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SCIENTIFIC COMMITTEE ON TOXICITY, ECOTOXICITY AND THE ENVIRONMENT (CSTEE)

Opinion on

the report:

Validation of methodologies for the release of diisononylphthalate (DINP) in saliva simulant from toys (2001 EUR 19826 EN)

Expressed at the 25th CSTEE plenary meeting

Brussels, 20 July 2001

Background

In the light of previous opinions of the CSTEE on test methods for phthalate migration from soft PVC toys and child care articles and in particular of the opinion of 5 September 2000 on a programme for validation of test methods for phthalate migration, the JRC has developed and co-ordinated a programme for validating methodologies for measuring the release of DINP in saliva simulant from toys.

Terms of reference

The Committee on the basis of examination of the following report: Validation of methodologies for the release of diisononyl phthalate (DINP) in saliva simulant from toys, European Commission DG Joint Research Centre, 2001 EUR 19.826 EN,

is to answer the following questions :

(1) is the programme for validating the test methods as carried out by the JRC of a good scientific quality ?

(2) is the head over heels method that is considered to be validated by the JRC suitable to verify compliance of soft PVC toys and child care articles containing phthalates with the migration limits set by the CSTEE for the phthalates?

In assessing the validity of the method, the CSTEE should also take into account differences in the exposure of children to phthalates, which could result from differences in the intended uses of the various categories of soft PVC toys and childcare articles.

CSTEE's answers to the questions

(1) The programme for validation of methods to measure migration rates of DINP from PVC articles to a saliva simulant has generally been carried with a good scientific quality. However, the feedback from the participating laboratories should have been included in the report. This shortcoming is probably explained by time constraints, but CSTEE anticipates that comments from the laboratories are taken into account in the continued work. Some further comments on the report are given below under “Detailed comments”.

(2) The head over heals method gives results with good repeatability (within lab variation) and acceptable reproducibility (between lab variation), and the results are comparable with the mean of the results obtained in earlier *in vivo* studies. The guidance value (6.7 $\mu g/10cm^2/min$) recommended by the CSTEE was set to protect the individual with the highest exposure. This value is, however, higher than the results obtained with the head over heal method. It may be possible to overcome this difference by the use of a factor.

The migration results obtained with these *in vitro* methods are not dependent on the intended use of the investigated items.

Detailed comments

To simulate the migration of phthalates, when infants and small children suck/chew soft PVC toys, TNO, NL and LGC, UK proposed 3 *in vitro* methods for dynamic migration of DINP from PVC toys:

- Head over Heals (HoH) method[1] proposed by TNO, NL,
- Horizontal Shaking, Mild conditions (HSM)[2], proposed by LGC, UK, and
- Horizontal Shaking, Stringent conditions (HSS)[2], proposed by LGC, UK

An inter-laboratory comparison of the determination of migration of DINP from PVC toys, with the aim to validate the *in-vitro* methods mentioned above, are described in the report presented to CSTEE (European Commission DG Joint Research Centre, 2001 EUR 19.826 EN).

The validation exercise was organised and performed according to the guideline ISO 5725[3] and IUPAC harmonised protocol[4], as recommended by CSTEE[5]. DINP was chosen as model plasticiser for the study. Fifteen laboratories from EU and USA participated in the validation exercise, in which migration of DINP from a reference PVC material and 5 PVC toys were determined. The reference PVC and the toys with known formulation and manufacturing process were especially prepared for the validation study. The CSTEE would have preferred to see the actual formulations and manufacturing process reported. It was anticipated that the rate of DINP release from at least one of the toy samples would be higher than the proposed guidance value: >6.7 μg/10cm^2/min. This toy should be identified in the report. Not all laboratories reported all of the results, but the comments from the participating laboratories (such as time constraints, apparatus breakdown, other problems…) are unfortunately not provided in the report.

All 3 methods are based on the principle that the phthalate from a toy sample is released in the artificial saliva under predefined mechanical agitation conditions, and that it is extracted in cyclohexane and determined by either HPLC and/or GC-MS. The chosen internal standard, butylbenzylphthalate (BBP), is far from perfect, especially as it may be used as a plasticiser in PVC. The artificial saliva used in the study is devoid of any organic substance. Only acceptable results (outliers determined by Coachran's and Grubb's test) have been used for the statistical evaluation of the method performance by one-way analysis of variance (ANOVA).

A standard operating procedure (SOP) and test samples were provided to the participating laboratories. To ascertain the minimum possible variation in the analysis in various laboratories, the same type of chromatography columns, shakers and other important materials were also provided to participants,.

The procedure adopted for the homogeneity testing of the reference material and toy samples was adequate. The results of homogeneity testing indicated that all samples were drawn from homogenous populations of test materials with respect to DINP content. The absolute recovery must be possible to determine, as the laboratory had received the DINP product used in the material. The concentration of DINP in the used product should have been reported, as well as the recovery and its range. The coefficient of variation (CV) of recoveries from test materials varied 1.7-5.5%, 2.75% for reference material.

The recoveries of DINP from artificial saliva fortified with this substance (5 μg/ml and 25 μg/ml) were acceptable when HoH (recovery 92-94%) and HSM (recovery 75-94%) were used. However,

DINP recovery from the artificial saliva was relatively low (65-84%) when the HSS method was used. The recoveries at the target DINP release rate 9μg/min (or close to target DINP release rate) corresponding to the concentration level 11 μg/ml should be reported (recoveries at 15 μg/ml have been investigated). The repeatability of DINP recovery experiments (mechanical agitation of the fortified saliva + extraction in cyclohexane + analytical determination) within individual laboratories (RSD_r) was 1-5% for all 3 methods. However, the inter-laboratory reproducibility (CV, RSD_R) of DINP recovery from fortified saliva were 12-16%, 14-43% and 13-41% respectively for HoH, HSM and HSS methods. As the relative standard deviation of repeatability for (RSD_r) all 3 methods are comparable (range 1-5%), the wider range of reproducibility by HSS and HSM methods compared to that by HoH might be associated with the differences in the principles of mechanical agitation used in the 3 methods.

The performance of the methods subjected to validation for the analysis of migration of DINP is described in Table 1. The HoH method was validated with enough number of acceptable (valid) results (from ≥8 laboratories) according to ISO and IUPAC guidelines. However, this was not the case when the horizontal shaking method was used (results from 6-9 laboratories were accepted for various toy samples). This means that the horizontal shaking methods cannot be regarded as validated by the collaborative trial. However, as the numbers of valid results obtained employing horizontal shaking methods are very close to that recommended by ISO/IUPAC, the performances of these methods have also been evaluated in the present document.

The average rate of DINP release from the reference PVC by HoH and HSS were comparable, and they were also similar to the DINP release rate from the reference PVC disk described in earlier studies[1,2,6]. However, the average rate of DINP release from reference PVC as well as from PVC toys by HSM were at least 3x lower compared to the rate of DINP release when HoH or HSS methods was used. The HSM method is, therefore, not suitable for the *in vitro* analysis of migration of DINP from PVC toys, and this will not be discussed further.

The repeatability (RSD_r) of DINP release from reference PVC as well as from PVC toys, measured within individual laboratories, both by HoH and HSS were <10%; and thus, they were acceptable. The inter-laboratory reproducibility of the HoH method (RSD_R, CV) was approximately 30% for the measurement of DINP release rate from reference PVC, and that was 35-65% for when toy samples were analysed. The reproducibility of the determination of DINP release rate from both reference PVC and toy samples by HSS was 64-140%. Thus, although the mean DINP release rates determined by HoH and HSS method appear to be similar (Table 1), the range of the results from different laboratories are relatively large when HSS method was employed (Table 2A and 2B, Annex 5 of the report). This may have a great influence on compliance testing of toys with respect to release of DINP. For example, all test samples may be approved (when the rate of release of DINP for compliance is considered to be 6.7 *μg/10 cm^2/min)* by all testing laboratories using HoH as test method. However, the toy samples of Duck and Gloworm will not be approved by some of the laboratories using HSS as the test method (Table 2A and Table 2 B, Annex 5 of the report).

Table 1: Summary of method performance - DINP release rates

Sample	Method	No. of valid results (laboratories)		Mean DINP release rate *µg/10 cm²/min*		RSD_r: repeatability relative standard deviation (%)		RSD_R: reproducibility relative standard deviation(%)		No. of outliers (laboratories)	
		GC-MS	HPLC	GC-MS	HPLC	GC-MS	HPLC	GC-MS	HPLC	GC-MS	HPLC
Reference PVC	HoH	14	12	3.72	4.06	6	6	28	33	0	0
	HSS	7	8	3.46	3.13	4	5	83	140	2	1
	HSM	8	9	0.40	0.67	8	13	79	101	2	1
PVC toy 001 Duck	HoH	10	11	5.17	6.07	6	4	58	48	1	1
	HSS	7	7	8.67	10.07	8	9	89	91	1	1
	HSM	8	7	1.70	2.00	12	9	110	92	1	2
PVC toy 002 Gloworm	HoH	14	10	6.15	4.19	6	5	43	35	0	2
	HSS	8	7	5.37	4.97	9	8	114	121	0	2
	HSM	8	7	0.72	0.83	12	11	73	81	2	2
PVC toy 004 Nikki	HoH	12	10	4.32	3.80	6	10	60	65	1	2
	HSS	8	8	4.04	3.09	4	4	64	106	0	1
	HSM	9	7	0.94	1.06	8	8	62	68	1	2
PVC toy 005 Betsy	HoH	11	10	1.74	2.36	8	7	41	53	1	2
	HSS	6	8	2.74	2.84	6	9	78	77	1	0
	HSM	7	7	0.46	0.57	12	20	51	122	2	2
PVC toy 006 Tiny	HoH	12	10	3.20	3.46	8	6	41	50	0	2
	HSS	7	8	3.36	4.03	5	8	100	85	0	0
	HSM	7	7	0.52	0.59	16	19	93	110	1	2

Table 2A: Range of DINP releases rate from test materials, when GC-MS was used as analytical technique for DINP determination.

Sample	Method	DINP release rate*, (average of 5 measurements in each laboratory) µg/10 cm^2/min	DINP release rate*, all measurements µg/10 cm^2/min
Reference PVC	HoH	2.5-4.5	2.2-5.2
	HSS	1.1-7.0	0.7-7.3
001 Duck	HoH	Not available	Not available
	HSS	3.0-15.8	2.5-17.6
002 Gloworm	HoH	3.7-8.4	3.1-9.9
	HSS	0.8-11.2	0.8-13.8
004 Nikki	HoH	1.9-7.0	1.8-7.3
	HSS	1.1-6.3	1.0- >10.0
005 Betsy	HoH	1.2-2.2	0.5-2.5
	HSS	0.0-4.0	0.0-4.5
006 Tiny	HoH	2.5-4.7	2.0-5.6
	HSS	0.2-6.4	0.0-7.5

*only accepted results, approximate values derived from Figures in Annex 5 (precise range should be reported)
Not available: Figures in page 59 of the report are missing!

Table 2B: Range of DINP releases rate from test materials, when HPLC was used as analytical technique for DINP determination.

Sample	Method	DINP release rate*, (average of 5 measurements in each laboratory) µg/10 cm^2/min	DINP release rate*, all measurements
Reference PVC	HoH	2.7-5.5	3.1-6.9
	HSS	0.5-7.2	0.5-7.8
001 Duck	HoH	Not available	Not available
	HSS	4.0-18.2	2.8- >20.0
002 Gloworm	HoH	3.0-6.3	2.1-6.5
	HSS	0.7-10.0	0.5-11.4
004 Nikki	HoH	1.2-5.6	1.0-8.5
	HSS	0.5-6.4	0.5-7.2
005 Betsy	HoH	1.7-4.2	1.3-4.8
	HSS	0.5-4.0	0.4-4.5
006 Tiny	HoH	2.0-5.7	1.5-6
	HSS	1.4-6.8	0.6-8.2

* only accepted results, approximate values derived from Figures in Annex 5 (precise range should is not described in the report)

Not available: Figures in page 59 of the report are missing!

The validation exercise has thus revealed the following:

- The HoH method for the determination of DINP release rate from PVC toys is reproducible with a relative standard deviation of the method (RSD_R) being 35-65%. None of the toys tested in the validation exercise revealed a DINP release > 6.7 µg/10 cm^2/min (the proposed guidance value), not even from the toy which was anticipated not to comply with this value (sample not identified in the report).

- The HSS method may appear to be comparable to HoH method with respect to average DINP release from various toys, but the reproducibility of the method (RSD_R 64-140%) is much inferior to HoH method. The large variations among the accepted results from various laboratories indicated that some of the participating laboratories may approve a toy while some others may not, considering 6.7 µg/10 cm^2/min as DINP release rate for compliance.

- The DINP release rate from reference PVC as well as from toy samples by HSM method were at least 3 fold lower to that by other two methods, and they were far lower than the DINP release rate observed in *in vivo* studies employing reference PVC. Therefore, this method may not be suitable for the testing of toys for DINP release rates.

In the table on page 21 there is a mistake in the unit for area specific migration, the correct unit should be µg/min/10 cm^2.

Discussion/Conclusions

The results of the validation exercise indicated that the release of DINP by sucking/chewing PVC toys cannot be simulated *in vitro* by the HSM method as the observed release of DINP from reference PVC as well as from toys was rather low and the reproducibility of the method was poor (62-110%). The reproducibility of DINP release rate from the reference PVC and the toys tested by the HSS method was 64-140%. As a consequence, some laboratories using the HSS method for testing of PVC toys may approve a toy while some others may not. Therefore, the HSS method may also not be suitable as a standard method for the *in vitro* analysis of DINP release from toys. The interlaboratory reproducibility (RSD_R) of DINP release by the HoH method for the reference PVC was approximately 30% and that was 35-65% for 5 different toys. Thus, the reproducibility of the HoH method was better than that for the other 2 methods investigated in the present validation exercise, but it may not be optimal. The implications of permitted maximum reproducibility of a standard t test method on the compliance of a product with respect to DINP release have been described in an earlier CSTEE document[7]. It was shown that test results with a permitted reproducibility (RSD_R) of 20% (with 1 SD) may exceed the regulatory limit (in other words TDI) by 50%, but it will pass the test. Allowance of 30% reproducibility (with 1 SD) will result in 90% excess of TDI.

The HoH method can thus only be used as a standard method if the rates of release determined by this method are corrected for the large variations among the laboratories. An additional reason for using a correction factor may also be as follows:

The DINP release rate from reference PVC by the HoH method was lower (5.2 μg/10 cm^2/min worst case by GC-MS analysis and 6.9 μg/10 cm^2/min worst case by HPLC analysis) than the worst case DINP release (8.9 μg/10 cm^2/min) observed in *in vivo* study[6.] This may be further supported by the fact that the mean DINP release rate over the proposed guidance value (6.7 μg/10cm^2/min) was not obtained for any of the toys tested, not even from the toy which was especially manufactured to release high amounts of DINP.

The following additional points should also be considered when recommending HoH as a standard method for the determination of phthalate release from PVC toys *in-vitro*:

- The method has only been validated for the release of DINP from PVC toys.
- BBP has been used an internal standard for the determination, but that is also included in the proposed regulation. A suitable internal standard has not yet been identified.
- The release rates obtained using HPLC analysis of DINP are reported to be similar to those by GC-MS analysis. As several phthalates elute in HPLC with the same retention time as the DINP, the HPLC cannot be used as a standard method of analysis unless it is documented that a test sample contains only this phthalate.
- The of calibration curve for DINP by GC-MS showed quadratic regression, but the method recommends use of linear regression.
- The method must recommend that 5 subsamples of each product should be analysed, as this has been used for the calculation of repeatability and reproducibility of the method.

Finally, the final version of the method (SOP) must include the suggestions from the experiences achieved by the participating laboratories.

References

1. CSTEE/97/1 - Add 149 [Validation of the method "Determination of Diisononylphthalate in saliva simulant" – TNO report V99.598 from Rinus Rijk & Karl Ehlert (TNO Nutrition and Food Research Institute) – 27 May 1999].

2. CSTEE/97/1 – Add 148D (Consumer Safety Research Report – LGC Technical Report Number : LGC/1999/DTI/004 – June 1999 "Inter-laboratory Validation of Laboratory-based Agitation Methods for the Determination of Phthalate Plasticiser Migration from PVC Toys and Childcare Articles").

3. ISO 5725: Accuracy (trueness and precision) of measurement methods and results, Part 1-6, (1994).

4. Horowitz, W. IUPAC: protocol for the design, conduct and interpretation of method performance studies., *Pure & Applied Chemistry* **67**, 331-343 (1995).

5. Opinion of the Scientific Committee on Toxicity, Ecotoxicity and the Environment (CSTEE) on validation of test methods for phthalate migration – Opinion expressed at the 17th CSTEE plenary meeting, Brussels, 5 September 2000

6. Report from Dutch Consensus Group: Phthalate release from soft PVC toys. RIVM report 613320 002, RIVM, NL. September 1998.

7. Addendum to Opinion on TNO, LGC and U.S. CPSC reports on phthalate migration test validation adopted at the 11th CSTEE plenary meeting on the 28th September 1999 – adopted at the 12th CSTEE plenary meeting on 25th November 1999.