CARBOFURAN (ADDENDUM)

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Explanation

Carbofuran is the ISO approved common name for 2,3-dihydro-2,2-dimethylbenzofuran-7-yl methylcarbamate, a broad spectrum *N*-methyl carbamate insecticide and nematicide that acts by inhibiting acetylcholinesterase activity in nervous tissues. Carbofuran was previously evaluated by the Joint Meeting in 1976, 1979, 1980, 1982, 1996, and 2002. In 1996, an acceptable daily intake (ADI) of 0–0.002 mg/kg bw was established based on the no-observed-adverse-effect level (NOAEL) for inhibition of erythrocyte acetylcholinesterase at 0.22 mg/kg bw per day in a 4-week dietary study in dogs, and using a safety factor of 100. In 2002, an acute reference dose (ARfD) of 0.009 mg/kg bw was established based on the NOAEL of 0.22 mg/kg bw per day in a 4-week study in dogs, and using a safety factor of 25, as the relevant toxic effects of carbofuran are dependent on the C_{max}.

The present Meeting evaluated newly submitted studies of acute toxicity in rats (adults and pups) and a newly submitted study in human volunteers (conducted in 1976), and re-examined relevant data from short-term studies of toxicity in dogs, which had been considered by previous Meetings. All pivotal studies were certified as complying with good laboratory practice (GLP) or an approved quality assurance programme.

Evaluation for acute reference dose

1. Toxicological studies

1.1 Acute toxicity

(a) Dose range-finding studies in rats aged 11 days

In a dose range-finding study of acute toxicity, groups of three male and three female Crl:CD®(SD) rats were given a single dose of carbofuran (purity, 99%) in corn oil by oral gavage at dose of 0.3, 0.6 or 1.0 mg/kg bw (dose volume, 5 ml/kg bw) on postnatal day 11; there was no concurrent control group. The pups were taken from two lactating dams with reconstituted litters (five males and five females per litter). After dosing, the pups were returned to their dams and examined for clinical signs of toxicity at approximately 15, 30, 60, 90, 120, 240 and 360 min after dosing. At 360 min after dosing, each pup was weighed, examined, terminated by carbon dioxide (CO_2) asphyxiation, and blood was collected via cardiac puncture. Also, the brain was removed, weighed and homogenized for analysis of acetylcholinesterase activity. The assays for acetylcholinesterase enzyme activity were performed by an Ellman method modified for use on an automated clinical chemistry analyser (for details, see Tyl et al., 2005c, below). The study was conducted in accordance with the principles of GLP.

There were no deaths and no pups were found in a moribund condition in any group up to termination at 6 h after dosing. Mean body weights of males and females at 0.3 and 0.6 mg/kg bw were slightly higher at termination than at dosing, while mean body weights at 1.0 mg/kg bw were slightly lower. Mean brain weights were similar across groups at termination for males and females.

Treatment-related clinical signs were first observed at 2 min after dosing. The number of affected rats initially increased with time after dosing as well as with dose. The severity of the signs observed also initially increased with dose and with time after dosing, with peak incidence and severity occurring approximately 10–19 min after dosing, with subsequent reductions in incidence and severity over time to no clinical signs after 42 min after dosing at 0.3 mg/kg bw, after 30 min at 0.6 mg/kg bw, and after 60 min at 1.0 mg/kg bw. The clinical observations were limited to fine tremors initially, then whole-body tremors, and then large head tremors. Over time after dosing, the tremors became intermittent, with large head tremors persisting for the longest time in all three groups receiving carbofuran. Since at age 11 days rat pups are just beginning to open their eyes (most of them have not yet opened their eyes), lacrimation was not observed. As the dams groomed the pups after dosing and while they were experiencing tremors, it was also not possible to observe salivation. The dams and their litters were housed in polycarbonate cages with bedding, and the dams kept their pups in tight piles and groomed them constantly. Therefore, urination and defaecation were also not observed. Once the pups stopped experiencing tremors, they began to nurse and then sleep in the nursing "pile". The slight loss of pup body weight in males and females at 1.0 mg/kg bw between dosing and termination 6 h later is most likely to be because thee pups experienced the longest period of tremors and, therefore, the shortest period of nursing before scheduled termination.

Erythrocyte acetylcholinesterase activity at termination was similar across groups for males and females. Brain acetylcholinesterase activity in males was statistically significantly reduced at 0.6 mg/kg (91% of the value at 0.3 mg/kg bw) and at 1.0 mg/kg (71% of the value at 0.3 mg/kg bw). For females, there were no statistically significantly differences in brain acetylcholinesterase activity between groups (Table 1).

The NOAEL in postnatal day 11 pups was < 0.3 mg/kg bw, based on treatment-related clinical signs (tremors) at a dose of 0.3 mg/kg bw and above. For brain acetylcholinesterase activity, a NOAEL could not be identified since there was no concurrent control group in this study by design (Tyl et al., 2005a).

In a dose range-finding study of acute toxicity, groups of five male and five female Crl:CD®(SD) rats were given a single dose of carbofuran (purity, 98.8%) at 0, 0.03, 0.1 or 0.3 mg/kg bw in corn oil by oral gavage (dose volume, 1 ml/kg bw) on postnatal day 11. Pups from five litters (of four male pups and four female pups per litter) were assigned by consecutive numerical order to the four dosing groups, one pup per sex per litter per group. Clinical observations were recorded before dosing, 1–9 min after dosing and/or 15 min after dosing (before sacrifice for pups from two out of five litters only, due to a deviation from the study protocol). Body weights were recorded once during the period before dosing and on the day of dosing. Samples of whole blood were collected approximately 15 min after dosing, by decapitation. The blood samples were processed for erythrocyte acetylcholinesterase determinations within 15 min and analysis began within 1 h after collection. Immediately after the collection of the blood sample, the brain was excised, weighed and assayed for acetylcholinesterase activity. The assays for acetylcholinesterase enzyme were performed by an Ellman method modified for use on an automated clinical chemistry analyser (for details see Hoberman, 2007c, below). The study was conducted in accordance with the principles of GLP.

There were no deaths. Two of the five males and two of the five females in the group at 0.3 mg/kg bw experienced slight to moderate whole body tremors after dosing, immediately before sacrifice. All other pups survived dosing with no adverse clinical observations. Body weights were comparable between the groups for male and female pups.

No statistically significant reductions in erythrocyte acetylcholinesterase activity occurred for either the male or female pups. Brain acetylcholinesterase activity was significantly reduced in groups at the 0.1 and 0.3 mg/kg bw compared with values for the control group (Table 2).

The NOAEL in pups on postnatal day 11 was 0.03 mg/kg bw on the basis of significant and > 20% inhibition of brain acetylcholinesterase activity at a dose of 0.1 mg/kg bw and above. Treatment-related clinical signs (tremors) were observed at 0.3 mg/kg bw only, the highest dose tested (Hoberman, 2007a).

(b) Time-course studies of acetylcholinesterase inhibition

In a study designed to identify the time of maximum inhibition and the time of complete recovery of acetylcholinesterase activity in erythrocytes and brain, groups of 35 male and 35 female pups at postnatal day 11 and groups of 35 male and 35 female adult (age 60 days) Crl:CD®(SD) rats

Table 1. Relevant findings in rat pups dosed with carbofuran on postnatal day 11

Finding	Dose (mg/kg bw)					
	Males			Females		
	0.3	0.6	1.0	0.3	0.6	1.0
Body weight at dosing (g)	27.34	28.44	25.05	24.70	28.80	25.40
Body weight at termination (g)	29.03	28.67	24.72	25.06	29.32	24.88
Incidence of tremors:						
No. of pups affected	3	2	3	3	2	3
Time-point of observation (min); pup No. 1	8, 19	9	8, 31, 54	15	7, 20	10
Time-point of observation (min); pup No. 2	2, 15	8, 30	13, 14, 19	21	15	8, 13, 35, 60
Time-point of observation (min); pup No. 3	42		2, 7, 10, 14, 16	8		13, 19
Erythrocyte acetylcholinesterase activity (U/l)	3307	3353	3400	2780	3393	3020
Brain acetylcholinesterase activity (U/g)	7.74	7.06**	5.47***	7.01	7.45	6.37

From Tyl et al. (2005a)
** $p < 0.01$; *** $p < 0.001$; compared with the value for the group at 0.3 mg/kg bw.

were given a single dose of carbofuran (purity, 99%) at 0.6 mg/kg bw by gavage in corn oil (dose volume, 5 ml/kg bw). In addition, 10 male and 10 female pups and 10 male and 10 female adults were used as untreated control groups. Clinical observations were recorded before dosing and at approximately 15, 30, 60, 90, 120, 240 and 360 min after dosing (for rats scheduled for sacrifice at each time-point and for rats scheduled for termination at later time-points). At termination, a blood sample (cardiac puncture) and the brain were collected from five male and five female pups and from five male and five female adults each at 15, 30, 60, 90, 120, 240 and 360 min after dosing, and from five male and five female pups and five male and five female adults in the untreated control groups at 0 and 360 min after dosing. The assays for acetylcholinesterase enzyme activity were performed by an Ellman method modified for use on an automated clinical chemistry analyser (for details see Tyl et al., 2005c, below). The study was conducted in accordance with the principles of GLP.

There was no mortality or moribundity in any pup or adult of either sex at 0.6 mg/kg bw at any time-point up to termination 6 h after dosing. In the pups at postnatal day 11, treatment-related clinical signs (whole-body tremors) were observed initially in one (out of thirty-five) males at 2 min after dosing. Tremors were observed in one male 3 min after dosing and in one male and one female at 4 min after dosing, with maximum incidence and severity at 15 min after dosing. Few or no treatment-related clinical observations (tremors) were recorded after 28 min unless the rat was touched or placed in the weighing pan or in the carbon dioxide chamber for termination. Such manipulation triggered tremors that persisted in pups for up to 119 min after dosing. The clinical observations were limited to fine tremors initially, then whole-body tremors, and then large head tremors. With time after dosing, the tremors became intermittent, with large head tremors persisting for the longest period of time. Since at age 11 days rat pups are just beginning to open their eyes (most of them have not yet opened their eyes), lacrimation could not be observed. As the dams groomed the pups after dosing and while they were experiencing tremors, salivation could not be observed. The dams and their litters were housed in polycarbonate cages with bedding, and the dams kept their pups in tight piles and groomed them constantly. Therefore, urination and defaecation could not be observed in the pups.

For the adult rats, the earliest reports of tremors (face) were in one female (out of thirty-five) at 6 min after dosing and one female (out of thirty-five) at 7 min after dosing. The incidences and

Table 2. Relevant findings for rat pups dosed with carbofuran by gavage on postnatal day 11

Finding	Dose (mg/kg bw)							
	Males				Females			
	0	0.03	0.1	0.3	0	0.03	0.1	0.3
Body weight (g)	23.5	24.4	23.9	22.8	21.9	23.4	22.8	23.0
No. of pups with tremors	0	0	0	2[a]	0	0	0	2[a]
Erythrocyte acetylcholinesterase activity (U/l)	1874	1627	1474	1354	2024	1962	1543	1551
Erythrocyte acetylcholinesterase activity (% of values for the control group)	100%	87	79	72	100	97	76	77
Brain acetylcholinesterase activity (U/g)	7.17	6.49	4.76**	2.84**	7.07	6.30	4.62**	3.78**
Brain acetylcholinesterase activity (% of values for the control group)	100	90	66	40	100%	89	65	54

From Hoberman (2007a)

[a] At 15 min after dosing, no clinical observations were recorded for 24 of the 40 pups, owing to a deviation from the study protocol.

** $p < 0.01$.

severities were highest at 15 min, and no tremors were observed after 60 min after dosing. Additional clinical signs included lethargy (in one male at 9 min, one male at 30 min and three males at 15 min after dosing), salivation (in one male at 11 min and in three males at 15 min after dosing), coprophagia (in one female at 15 min, one female at 60 min and two females at 30 min, and in three males at 60 min after dosing), soft faeces (in one male at 30 min), slow respiration (in one male at 60 min), abdominal twitching (in one female at 120 min), and piloerection (in one female at 30 min and in three females at 240 min).

There were no statistically significant effects on erythrocyte acetylcholinesterase activity in pups aged 11 days or adults compared with controls at any time-point (Table 3). However, there was a high variability in erythrocyte acetylcholinesterase activity even in the control group (e.g. means were 3000 ± 466 and 3988 ± 1061 U/l for male pups, and 3192 ± 482 and 4348 ± 1213 U/l for female pups at 0 or 360 min, respectively); this casts doubt on the validity of combining the data for the control group at 0 and 360 min for the statistical analysis. Thus, regarding the quite variable data and the unfavourable assay conditions (see Tyl et al., 2005c, below), the reliability of the assessment of erythrocyte acetylcholinesterase activity in this study is questionable.

Brain acetylcholinesterase activity in male and female pups aged 11 days was statistically significantly inhibited at all time-points examined, with maximum inhibition at 15 min after dosing. Full recovery of brain acetylcholinesterase activity was not achieved in postnatal day 11 pups by 6 h after dosing with a single oral dose of carbofuran at 0.6 mg/kg bw. In adult rats, brain acetylcholinesterase activity was significantly reduced at most time-points, and the time of maximum inhibition was at 15 min after dosing. Recovery of brain acetylcholinesterase activity was achieved in males by 360 min after dosing and in females by 240 min after dosing.

Based on these results, the time of maximum inhibition of brain acetylcholinesterase activity was determined to be 15 min after dosing, in pups and adults. Since there was not full recovery of brain acetylcholinesterase activity in postnatal day 11 pups at 360 min after dosing, the time for full

Table 3. Acetylcholinesterase activity in postnatal day 11 pups and adults given a single dose of carbofuran at 0.6 mg/kg bw

Acetylcholinesterase activity	Time of sacrifice (minutes after dosing)							
	0[a]	15	30	60	90	120	240	360
Pups								
Erythrocytes (U/l):								
Males	3494	2988	3056	4092	3052	3896	3340	4320
Females	3770	3100	3232	4444	3048	3928	3208	5064
Brain (U/g):								
Male	7.33	3.50***	4.02***	4.29***	5.31***	4.62***	5.54***	6.18*
Female	8.38	3.43***	4.45***	4.13***	4.71***	4.99***	6.87*	6.36**
Adults								
Erythrocytes (U/l):								
Male	4208	4332	4456	5124	4228	4732	4796	5164
Female	4760	4088	4432	5272	4908	4540	3856	4536
Brain (U/g):								
Male	12.98	8.04***	8.47***	11.28***	10.74***	11.68**	11.78*	12.00
Female	13.06	8.67***	9.18***	11.38***	11.18***	12.12***	13.04	12.24**

From Tyl et al. (2005b)

[a] The two untreated groups (0 and 360 min sacrifice time) were combined into a single control group.

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$;

recovery was selected to be 720 min for a study of the dose–response relationship (see Tyl et al., 2005c, below) (Tyl et al., 2005b).

In a study of acute toxicity designed to identify the time of maximum inhibition and the time of complete recovery of acetylcholinesterase activity in erythrocytes and brain, groups of 60 male and 60 female pups at postnatal day 11and groups of 60 male and 60 female adult Crl:CD®(SD) rats were given a single dose of carbofuran (purity, 98.8%) at 0 or 0.1 mg/kg bw (dose volume, 1 ml/kg bw) in corn oil by gavage. Groups of 10 males and 10 females in the control group and among the rats given carbofuran were assigned to one of six time-points (time between dose administration and sacrifice): 15 min, 30 min, 1 h, 2 h, 4 h and 6 h. The adults and pups were examined for routine clinical observations within the 15 min before dosing and at approximately 15 min, 30 min, 1 h, 2 h, 4 h and 6 h after dosing. Body weights were recorded on the day of dosing. Adults were sacrificed by exsanguination under isoflurane/oxygen anaesthesia during the collection of blood samples at the specified time-points and the pups were sacrificed by decapitation for blood collection. The brains from pups and adults were then excised and weighed. Blood and brain samples were processed and analysed for cholinesterase activity within 2 h of collection. The assays for cholinesterase enzyme activity were performed by an Ellman method modified for use on an automated clinical chemistry analyser (for details, see Hoberman, 2007c, below). The study was conducted in accordance with the principles of GLP.

There were no mortalities. No adverse clinical observations related to the test substance occurred in any adult or pup. There were no treatment-related effects on body or brain weights for adults.

For adults, the time of peak effect on brain acetylcholinesterase activity was determined to be 30 min after dosing for males and females (Table 4). Although erythrocyte acetylcholinesterase activity was increased at 30 min in females, the maximum inhibition of erythrocyte acetylcholinesterase

Table 4. Acetylcholinesterase activity in pups at postnatal day 11 and adults given a single dose of carbofuran at 0.1 mg/kg bw by gavage

Acetylholinesterase activity	Time of sacrifice (minutes after dosing)					
	15	30	60	120	240	360
Pups						
Erythrocyte (% of control):						
Males	70.7	88.4	79.1**	91.0	103.2	123.2
Females	83.9	92.2	85.1	105.2	135.4	102.5
Brain (% of control):						
Males	79.7**	78.7**	73.1**	99.3	101.3	98.5
Females	84.7**	67.7**	67.1**	82.5**	100.4	98.5
Adults						
Erythrocyte (% of control):						
Males	88.0	78.3**	99.7	114.6	81.9**	89.2
Females	83.4**	102.1	89.9	88.9	88.2	114.3
Brain (% of control):						
Males	83.4**	79.1**	83.8*	88.4**	91.4**	95.1
Females	92.1	82.2**	97.3	90.7**	94.5*	97.8

From Hoberman (2007b)
* $p < 0.05$; ** $p < 0.01$.

activity was at 30 min in males, thus supporting the selection of 30 min as the time of peak effect for a subsequent study on dose–response relationship (see Hoberman, 2007c, below).

For pups, the time of peak effect on erythrocyte acetylcholinesterase was between 15 min and 1 h. The time of maximum inhibition of acetylcholinesterase activity in pup brains was at 1 h, although the values for 30 min and 1 h for female pup brain were nearly the same.

The time of peak effect selected for the study on the dose–response relationship was 30 min on the basis of the data for adults and pups presented above. The time of recovery was selected as 4 h for the study on the dose–response relationship, since by this time the pups had recovered and brain acetylcholinesterase activity in adults had reached > 90% of values for the controls (Hoberman, 2007b).

(c) Inhibition of acetylcholinesterase activity in juvenile and adult rats

In a study of acute toxicity designed to determine the dose–response relationship at the times of maximum inhibition and of full recovery of acetylcholinesterase activity in erythrocytes and brain, groups of 10 male and 10 female pups at postnatal day 11 and groups of 10 male and 10 female adult (age 60 days) Crl:CD®(SD) rats were given a single dose of carbofuran (purity, 99%) at 0, 0.3, 0.6 or 1.0 mg/kg bw (dose volume, 5 ml/kg bw) in corn oil by gavage and terminated at approximately 15 min or 720 min after dosing. After dosing, the pups were returned to their dams (18 dams with 5 males and 5 females per litter), and pups and adults were examined for clinical signs of toxicity at 0, 15, 30, 60, 120, 240 and 720 min after dosing. At 15 min or 720 min after dosing, a group of 10 male and 10 female pups and a group of 10 male and 10 female adults per time-point were weighed, examined, terminated by CO_2 asphyxiation, and blood was collected via cardiac puncture. The brain was also removed and weighed. For the assessment of cholinesterase activity, the samples of erythrocytes were washed once and diluted with an equal volume of phosphate buffer at 0.1 mol/l (pH 8.0) containing 1% Triton-X-100, while brains were homogenized in five volumes by weight of phosphate buffer. All samples were stored at -70 °C until analysis. Immediately before analysis, the erythrocyte lysates were further diluted with phosphate buffer, resulting in a final dilution of 1 : 20. The assays for cholinesterase enzyme activity were performed by an Ellman method (Ellman et al., 1961) modified for use on an automated, random-access COBAS MIRA® Plus CC clinical chemistry analyser (Roche Diagnostics, Indianapolis, IN, USA). The assay was conducted at 37 °C with dithiobisnitrobenzoate (DNTB) at 0.25 mmol/l and acetylthiocholine iodide (ACT) at 1 mmol/l, and absorbance was measured at 405 nm. The study was conducted in accordance with the principles of GLP.

All male and female pups and adults survived to the scheduled termination times.

For pups terminated 15 min after dosing, head tremors and tremors not otherwise specified were observed initially in three females 5 min after dosing at 0.3 or 0.6 mg/kg bw. By 6 min after dosing, there was a clear dose–response relationship for incidence of tremors, with maximum incidence and severity until 9 min after dosing. In addition, one male at 0.6 mg/kg bw exhibited gasping and moribundity at 14 min after dosing and was sacrificed on schedule at 15 min after dosing.

For the pups sacrificed 720 min after dosing, tremors were first observed at 5 min after dosing, with a clear dose–response pattern in incidence and severity. The time of maximum response was observed at 15–30 min after dosing in all groups receiving carbofuran. Treatment-related clinical signs (tremors) were present at 0.6 (two rats) and 1.0 mg/kg (eight rats) at 60 min (with nine out of ten rats exhibiting tremors only when touched) and at 1.0 mg/kg (thirteen rats) at 120 min (with twelve out of thirteen rats exhibiting tremors only when touched).

For the adults sacrificed 15 min after dosing, ataxia was observed in one (out of ten) females at 6 min after dosing and in one male at 15 min after dosing, both at 1.0 mg/kg bw, and in one female at 15 min at 0.6 mg/kg bw. Tremors (body, head, facial) began at 6 min at 1.0 mg/kg bw and at 8 min at 0.6 mg/kg bw. The highest incidence and severity of tremors were present at 15 min after dosing, with two males and five females at 1.0 mg/kg bw and one male and two females at 0.6 mg/kg bw.

Piloerection was observed in four males and one female at 0.6 mg/kg bw and in five males and three females at 1.0 mg/kg bw. Lethargy, hindlimb splay, and prone positioning were observed in females at 15 min after dosing at 0.6 mg/kg bw and 1.0 mg/kg bw in a dose-related pattern. There were no treatment-related clinical signs at any time at 0.3 mg/kg bw.

For the adults sacrificed 720 min after dosing, facial tremors were first observed at 7 min in males and females at 1.0 mg/kg bw. Tremors were first observed at 9 min at 0.6 mg/kg bw and at 10 min at 0.3 mg/kg bw. There was a clear dose–response relationship for increased severity of treatment-related clinical observations, with the time of maximum effect being 15–30 min after dosing. Tremors were also observed after 60 min at 0.3, 0.6, and 1.0 mg/kg bw. Other observations included chromodacryorrhoea, lethargy, prone positioning, uneven respiration, slow respiration, coprophagia, salivation, facial fasciculations, jerking head movements, and piloerection at 1.0 mg/kg bw; piloerection, slow respiration, and coprophagia at 0.6 mg/kg bw; and slow respiration, coprophagia, and piloerection at 0.3 mg/kg bw.

Table 5. Clinical signs and acetylcholinesterase activity in pups (postnatal day 11) and adult rats given a single dose of carbofuran by gavage

Finding	Dose (mg/kg bw)							
	Males				Females			
	0	0.3	0.6	1.0	0	0.3	0.6	1.0
Sacrifice at 15 min after dosing								
Pups:								
No. with clinical signs	0	10	10	10	0	10	10	10
Erythrocyte AChE (U/l)	5436	5746	4944	5260	4782	6672	4646	5064
Brain AChE (U/g)	6.62	3.47***	3.13***	2.53***	6.90	3.56***	3.27***	2.42***
Brain AChE (% of control values)	100	52	47	38	100	52	47	35
Adults:								
No. with clinical signs	0	0	5	9	0	0	3	8
Erythrocyte AChE (U/l)	4774	5110	4880	5276	5390	5754	4718	5200
Brain AChE (U/g)	12.46	9.22***	8.44***	7.01***	12.88	9.84***	8.54***	7.16***
Brain AChE (% of control values)	100	74	68	56	100	76	66	56
Sacrifice at 720 min after dosing								
Pups:								
No. with clinical signs	0	10	10	10	0	10	8[a]	10
Erythrocyte AChE (U/l)	4791	4034	6093	4062	4558	4166	5427	4092
Brain AChE (U/g)	7.03	6.49	6.55	6.53	7.17	6.87	7.10	7.20
Brain AChE (% of control values)	100	92	93	93	100	96	99	100
Adults:								
No. with clinical signs	3[b]	5	8	10	0	5	6	10
Erythrocyte AChE (U/l)	5470	5570	5246	6884	5058	5146	5492	5910
Brain AChE (U/g)	13.00	12.29*	12.41	12.18*	13.29	12.74	12.62*	12.55*
Brain AChE (% of control values)	100	95	95	94	100	96	95	94

From Tyl et al. (2005c)
AChE, acetylcholinesterase.
[a] One pup was removed owing to a dosing error.
[b] Piloerection, rough coat.
* $p < 0.05$; ** $p < 0.01$; ** $p < 0.001$.

For pups and adults, there were no statistically significant differences in erythrocyte acetylcholinesterase activity between the control group and any treated group at 15 or 720 min after dosing in males or females. However, in view of the non-optimal assay conditions used in this study (e.g. dilution of samples, incubation time, incubation temperature) which may contribute to significant spontaneous enzyme reactivation (Nostrandt et al., 1993; Wilson et al., 1996; Hunter et al., 1997), the reliability of the assessments of erythrocyte acetylcholinesterase activity in this study is questionable. This conclusion is supported by the results from a recent comparison of two assay methods indicating that the commonly used spectrophotometric method (Ellman et al., 1961) tends to underestimate inhibition of cholinesterase activity in tissues from animals treated with carbamate when compared with the radiometric method (Padilla et al., 2007).

Regarding pup and adult brain acetylcholinesterase activity at the 15-minute time-point, mean values for males and females were statistically significantly reduced at all doses compared with the control group (Table 5). At 720-min, brain acetylcholinesterase activity of the pups was similar in all groups, while brain acetylcholinesterase activity was slightly (≤ 6%) but statistically significantly reduced at 0.3 and 1.0 mg/kg bw in male adults and at 0.6 and 1.0 mg/kg bw in female adults. These differences were not considered to be biologically relevant.

The NOAEL in pups aged 11 days and in adult rats was < 0.3 mg/kg bw on the basis of treatment-related clinical signs and inhibition of brain acetylcholinesterase activity at a dose of 0.3 mg/kg bw and above (Tyl et al., 2005c).

In a study of acute toxicity designed to determine the dose–response relationship at the times of maximum inhibition and of full recovery of acetylcholinesterase activity in erythrocytes and brain, groups of 10 male and 10 female pups at postnatal day 11 and groups of 10 male and 10 female adult Crl:CD®(SD) rats were given a single dose of carbofuran (purity, 98.8%) at 0, 0.03, 0.1 or 0.3 mg/kg bw (dose volume, 1 ml/kg bw) in corn oil by gavage and terminated at approximately 30 min or 240 min after dosing. The pups used for the study were selected from twenty litters; eight pups in each litter were assigned in consecutive numerical order to the four dose groups, one pup per sex per litter per group. The study was conducted in accordance with the principles of GLP.

Adults were examined for clinical observations before and approximately 15 min after dosing, and before sacrifice, at either 30 min or 4 h after dosing. Pups were observed for clinical signs before and at 15 min, 30 min, 1 h and 2 h after dosing. Body weights were recorded on the day of dosing. Motor activity was evaluated 30 min after dosing only for the adult rats designated for sacrifice at 4 h.

Adults were killed by exsanguination under isoflurane/oxygen anaesthesia during collection of blood samples and pups were killed by decapitation via guillotine and blood was collected. Brains were then excised and weighed. For the assessment of cholinesterase activity, blood and brain samples were processed within 10 or 20 min of collection, respectively. Brains were homogenized in 5 ml of 0.1% Tween 80 buffer (pH 8.0), followed by a secondary dilution of 1 : 2 or 1 : 5 for a pup brain or an adult brain, respectively. For erythrocyte samples, the dilution with 0.1% Tween 80 buffer was approximately 1 : 15. All samples were stored on crushed ice during processing and all sample analysis was initiated within approximately 1 h of sampling processing. The assays for cholinesterase enzyme activity were performed by an Ellman method (Ellman et al., 1961) modified for use on an automated clinical chemistry analyser (SpectraMax® 190 microplate reader). The assay was conducted at 26 °C with DNTB at 0.65 mmol/l and ACT at 3.5 mmol/l using a preincubation period of 2 min for erythrocyte and brain homogenates. Absorbance was read at 435 nm at intervals of 15 s, with a run time of 12 min. All samples were routinely analysed in duplicate. Criteria for acceptance of cholinesterase measurements included a duplication rate of 85% for brain and 80% for erythrocyte samples. Samples that did not meet these criteria were noted as “does not replicate” and were re-analysed as soon as possible. Samples that were still coded as “does not replicate” after the second

analysis were re-analysed up to five times, with the final measurement being used in the overall group mean. However, the length of time from collection of blood to the final analysis for those samples requiring additional measurements could not be determined.

There were no mortalities or clinical observations related to dosing with the test substance. There were no effects on body weights, brain weights, or motor activity for either adults or pups.

Adult erythrocyte acetylcholinesterase activity was reduced in a dose-dependent manner at 30 min after dosing and returned to control values 4 h after dosing (Table 6). Pup erythrocyte acetylcholinesterase activity was less sensitive than adults and did not show significant differences from control values at 30 min or 4 h after dosing at any dose. However, additional information from the laboratory performing the assessment revealed that large dilutions, lack of adequate sample mixing, and the time necessary to analyse samples were all likely to have contributed to the high variability and large duplication errors in the study. Therefore, the Meeting considered the data on erythrocyte acetylcholinesterase from this study to be unreliable.

Pup and adult brain acetylcholinesterase activity values were reduced in a dose-dependent manner at 30 min and had essentially recovered by 4 h after dosing, with the exception of male and

Table 6. Acetylcholinesterase activity in pups (postnatal day 11) and adult rats given a single dose of carbofuran by gavage

Finding	Dose (mg/kg bw)							
	Males				Females			
	0	0.03	0.1	0.3	0	0.03	0.1	0.3
Sacrifice at 30 min after dosing								
Pups:								
Erythrocyte AChE (U/l)	826	889	708	617	965	808	609	585
Erythrocyte AChE (% of control values)	100	108	86	75	100	84	63	61
Brain AChE (U/g)	6.00	5.19*	3.88**	2.77**	6.26	5.02**	3.32**	2.67**
Brain AChE (% of control values)	100	87	65	46	100	80	53	43
Adults:								
Erythrocyte AChE (U/l)	1148	1066	815**	707**	1077	906*	854**	664**
Erythrocyte AChE (% of control values)	100	93	71	62	100	84	79	62
Brain AChE (U/g)	14.8	13.1**	10.0**	7.50**	15.1	15.1	12.1**	8.99**
Brain AChE (% of control values)	100	88	68	51	100	100	80	60
Sacrifice at 240 min after dosing								
Pups:								
Erythrocyte AChE (U/l)	518	468	655	532	612	601	589	515
Erythrocyte AChE (% of control values)	100	90	126	103	100	98	96	84
Brain AChE (U/g)	6.23	5.68	5.04**	2.94**	6.37	6.05	4.96**	3.39**
Brain AChE (% of control values)	100	91	81	47	100	95	78	53
Adults:								
Erythrocyte AChE (U/l)	1034	1079	1167	1126	1162	1190	1151	1114
Erythrocyte AChE (% of control values)	100	104	113	109	100	102	99	96
Brain AChE (U/g)	15.7	14.6	13.6**	13.1**	15.9	15.2	14.9	14.5
Brain AChE (% of control values)	100	93	87	84	100	95	94	91

From Hoberman (2007c)
AChE, acetylcholinesterase.
* $p < 0.05$; ** $p < 0.01$.

female pups in the groups at 0.1 and 0.3 mg/kg bw. Statistically significant inhibition of brain acetylcholinesterase activity in the group of pups at 0.03 mg/kg bw recovered to control levels by 4 h.

The NOAEL in pups aged 11 days and in adult rats was 0.03 mg/kg bw on the basis of significant and > 20% inhibition of brain acetylcholinesterase activity at a dose of 0.1 mg/kg bw and above (Hoberman, 2007c).

In a study of acute toxicity designed to compare the sensitivity of inhibition of acetylcholinesterase activity in juvenile and adult rats, groups of 10 male pups (postnatal day 17) and groups of 10 male adult Long-Evans hooded rats were given a single dose of carbofuran (purity, 99%) at 0, 0.1, 0.3, 0.6 or 1.0 mg/kg bw for the pups, or at 0, 0.1, 0.3, 0.5, 0.75 or 1.5 mg/kg bw for the adults, in corn oil by gavage (dose volume, 2 ml/kg bw). The data for the adults were taken from an earlier study (McDaniel et al., 2007), which was carried out under essentially similar conditions. The studies were conducted under an approved intramural research protocol, and data were subjected to a quality assurance review.

At 15 min after dosing, the rats were placed in figure-eight chambers for a 20-min activity session. At approximately 40 min after dosing, the rats were anaesthetized by CO_2 asphyxiation, decapitated, trunk blood was collected and the brain was removed. Erythrocytes and brain tissue were diluted 1 : 3 and homogenized. The measurement of cholinesterase activity was performed using a radiometric cholinesterase assay (Johnson & Russell, 1975). The assay was conducted at 26 °C using an incubation period of 4 min for erythrocytes and 30 s for brain homogenate. After the reaction had been stopped and scintillant had been added, activity was measured using a liquid scintillation counter. Counting efficiency, as determined by an external quench standard, was approximately 62%.

There were no unanticipated deaths or cases of severe toxicity; however, no further details on clinical signs attributed to treatment were reported. For the pups, brain and erythrocyte acetylcholinesterase activity was significantly decreased at all doses of carbofuran, while motor activity was decreased only at doses of 0.3 mg/kg bw and above. Erythrocyte acetylcholinesterase activity was inhibited to a greater degree than brain acetylcholinesterase activity (Table 7).

When compared with adult rats (Table 8), pups aged 17 days showed greater inhibition of acetylcholinesterase activity, even at the lowest dose tested. The effect was slightly greater in erythrocytes than in brain. There was no age-related difference in the inhibition of motor activity by carbofuran at lower doses.

Pearson correlation coefficients of within-subject data were higher for brain acetylcholinesterase and motor activity when compared with erythrocyte acetylcholinesterase activity. This result was in general agreement with the overall conclusion of a comparison of acute neurobehavioral

Table 7. Relevant findings in male rat pups (postnatal day 17) given a single dose of carbofuran by gavage[a]

Finding	Dose (mg/kg bw)				
	0	0.1	0.3	0.6	1.0
Erythrocyte AChE (µmol ACh hydrolysed/min/ml)	0.886	0.446	0.243	0.171	0.153
Erythrocyte AChE (% of control values)	100	50	27	19	17
Brain AChE (µmol ACh hydrolysed/min/g)	5.096	3.686	2.626	2.311	1.812
Brain AChE (% of control values)	100	72	52	45	36
Motor activity (counts/20 min)	80.5	93.7	47.8	28.6	3.7
Motor activity (% of control values)	100	116	59	36	5

From Moser et al. (2007a)
AChE, acetylcholinesterase; ACh, acetylcholine.
[a] Statistical analyses were not performed.

(motor activity) and cholinesterase inhibitory effects conducted for a range of *N*-methyl carbamate pesticides, including carbofuran, in rats (McDaniel et al., 2007).

The NOAEL for pups aged 17 days was < 0.1 mg/kg bw on the basis of > 20% inhibition of brain and erythrocyte acetylcholinesterase activity at a dose of 0.1 mg/kg bw and above. The NOAEL for adult rats was 0.1 mg/kg bw on the basis of > 20% inhibition of brain and erythrocyte acetylcholinesterase activity at a dose of 0.3 mg/kg bw and above (Moser et al., 2007a; McDaniel et al., 2007).

In a study of acute toxicity designed to compare the sensitivity of acetylcholinesterase inhibition in juvenile and adult rats, groups of eight male pups (postnatal day 11) and groups of six male adult Long-Evans hooded rats were given a single dose of carbofuran (purity, 99%) at 0, 0.1, 0.3, 0.6 or 1.0 mg/kg bw (dose volume, 2 ml/kg bw) in acetone/corn oil by gavage. At approximately 40 min after dosing, the rats were anaesthetized by CO_2 asphyxiation, decapitated, trunk blood was collected and the brain was removed. Erythrocyte and brain tissue were diluted 1 : 3 and homogenized. The measurement of cholinesterase activity was performed by a radiometric cholinesterase assay (Johnson & Russell, 1975). The assay was conducted at 26 °C using an incubation period of 2 min for erythrocytes and 30 s for brain homogenate. After the reaction was stopped and scintillant was added, activity was measured using a liquid scintillation counter. Counting efficiency, as determined by an external quench standard, was approximately 62%. The study was conducted under an approved intramural research protocol, and data were subjected to a quality assurance review.

There were no unanticipated deaths or cases of severe toxicity; however, no further details on clinical signs attributed to treatment were reported. As a percent of the respective control groups, inhibition of both brain and erythrocyte acetylcholinesterase activity was greater at all doses tested in the pups than in the adults. In both age groups, erythrocyte acetylcholinesterase activity was inhibited to an equal or greater extent than brain (Table 9).

The NOAEL for pups at postnatal day 11 was less than 0.1 mg/kg bw on the basis of > 20% inhibition of brain and erythrocyte acetylcholinesterase activity at a dose of 0.1 mg/kg bw and above. The NOAEL for adult rats was 0.1 mg/kg bw on the basis of > 20% inhibition of brain and erythrocyte acetylcholinesterase activity at a dose of 0.3 mg/kg bw and above (Moser et al., 2007b).

Using the reliable data on brain acetylcholinesterase from three studies conducted with postnatal day 11 pups (Tyl et al., 2005c; Hoberman, 2007c; Moser et al., 2007b), the estimated oral dose resulting in 10% inhibition of brain acetylcholinesterase activity (benchmark dose at the 10% effect level, BMD_{10}) was 0.04 mg/kg bw, while the lower 95% confidence limit for the BMD_{10} ($BMDL_{10}$) was 0.03 mg/kg bw (US EPA, 2008a and 2008b).

Table 8. Relevant findings in adult rats given a single dose of carbofuran by gavage[a]

Finding	Dose (mg/kg bw)					
	0	0.1	0.3	0.5	0.75	1.5
Erythrocte AChE (µmol ACh hydrolysed/min/ml) [b]	0.428	0.366	0.319	0.301	0.252	0.142
Erythrocyte AChE (% of control values) [b]	100	86	75	70	59	33
Brain AChE (µmol ACh hydrolysed/min/g) [b]	7.158	5.895	4.812	4.544	4.411	2.873
Brain AChE (% of control values) [b]	100	82	67	63	62	40
Motor activity (counts/20 min) [c]	229	179	93	114	85	29
Motor activity (% of control values) [c]	100	78	41	50	37	13

From McDaniel et al., (2007); Moser et al. (2007a)
ACh, acetylcholine; AChE, acetylcholinesterase.
[a] Statistical analyses were not performed.
[b] $n = 5$
[c] $n = 10$

1.2 Short-term studies of toxicity

In a study evaluated by the 1996 and 2002 JMPR, groups of four male and four female beagle dogs were given diets containing carbofuran (purity, 99.6%) at a concentration of 0, 10, 70 or 500 ppm, the highest concentration being reduced to 250 ppm after 6 days owing to toxicity, for 13 weeks. These dietary concentrations resulted in mean intakes of 0, 0.43, 3.1 and 11 mg/kg bw per day for males and females combined. Two additional males and two females given the control diet and the diet containing carbofuran at the highest concentration were kept for a further 4 weeks in order to study the reversibility of the effects.

The dogs were examined daily for deaths and clinical signs. Food consumption was recorded daily and food consumption weekly. Tests of hearing were carried before the start of the study, at the end of dosing and at the end of the recovery period. Ophthalmoscopic examinations and electrocardiography were also carried out, with an additional examination after 6 weeks of dosing. Blood samples (for haematology and clinical chemistry) and urine were taken 7 days before the start of the treatment, at 6 and 13 weeks and after the recovery period. For estimation of plasma and erythrocyte cholinesterase activity, blood samples were taken 30 min after the daily 2 h feeding period at 7 days before the start of the treatment, on test days 1 and 3 and at the end of weeks 1, 2, 6 and 13 of the study (also 11 days later for males); and after the recovery period. The cerebellum was taken at autopsy and immediately assayed for acetylcholinesterase activity. Cholinesterase activity was estimated by an Ellman method (Ellman et al., 1961), modified for use on a Technicon AutoAnalyser II. The assay was conducted at 37 °C. However, no further details of the method were given, and it was not clear whether the experimental conditions were appropriate to minimize spontaneous reactivation of the enzyme. Selected organs were processed for histopathological examination.

One dog at 500 ppm died after 5 days, possibly owing to intussusception of the jejunum. Dogs at all doses showed hyperaemia (of the ear pinnae, abdominal skin and oral mucous membranes) and salivation, these signs being most severe early in the study. The clinical signs at the highest dietary concentration included ataxia, vomiting and tachypnoea. At this concentration, there was loss of body weight and decreased food consumption, which recovered when the concentration of carbofuran in the diet was reduced. No treatment-related differences were seen in ophthalmic, electrocardiographic, haematological or clinical chemical parameters, other than cholinesterase activity.

Table 9. Relevant findings for pups (postnatal day 11) and adult rats given a single dose of carbofuran by gavage[a]

Finding	Dose (mg/kg bw)				
	0	0.1	0.3	0.6	1.0
Pups:					
Erythrocte AChE (µmol ACh hydrolysed/min/ml)	0.871	0.407	0.260	0.139	0.096
Erythrocte AChE (% of control values)	100	47	30	16	11
Brain AChE (µmol ACh hydrolysed/min/g)	3.382	2.028	1.435	0.993	0.760
Brain AChE (% of control values)	100	60	42	29	22
Adults:					
Erythrocte AChE (µmol ACh hydrolysed/min/ml)	0.599	0.523	0.307	0.251	0.155
Erythrocte AChE (% of control values)	100	87	51	42	26
Brain AChE (µmol ACh hydrolysed/min/g)	6.690	5.834	4.786	4.325	3.312
Brain AChE (% of control values)	100	87	72	65	50

From Moser et al. (2007b)
ACh, aceylcholine; AChE, acetylcholinesterase.
[a] Statistical analyses were not performed.

Table 10. Plasma, erythrocyte and brain cholinesterase activity in dogs given diets containing carbofuran for 13 weeks

Cholinesterase activity	Dietary concentration (ppm)							
	Male				Female			
	0	10	70	500/250	0	10	70	500/250
Plasma cholinesterase activity *(µmol-SH/ml)*								
Pre-test, day -7	6.65	7.83	7.13	7.83	6.83	5.74	6.01	7.08
% [a]	100	118	107	118	100	84	88	104
Day 1	5.83	5.47	1.93*	0.93*	5.61	4.45	1.82*	1.22*
% [a]	100	94	33	16	100	79	32	22
Day 3	6.11	6.17	2.63*	3.43*	6.36	4.72	2.52	3.22
% [a]	100	101	43	56	100	74	40	51
Day 7	6.04	5.90	2.84*	1.50*	6.11	4.67	2.41*	1.20*
% [a]	100	98	47	25	100	76	39	20
Day 14	6.61	6.49	3.16*	1.79*	6.94	5.52	2.84*	1.80*
% [a]	100	98	48	27	100	80	41	26
Week 6	7.72	7.19	3.81*	1.61*	8.04	6.38	3.49*	2.06*
% [a]	100	93	49	21	100	79	43	26
Week 13	6.86	6.60	3.38*	1.54*	7.76	5.63	3.16*	1.63*
% [a]	100	96	49	22	100	73	41	21
Erythrocyte acetylcholinesterase (µmol-SH/ml)								
Pre-test (day -7)	2.49	2.50	2.78	2.95	2.85	3.06	2.18	2.19
% [a]	100	100	112	118	100	107	76	77
Day 1	2.34	1.71	0.81*	0.31*	2.64	2.18	0.73*	0.37*
% [a]	100	73	35	13	100	83	28	14
Day 3	2.61	2.07	1.15*	1.20*	3.11	2.52	1.05*	0.80*
% [a]	100	79	44	46	100	81	34	26
Day 7	2.66	1.95	1.07*	0.57*	2.96	2.46	1.03*	0.53*
% [a]	100	73	40	21	100	83	35	18
Day 14	2.68	2.05	1.15*	0.68*	3.12	2.54	1.00*	0.63*
% [a]	100	76	43	25	100	81	32	20
Week 6	3.15	2.35	1.33*	0.67*	3.53	3.12	1.30*	0.62*
% [a]	100	75	42	21	100	88	37	18
Week 13	2.37	1.54	0.81*	0.50*	2.22	2.09	0.92*	0.46*
% [a]	100	65	34	21	100	94	41	21
Brain acetylcholinesterase (µmol-SH/g)								
Week 14	4.97	5.43	5.45	4.70	4.45	4.78	4.41	5.26*
% [a]	100	109	110	95	100	107	99	118

From Bloch et al. (1987a)

[a] Percentage of value for control group.

* $p < 0.05$; compared with control group.

Inhibition of plasma cholinesterase activity was observed at the two higher dietary concentrations, starting from day 1 in males, but only exiguous, insignificant inhibition was observed at the lowest concentration (Table 10). In females, biologically significant inhibition of plasma cholinesterase activity was observed at all dietary concentrations. Inhibition of erythrocyte acetylcholinesterase activity was also seen at all three doses, representing 73%, 35% and 13% of that of concurrent controls at 10, 70 and 500 ppm on day 1 in males, when inhibition was generally maximal. In females at 10, 70 and 500 ppm, the activity on day 1 was 83%, 28% and 14% of that of concurrent controls, respectively. No significant inhibition of brain acetylcholinesterase activity was observed. However, since administration of the test substance was terminated on the day before necropsy, the data on brain acetylcholinesterase activity were not considered to be reliable owing to significant spontaneous reactivation of the enzyme.

As biologically significant depression of erythrocyte acetylcholinesterase activity was observed at the lowest dietary concentration (0.43 mg/kg bw, average intake in males and females), at which clinical signs (salivation, hyperaemia) were also observed, no NOAEL could be identified (Bloch et al., 1987a).

In a subsequent study conducted in order to identify a NOAEL with respect to clinical signs and inhibition of cholinesterase activity, groups of four male beagle dogs were fed diets providing carbofuran (purity, 99.6%) at a concentration of 0 or 5 ppm for 4 weeks. The dogs were examined daily for deaths and clinical signs. Blood samples for measurement of plasma and erythrocyte cholinesterase activity were collected within 30 min after food withdrawal. Cholinesterase activity was estimated by an Ellman method (Ellman et al., 1961), modified for use on a Technicon Auto Analyser II. The assay was conducted at 37 °C. However, no further details of the method were given, and it was not clear whether the experimental conditions were appropriate to minimize spontaneous reactivation

Table 11. Plasma and erythrocyte cholinesterase activity in male dogs fed diets containing carbofuran for 4 weeks

Cholinesterase activity	Dietary concentration (ppm)		
	0		5
Plasma cholinesterase	*Units (µmol-SH/ml)*	% of pre-test value	
Pre-test	6.17	100.0	7.13 (100%) [115.6%][b]
Day 1	5.95	96.4	6.17 (86.5%)[a] [103.7%][b]
Day 3	5.90	95.6	5.95 (83.5%)[a] [100.8%][b]
Day 7	6.06	98.2	6.27 (87.9%)[a] [103.5%][b]
Day 14	5.95	96.4	6.27 (87.9%)[a] [105.4%][b]
Day 28	5.79	93.8	5.95 (83.5%)[a] [102.8%][b]
Erythrocyte acetylcholinesterase (µmol-SH/ml)			
Pre-test	3.14	100.0	2.48* (100%) [79.0%][b]
Day 1	2.99	95.2	2.24* (90.3%)[a] [74.9%][b]
Day 3	2.86	91.1	1.80* (72.6%)[a] [62.9%][b]
Day 7	3.23	102.9	2.37* (95.6%)[a] [73.4%][b]
Day 14	3.27	104.1	2.44* (98.4%)[a] [74.6%][b]
Day 28	3.14	100.0	2.18* (87.9%)[a] [69.4%][b]

From Bloch et al. (1987b)

* $p < 0.05$; compared with control group

[a] (% of pre-test value)

[b] [% of control group value]

of the enzyme. Haematology and clinical chemistry investigations were not performed. Also, gross necropsy and histopathology examinations and measurements of brain acetylcholinesterase activity were not conducted since the dogs were not sacrificed at the end of the test.

Treatment with carbofuran had no effects on clinical signs, mortality, body weight, food consumption, or plasma and erythrocyte cholinesterase activity (Table 11). At the pre-test investigation, erythrocyte acetylcholinesterase activity in dogs to be treated with carbofuran was slightly lower than the value for the control group. Similar differences were seen on days 1, 7, 14 and 28. On day 3, a stronger increase in this difference was noted, but this isolated finding was considered to be of no toxicological significance.

The NOAEL in dogs fed diets containing carbofuran was 5 ppm, equal to 0.22 mg/kg bw per day, the highest dose tested (Bloch et al., 1987b).

2. Observations in humans

In a study to determine the threshold toxicity level in humans, groups of healthy male volunteers (Caucasian; age 23–47 years) received capsules containing carbofuran (purity not specified) as a single oral dose at 0.05, 0.10 or 0.25 mg/kg bw. The number of subjects in the assigned dose groups was two, two and four, respectively, while one male received placebo only. The study was conducted in two parts. The first part was an open study (subjects and investigators knew that carbofuran was ingested) to which subjects were admitted in groups of two. The first group received carbofuran at a dose of 0.05 mg/kg bw, and subsequent groups were introduced until intolerance was demonstrated at a dose of 0.25 mg/kg bw. The second part of the study followed a randomized double-blind design, and evaluated placebo in one subject and the threshold toxic dose of 0.25 mg/kg bw in two subjects. The study was conducted in accordance with the ethical standards current at the time (1976). The nature and purpose of this study were explained to the subjects and their written informed consent was obtained before participation in the programme. The consent procedure and form, as well as the protocol outlining the conduct of this study were approved by the Community Review Committee, Inc., a non-profit corporation organized for the protection of subjects participating in human research.

Subjects were chosen on the basis of a medical history review, physical examination, and laboratory determinations conducted within 14 days before the study. Carbofuran was administered as a single dose immediately after breakfast, after which the subjects remained under observation for 24 h and were monitored for clinical signs and symptoms. The medical examination included tests for heart function (electrocardiogram), vitals signs (blood pressure, temperature, respiration rate), vestibular function, vision (pupil size and accommodation), blood analysis (cholinesterase activity in plasma and erythrocytes) and urine analysis. Plasma and erythrocyte cholinesterase activity were determined using a modification of the Ellman method (Ellman et al., 1961) with propionylthiocholine as substrate. Whole blood was not washed but analysed directly from samples collected from finger tips, and the assay was run at 30 °C for 10 min. Subjects were allowed to smoke during the 24 h sample collection period.

In the two subjects receiving carbofuran at a dose at 0.05 mg/kg bw, no clinical signs were noted. The maximum percentage decreases in cholinesterase activity were 11–22% in erythrocyte and 32–36% in plasma, respectively. Erythrocyte acetylcholinesterase activity recovered within 3 h after administration of carbofuran.

The two subjects subsequently given carbofuran at a dose at 0.1 mg/kg bw presented with mild symptoms including headache (subject No. 3) or lightheadedness (subject No. 4). Subject No. 4 also showed changes in cardiovascular parameters including sinus bradycardia and sinus arrhythmia, and exhibited an abnormal vestibular mechanism before dosing and further deterioration at 3, 5 and 24 h after exposure to carbofuran. Since the time at which these symptoms were reported did not correlate with the time when there was inhibition of acetylcholinesterase activity, the symptoms were considered to be unrelated to treatment. The maximum percentage decreases in cholinesterase activity

were 31–33% in erythrocytes and 35–56% in plasma, respectively. Erythrocyte acetylcholinesterase activity recovered by 3 h after administration of carbofuran.

All subjects at 0.25 mg/kg bw exhibited clinical signs characteristic of acetylcholinesterase inhibition. Individual reactions, however, ranged from one brief episode of mild nausea in subject No. 8, multiple symptoms of mild to moderate severity (diaphoresis, unsteady, drowsy; dry mouth, salivation, abdominal pain) in subject No. 5, multiple symptoms of moderate severity (dizziness, diaphoresis, nervous, weakness, nausea) in subject No. 7, to multiple symptoms of mild to severe intensity (salivation, nervous, dry mouth, abdominal pain; drowsy, weakness, vomiting; diaphoresis, nausea, unsteady) in subject No. 6. Regarding examination of vitals signs, a trend toward lower heart rates after treatment was evident among volunteers at 0.25 mg/kg bw. This trend was most pronounced in subject No. 6, who also displayed the most severe signs and symptoms of toxicity. Also, the two volunteers with the severest symptoms (subjects No. 5 and 6) experienced a marked reduction in body temperature, and subject No. 6 revealed a reduction in respiration rate. In subjects No. 7 and No. 8 at 0.25 mg/kg bw, an effect on pupil size with 2 mm miosis manifested within 2 h after treatment. The maximum percentage decreases in cholinesterase activity were 46–63% in erythrocytes and 33–100% in plasma, respectively. Erythrocyte acetylcholinesterase activity recovered by 6 h after administration.

Plasma cholinesterase activity was depressed in virtually all the subjects, including subject No. 9 who received placebo, and no clear dose–effect relationship could be established for this parameter, although at the highest dose, inhibition was present more consistently. In contrast, inhibition of erythrocyte acetylcholinesterase activity showed a clear dose–response relationship, with maximum percentage decreases of 10% for the subject receiving placebo and up to 46–63% in the subjects given carbofuran at a dose of 0.25 mg/kg bw (Table 12).

Table 12. Relevant findings for men given a single dose of carbofuran as capsules

Finding	Dose (mg/kg bw)								
	0	0.05		0.10		0.25			
Subject No.	9	1	2	3	4	5	6	7	8
Age (years)	28	34	47	29	23	44	47	41	37
Body weight (kg)	75.5	61.3	76.0	71.2	60.0	72.2	77.0	81.6	95.0
Height (cm)	183	165	180	173	169	167	169	164	179
Clinical signs; relationship to treatment	None	None	None	Probably unrelated	Probably unrelated	Related	Related	Related	Related
Headache [a]	—	—	—	Mild (9 h and 45 min)	—	—	—	—	—
Lightheadedness [a]	—	—	—	—	Mild (15 min, 10 h and 45 min)	—	—	—	—
Nausea [a]	—	—	—	—	—	—	Severe (1 h)	Moderate (2 h 30 min)	Mild (30 min)
Diaphoresis [a]	—	—	—	—	—	Mild (1 h)	Severe (1 h)	Moderate (1 h)	—
Dry mouth [a]	—	—	—	—	—	Moderate (1 h)	Mild (3 h)	—	—
Salivation [a]	—	—	—	—	—	Moderate (1 h)	Mild (1 h)	—	—
Abdominal pain [a]	—	—	—	—	—	Moderate (1 h)	Mild (3 h)	—	—
Unsteady [a]	—	—	—	—	—	Mild (3 h)	Severe (1 h)	—	—

Drowsy [a]	—	—	—	—	—	Mild (2 h)	Moderate 45 min)	—	—
Nervous [a]	—	—	—	—	—	—	Mild (1 h)	Moderate (1 h)	—
Weakness [a]	—	—	—	—	—	—	Moderate (1 h)	Moderate (1 h)	—
Vomiting [a]	—	—	—	—	—	—	Moderate (2 h)	—	—
Dizziness [a]	—	—	—	—	—	—	—	Moderate (1 h)	—
ECG findings; time (hours after treatment)	SB (1–4 h)	—	SB (0–24 h)	SA (1 h) SB (0–4)	SA (0–24 h) SB (1–4 h)	SB (3–4 h)	SB (0–24 h)	SA (1 h) SB (2–4 h)	—
Heart-rate depression	—	—	—	—	—	Yes	Yes	Yes	—
Body-temperature depression	—	—	—	—	—	Yes	Yes	—	—
Respiration-rate reduction	—	—	—	—	—	—	Yes	—	—
Miosis	—	—	—	—	—	—	—	Yes	Yes
Vestibular function	AR	Normal	Normal	Normal	AR	Abnormal	Abnormal	AR	AR
Erythrocyte acetylcholinesterase activity (units not given)									
0 h	342	375	359	462	303	322	237	260	251
0 h (%)	100	100	100	100	100	100	100	100	100
0.5 h (%)	+6	-8	+5	-29	-15	-21	-5	+12	-55
1 h (%)	-10	-22	-11	-33	-31	-58	-63	-46	-59
2 h (%)	+3	ND	ND	ND	ND	-46	-49	-39	-52
3 h (%)	-3	+5	+3	-4	-2	-62	-57	-32	-26
6 h (%)	+28	+8	+7	±0	+12	-8	±0	+6	+5
24 h (%)	+40	+12	+5	+1	+5	+24	+27	+51	+44
Plasma cholinesterase activity (units not given)									
0 h	182	143	202	97	168	165	242	106	177
0 h (%)	100	100	100	100	100	100	100	100	100
0.5 h (%)	-42	-27	-20	-56	-35	-20	-15	-40	-47
1 h (%)	-52	-36	-32*	+18	+2	-12	-12	-67	-71
2 h (%)	-51	ND	ND	ND	ND	-14	-18	-100	-81
3 h (%)	-38	-15	-16	+9	+5	-2	-9	-52	-46
6 h (%)	+10	-26	-24	+5	+2	-53	-33	-5	-7
24 h (%)	-9	+2	-3	+22	+14	-7	-10	-28	-32

From Arnold (1976)

AR, abnormal response after treatment; however, evaluation obscured by abnormal responses before treatment; ECG, electrocardiogram; ND, not determined, SA, sinus arrhythmia; SB, sinus bradycardia.

[a] Severity of clinical signs and onset time (hours after treatment)

In conclusion, when given as a single oral dose at 0.05 mg/kg bw, carbofuran inhibited erythrocyte acetylcholinesterase activity by up to 22% in one of two subjects; at 0.1 mg/kg bw, erythrocyte ace-

tylcholinesterase activity was inhibited by up to 31–33% in two out of two subjects; while at 0.25 mg/kg bw, erythrocyte acetylcholinesterase activity was inhibited by up to 46–63% and treatment-related clinical signs were seen in all four subjects. However, owing to the small sample size, the study could not be used for identification of a NOAEL or lowest-observed-effect level (LOAEL) (Arnold, 1976).

Comments

Toxicological data

Carbofuran is highly toxic after a single oral dose; the median lethal dose (LD_{50}) values in rats ranging from 6 to 18 mg/kg bw and in various other species (including mouse, guinea-pig, rabbit, cat and dog) from 3 to 19 mg/kg bw. The clinical signs of toxicity observed were typical of inhibition of acetylcholinesterase activity. In rats, clinical signs were observed from about 5 min after dosing, and mortality generally occurred within 1 h after dosing.

Two studies of the time-course of inhibition of acetylcholinesterase activity were carried out in adult rats and pups aged 11 days (postnatal day 11). In the first study, after a single dose of carbofuran at 0.6 mg/kg bw, the time of maximum incidence and severity of clinical signs and of maximum inhibition of brain acetylcholinesterase activity was at 15 min after dosing for adults and pups. Recovery of brain acetylcholinesterase activity was achieved in adult males and females within 360 or 240 min after dosing, respectively, while the pups had not fully recovered by 360 min after dosing. In the second study, after a single dose of carbofuran at 0.1 mg/kg bw, no clinical signs were observed, and the time of maximum inhibition of brain acetylcholinesterase activity was at 30 min after dosing for adult rats and at 60 min after dosing for pups aged 11 days. Recovery of brain acetylcholinesterase activity was achieved in adults and in the pups within 240 min after dosing.

Two studies of acute toxicity were conducted to compare inhibition of acetylcholinesterase activity in pups aged 11 days (postnatal day 11) and adult rats, and two range-finding studies of acute toxicity were carried out in rats aged 11 days given carbofuran at doses ranging from 0.03 to 1.0 mg/kg bw. In these studies, a spectrophotometric assay for cholinesterase activity was used. While data on erythrocyte acetylcholinesterase were considered to be unreliable because of non-optimal experimental conditions that led to significant spontaneous enzyme reactivation, the data on brain acetylcholinesterase were considered to be suitable for use in risk assessment because the degree of inhibition of acetylcholinesterase activity agreed with that obtained using the more reliable radiometric assay for cholinesterase activity (see below). Clinical signs (tremors) were observed at doses of 0.3 mg/kg bw and above. On the basis of inhibition of acetylcholinesterase activity in brain (pups, 35–47%; adults, 20–32%) at 0.1 mg/kg bw and above, the overall NOAEL for pups and adults was 0.03 mg/kg bw.

In two studies of acute toxicity designed to compare inhibition of acetylcholinesterase activity in pups (postnatal day 11 or postnatal day 17) and adult rats given carbofuran at doses ranging from 0.1 to 1.5 mg/kg bw and using a radiometric assay for cholinesterase activity, the overall NOAEL for pups (both postnatal day 11 and postnatal day 17) was < 0.1 mg/kg bw on the basis of inhibition of acetylcholinesterase activity in brain (28–40%) and erythrocytes (50–53%) at 0.1 mg/kg bw and above. The overall NOAEL for adult rats was 0.1 mg/kg bw on the basis of inhibition of acetylcholinesterase activity in brain (28–33%) and erythrocytes (25–49%) at 0.3 mg/kg bw and above.

Using the data from three studies in rat pups aged 11 days, the BMD_{10} was 0.04 mg/kg bw, while the $BMDL_{10}$ was 0.03 mg/kg bw.

In the latter two studies, inhibition of erythrocyte acetylcholinesterase activity appeared to be a more sensitive end-point than did inhibition of brain acetylcholinesterase activity. In the absence of data on inhibition of acetylcholinesterase activity in peripheral target tissues, the use of data on erythrocyte acetylcholinesterase activity might thus be considered as surrogate for data on the peripheral nervous system. However, given the quantitative dose–response correlation between clinical signs

of cholinergic toxicity and inhibition of brain acetylcholinesterase activity by a range of *N*-methyl carbamates including carbofuran, the Meeting concluded that the current data support the use of inhibition of brain acetylcholinesterase activity rather than the surrogate measure of erythrocyte acetylcholinesterase activity as the end-point for the risk assessment of carbofuran.

In a 13-week dietary study in dogs, which was evaluated by the Joint Meeting in 1996 and 2002 and re-evaluated by the present Meeting, the LOAEL was 10 ppm, equal to 0.43 mg/kg bw per day. A NOAEL was not identified since significant inhibition of erythrocyte acetylcholinesterase activity and clinical signs were seen on the first day of dosing at the lowest dose. The data on brain acetylcholinesterase activity in this study were not reliable owing to significant recovery of acetylcholinesterase activity at the time-point of necropsy. In a supplementary 4-week study in male dogs, which was evaluated by the Joint Meeting in 1996 and re-evaluated by the present Meeting, brain acetylcholinesterase activity was not examined. The NOAEL for clinical signs and inhibition of erythrocyte acetylcholinesterase activity was 5 ppm in the diet, equal to 0.22 mg/kg bw per day, the highest dose tested. However, since a spectrophotometric assay for cholinesterase activity was used in both studies in dogs and it was not clear whether the experimental conditions were appropriate to minimize reactivation of the enzyme, the reliability of the data on erythrocyte acetylcholinesterase activity is questionable. The Meeting noted that, on the basis of the data on acute toxicity, dogs are not expected to be more sensitive than other species.

In a study in human volunteers, which met the ethical standards prevalent at the time when the research was conducted (1976), groups of two to four men received carbofuran as a single oral dose at 0.05, 0.1 or 0.25 mg/kg bw, while one man received placebo only. At 0.05 mg/kg bw, erythrocyte acetylcholinesterase activity was inhibited by 22% in one of two subjects; at 0.1 mg/kg bw, erythrocyte acetylcholinesterase activity was inhibited by 31–33% in both subjects; and erythrocyte acetylcholinesterase activity was inhibited by 46–63% and treatment-related clinical signs were seen in all four subjects at 0.25 mg/kg bw. Owing to the small sample size, the study could not be used for identification of a NOAEL or LOAEL, but provided information that was useful for the interspecies comparison of sensitivity for the risk assessment.

Toxicological evaluation

The Meeting established an ARfD of 0.001 mg/kg bw based on the overall NOAEL of 0.03 mg/kg bw per day identified on the basis of inhibition of brain acetylcholinesterase activity in rat pups aged 11 days and a safety factor of 25. This NOAEL was supported by the $BMDL_{10}$ of 0.03 mg/kg bw extrapolated from data on inhibition of brain acetylcholinesterase activity from three studies in rat pups aged 11 days. A safety factor of 25 was considered to be appropriate because the acute toxic effects of carbofuran are dependent on C_{max} rather than area under the curve of concentration–time (AUC) and data indicated that the sensitivity of humans and laboratory animals (rats, dogs) to inhibition of acetylcholinesterase activity by carbofuran was similar (see general item of the report for JMPR 2008: *Safety factors for acute C_{max}-dependent effects; specific considerations with respect to carbamates such as carbofuran*). Given the apparent higher sensitivity of younger animals, the ARfD was considered to be adequately protective of infants and children since it was based on the NOAEL from a study in pups aged 11 days.

The Meeting noted that this ARfD was lower than the current acceptable daily intake (ADI) of 0–0.002 mg/kg bw. This is plausible in view of the toxicological characteristics of inhibition of acetylcholinesterase activity by carbofuran, which shows very rapid recovery; long-term exposure can thus be likened to a series of acute exposures. The Meeting therefore concluded that the ADI and ARfD for carbofuran should be based on the same NOAEL and revised the ADI to 0–0.001 mg/kg bw based on the overall NOAEL of 0.03 mg/kg bw from the new studies of acute toxicity in rats and using a safety factor of 25.

Levels relevant to risk assessment

Species	Study	Effect	NOAEL	LOAEL
Rat	Acute study of toxicity (pups aged 11 days and adults) [a, b]	Inhibition of pup brain acetylcholinesterase activity	0.03 mg/kg bw [c]	0.1 mg/kg bw
		Clinical signs	0.1 mg/kg bw	0.3 mg/kg bw

[a] Gavage administration.
[b] Results of several studies combined.
[c] Supported by a $BMDL_{10}$ of 0.03 mg/kg bw, based on inhibition of brain acetylcholinesterase activity in pups aged 11 days (postnatal day 11).

Estimate of acceptable daily intake for humans

0–0.001 mg/kg bw

Estimate of acute reference dose

0.001 mg/kg bw

Information that would be useful for continued evaluation of the compound

Results from epidemiological, occupational health and other such observational studies of human exposure

Critical end-points for setting guidance values for exposure to carbofuran

Summary			
	Value	*Study*	*Safety factor*
ADI	0–0.001 mg/kg bw	Rat, study of acute toxicity	25
ARfD	0.001 mg/kg bw	Rat, study of acute toxicity	25

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