically significant. However, in the highest tertile (20.2 to 433.9 µg.L^{-1}), decreases in sperm motility were three times more common with a 95% confidence interval, which was statistically significant.

The study by Hauser et al. (2006) with a larger population size (increased statistical power) confirmed the dose-response relationship between increased urinary concentrations of MnBP and decreased sperm motility. Decreases in sperm motility were 2 times more common from 30 µg.L^{-1}. This study, unlike that of Duty et al. (2003), showed a dose-response relationship between increased urinary concentrations of MnBP and lower sperm concentration (3 times more frequent in the 3rd exposure tertile).

Another study did not show any changes in sperm parameters or hormone levels in men around the age of 20 years whose median urinary concentration of MnBP was 78 µg.L^{-1} and whose 95th percentile concentration was 330 µg.L^{-1} (Jönsson et al., 2005).

In 2004, the team of Duty et al. showed a non-statistically significant negative relationship between urinary concentrations of MnBP and sperm motility (linear and curvilinear velocity) parameters.

Duty et al. (2005) showed a positive relationship, at the limit of significance, between concentrations of MnBP and inhibin B (a hormone playing a role in spermatogenesis). According to the authors, these results were not expected since previous studies had shown a statistical relationship between changes in certain sperm parameters and increased urinary concentrations of MnBP. This study therefore should have shown a decrease in inhibin B concentrations. According to the authors, it is difficult to know whether the results of this study reflected biological alterations or were obtained because multiple comparisons had been made.

Furthermore, no reduction in testosterone associated with increased concentrations of MnBP was found in the study by Duty et al. (2005). Pan et al. (2006) showed a significant increase in free testosterone concentrations in workers exposed to DnBP.

Studies in adult women

Few studies have assessed the possible role that exposure to phthalates may play in female reproductive toxicity. In the study by Weuve et al. (2009), with over 1200 women, the authors showed a dose-response relationship between increased urinary concentrations of MnBP and the prevalence of uterine diseases (pooled cases of endometriosis and leiomyomata). However, the study did not show a significant increase in uterine diseases and this dose-response relationship was not found in the study of separate data (endometriosis or leiomyomata). It is therefore difficult to interpret the results of this study in terms of a causal link. In another study, with a smaller population size (137 women), Itoh et al. (2009) also did not find an increase in the prevalence of endometriosis cases.

Studies in children in relation to maternal exposure

A research team studied the possible relationship between decreased anogenital distance at birth and concentrations of phthalate metabolites in the urine of mothers (Swan et al., 2005). Swan's team found an increased frequency of decreased anogenital distance in newborns statistically associated with an increase in maternal urinary concentrations of MnBP (and of MEP, MEHP, MEHHP and MEOHP)[2].

[2] Mono-ethylphthalate MEP, mono-ethylhexylphthalate MEHP, mono-2-ethyl-5-hydroxyhexylphthalate MEHHP and mono-2-ethyl-5-oxohexylphthalate MEOHP

Table 1: summary of the data of epidemiological studies comparing urinary concentrations of MnBP and effects on reproduction in adults and development

Reference	Population and size	Urinary concentrations of MnBP **Median** (95th percentile)			Reproduction parameter	Levels of exposure associated with significant effect ($\mu g.L^{-1}$)
		$\mu g.L^{-1}$		$\mu g.g^{-1}$ cr		
		Without adjustment	Adjusted to urinary density[3]			
Duty et al., 2003	143 men, infertile couples	**15.9** (73.1)	**16.2** (58.5)	-	Decrease in sperm motility	20.16 – 433.93 (adjusted for specific gravity)
Duty et al., 2004	220 men, infertile couples	**17.8** (90)	**18.0** (73.9)	-	Decrease in sperm motility	-
Duty et al., 2005	295 men, infertile couples	**14.3** (75.4)	**16.2** (69.9)	-	Increase in the level of inhibin B (hormone)	-
Hauser et al., 2006	463 men, infertile couples	-	**17.7** (69.9)	-	Decrease in sperm concentration and motility	31.7 – 14 459 (adjusted for specific gravity)
Jönsson et al., 2005	234 Swedish men (military service)	**78** (330)	-	**47** (159)	No association with sperm counts or hormones	-
Swan et al., 2005	85* mothers	**13.5** (75th p 30.9)	-	-	Decreased anogenital distance in boys Frequency multiplied by 4	7.2 – 30.9
					Decreased anogenital distance in boys Frequency multiplied by 10	> 30.9
Pan et al., 2006	63 unexposed controls	-	-	**113.5** (434.5)	Significant correlation with reduction in free testosterone (fT) in exposed workers	-
	74 exposed workers (PVC)	-	-	**548.4** (8781.2)		-

* The article does not clearly state if the population size is 85 or fewer newborn boys

Study of correlations between urinary concentrations of MnBP and atmospheric concentrations

No field studies have examined the correlation between DnBP exposure by inhalation and biological levels of MnBP in urine.

[3] 1.024 as reference value

Only two studies in the workplace were identified. These report urinary concentrations of MnBP but not the corresponding atmospheric concentrations.

		MnBP		*References*
		Median - maximum value, *in µg/L and [µg/g of creatinine]*		
Business sector	n	Mid shift	End of shift	
Phthalate manufacturing	9	**230 [121]** 1240 [647]	**1010 [363]** 4680 [1750]	*Hines et al. 2009*
PVC film manufacturing	25	**26 [13]** 152 [39]	**26 [15]** 116 [86]	
Vehicle filters	18	**18 [16]** 202 [101]	**31 [21]** 360 [163]	
PVC compounding	12	**38 [24]** 196 [92]	**63 [29]** 169 [92]	
Rubber hoses	25	**211 [130]** 1580 [1370]	**257 [135]** 1790 [1810]	
Rubber boots	21	**39 [26]** 326 [592]	**57 [32]** 321 [313]	
Rubber gaskets	20	**334 [269]** 1930 [623]	**643 [403]** 2010 [1320]	
Nail-only salons	25	**29 [30]** 114 [77]	**38 [38]** 147 [119]	

		MnBP		*References*
		Median (25^{th} percentile – 75^{th} percentile) ($\mu g.L^{-1}$)		
Business sector	n	Start of shift	End of shift	
Nail salons	37	**58.5** (32.3 – 10.7*)	**87.2** (33.8 – 160)	*Kwapniewski et al. 2008*

* the value entered for the 75th percentile does not seem right

Experimental data

Due to the lack of relevant studies in humans, the OEL Committee used a study in animals to establish an 8h-OEL (reduction in foetal testosterone concentrations). Moreover, it was demonstrated that the critical exposure window for anti-androgenic effects on offspring (generation F1), identified in animal studies[4], could be transposed to occupational exposure (exposure in the 1^{st} trimester of pregnancy).

The OEL was established based on an NOAEL in animals (oral route) of 10 $mg.kg^{-1}.day^{-1}$. This critical dose in animals was extrapolated to humans through allometric adjustment. An equivalent oral dose for humans of 2.44 $mg.kg^{-1}.day^{-1}$ was thus obtained. In 2001, Anderson et al. reported data on urinary excretions of MnBP in humans that could be used in a mass conservation equation. This type of equation can calculate urinary concentrations of MnBP based on ingested doses (Kohn et al, 2000).

[4] Effects observed in rats at birth when the mothers had been exposed during gestation.

Establishment of BLVs and choice of biological reference values

The epidemiological studies in which BMEs for MnBP have been assessed in relation to health effects have primarily involved the general population.

Epidemiological studies on the fertility parameters of men of reproductive age are in favour of a relationship between urinary concentrations of DnBP and sperm quality (Duty et al., 2003; Duty et al. 2004; Hauser et al., 2006). However, they do not quantify a potential dose-response relationship (exposure classes too broad and too few studies considering that these studies are not independent).

The study results of Duty et al. (2005) (population of patients in an andrology laboratory) and Pan et al. (2006) on the hormonal levels of exposed workers should be taken into account in terms of possible toxicity to male fertility but alone do not draw any quantitative conclusions.

The results of the two studies by Swan et al. (2005 and 2008[5]) showing an increase in the prevalence of newborn boys with decreased anogenital distance linked to increased urinary concentrations of MnBP need to be confirmed by other studies. Furthermore, this study, which examines maternal environmental exposure to several phthalates, shows significant relationships between reduction in anogenital distance and concentrations of all of the measured phthalate metabolites, considered separately or with an overall exposure score. Lastly, the highest Odds Ratios are those obtained with MnBP, with no adjustment for concentrations of other metabolites.

In conclusion, the results of studies in humans suggest a reprotoxic effect of DnBP as found in studies in men and boys. While some reprotoxicity parameters are linked to urinary concentrations of MnBP, most results on impaired sperm quality come from one and the same study population and other fertility parameters still require further investigation. Additional data would be required to interpret these results and especially to choose a critical concentration of MnBP.

It is therefore not possible, in the current state of knowledge, to recommend a biological limit value on the basis of health effects.

There are no workplace studies reporting both urinary concentrations of MnBP and atmospheric concentrations of DnBP. Therefore, it is not possible to establish a BLV based on exposure to the 8h-OEL.

The calculations proposed by Kohn et al. (2000) cannot be used to extrapolate the oral human equivalent dose to urinary concentrations because they include too many uncertainties. The kinetic parameters of MnBP and its metabolites were described for oral absorption of only 2 different doses of DnBP, and excretion fractions were reported over 24 hours (Anderson et al. 2001). Therefore, no biological value can be established based on the experimental data.

Thus, since it is not possible to recommend a biological limit value, biological reference values can be proposed.

[5] The study by Swan et al. (2008) is not reported here since no BME measurements are reported in the publication (the results cannot be used in this context).

Proposed biological reference values

There are no French data reporting urinary levels of MnBP for large samples of the general population[6].

Furthermore, the data from other European countries such as Sweden (Jönsson et al., 2005) and Germany (Wittassek et al., 2007 and Göen et al., 2011) involve specific populations such as students only or men only, and cannot be used as a basis for recommending biological reference values.

Only the data in the American national NHANES survey can be used to establish biological reference values. The data reported in this survey show that urinary concentrations of MnBP vary significantly based on gender and age. And yet this study's reports present the results either by age or by gender and so it was not possible to identify, in the age group of interest (over 20 years), urinary concentrations for men and women separately.

The urinary concentration of 70 µg.L^{-1} or 50 µg.g^{-1} of creatinine for MnBP corresponding to the 95th percentile of the distribution of urinary concentrations for men and women, taken from the 2009-2010 campaign, has been proposed as the biological reference value, but it should be noted that urinary concentrations in women can be higher than those in men.

Conclusions of the collective expert appraisal

The biological values proposed for monitoring exposure to di-n-butylphthalate are:

Urinary mono-n-butyl phthalate

BLV based on health effect	None
BLV based on exposure to the 8h-OEL	None
Biological reference value	70 µg.L^{-1} or 50 µg.g^{-1} creatinine

Sampling method and factors that may affect the interpretation of results

Samples can be taken at end of shift.

Urine samples must be taken in tubes that have been tested and that have no detectable levels of phthalates. Urine samples must be frozen after they are collected at –20°C (Blount et al. 2000a) and –70°C (Silva et al., 2008) in order to be stored for several years.

In addition to expected differences in concentrations between men and women, some medications can cause urinary concentrations of MnBP to increase since they contain DnBP (excipient).

[6] There are no results involving BME assays for phthalates in French national studies (ENNS and Esteban). One cohort (ELFE) is intended to measure BME concentrations (particularly for phthalates) in young children (from birth to adulthood) and their mothers, but the results have not yet been published.

Biomonitoring

urinary MnBP		
Inter-laboratory quality control	NS	
	Method 1	**Method 2**
Analytical technique	LC-APCI-MS/MS	LC-ESI-MS/MS
Limit of detection	0.94 µg. L^{-1}	0.50 µg. L^{-1} (signal/noise = 3)
Limit of quantification	NS	5 µg. L^{-1} (signal/noise = 10)
Trueness	NS	
Precision		
Reference standard	Standard solution of $^{13}C_4$ MBP	Standard solution: d_4-MBP
Treatment before analysis	1:30 treatment at 37°C with ß-glucuronidase[7]. SPE extraction of metabolites.	1 hr. treatment at 37°C with ß-glucuronidase. SPE extraction of metabolites.
References	Silva et al 2003	Pan et al. 2006

NS = Not specified

[7] *ß-glucuronidase from Escherichia Coli, since it has no lipase activity on phthalate diesters. Deconjugation is monitored by incorporating 4-methyl-umbelliferryl-glucuronide ($^{13}C_4$) in the samples.*

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