

The evaluation of humane endpoints in pertussis vaccine potency testing

Coenraad F. M. Hendriksen¹, Björn Steen¹, John Visser¹, Klaus Cussler², David Morton³ & Femke Streijger⁴

¹National Institute of Public Health and the Environment (RIVM), PO Box 1, 3720 BA Bilthoven, The Netherlands,

²Paul-Ehrlich-Institut, Paul-Ehrlich Strasse 51–59, D-63225 Langen, Germany,

³Centre for Biomedical Ethics, University of Birmingham, Birmingham B15 2TT, UK and

⁴Van Hall Institute, PO Box 1528, 8901 BV Leeuwarden, The Netherlands

Summary

One particular approach to reduce pain and suffering in laboratory animals is the introduction of humane endpoints. Vaccine potency tests, as specified in the pharmacopoeias, are frequently based on an immunization-challenge procedure in rodents or in the target species, using survival of the animals as a parameter for the estimation of the batch potency. An example of such a lethal challenge procedure is the pertussis vaccine potency test. We have been evaluating the use of clinical signs and the decrease of body temperature and body weight as potential alternatives to death as the endpoint of this test. From the results we conclude that loss of muscle coordination of the animal and a decrease of body temperature $< 34.5^{\circ}\text{C}$ could be used as an endpoint. A decrease in body weight, although being the first sign of the disease, appeared to be a poor predictor for lethality.

A key element in our approach to laboratory animals is the concept of refinement. It is the guiding principle in most of the existing national and international laws and guidelines on animal experimentation (e.g. Council Directive 86/609/EEC 1986 and the European Convention 1986). If procedures can be identified which limit or reduce the adverse effects on animals, this is considered to be of advantage to the animal. In addition, it is also seen as a way to improve the quality of the scientific results (Morton 1995, Svendsen *et al.* 1997). A large number of approaches can lead to refinement: the use of proper anaesthesia and analgesia; replacement of invasive by non-invasive procedures; improvement of surgical techniques; and environmental enrichment, etc. One particular approach to reduce animal pain and suffering is the introduction of humane endpoints. This approach is of particular importance as it deals with the most severe

experiments in which animals are allowed to die as a result of the experimental manipulations. Research protocols that may include death as an endpoint are carcinogenesis studies, toxicity tests, drug comparison studies, infection models and vaccine potency tests (Hamm 1995).

The study presented here focuses on vaccine potency testing. Frequently, these tests are based on an immunization-challenge procedure in rodents or in the target species, using survival of the animals as a parameter for the estimation of the batch potency. In the last decade much effort has been put into the search for alternative methods, but emphasis was given to the introduction of *in vitro* techniques, rather than to refinement of existing procedures (Brown *et al.* 1995). So far, progress has been limited and it is likely that challenge tests will continue to be used to estimate the potency of certain types of vaccines for human and veterinary applica-

tion. It is, therefore, important to evaluate the possibilities of humane endpoints in order to minimize the suffering these animals endure.

Quality control tests for vaccine batch release have to be performed according to the requirements of the national and international pharmacopoeias, such as the *Pharmacopoeia European (Ph.Eur.)* and the World Health Organization guidelines. As a consequence, any proposed refinement has to be validated against the old standard before being accepted. This makes work more tedious. On the other hand, if the refinement method is incorporated and mandated in the official requirements, the impact will be substantial.

We studied the use of humane endpoints in the potency test for the whole cell pertussis vaccine. This test is routinely performed in our laboratories with an average annual use of 15 000 mice. Animals (about 140 per test) are immunized and 14 days thereafter challenged intracerebrally with a bacterial suspension of virulent *Bordetella pertussis*. According to the *Ph.Eur.* monograph *Vaccinum Pertussis (Adsorbatum)*, (3rd edition, 1997), lethality is the outcome measured.

Ideally, a humane endpoint to replace death in a routine potency test should: be easy to monitor; be reproducible; have a high predictive power for oncoming death; and have the highest reduction in suffering both in time and intensity. Various humane endpoints for infection models have been suggested, such as clinical signs (Acred *et al.* 1994) as well as pathophysiological parameters including loss of body weight and decrease or increase of body temperature (Soothill *et al.* 1992). This paper focuses on the evaluation of these parameters for the pertussis potency test. As far as possible experiments were performed as part of the ongoing routine vaccine potency and safety testing procedures.

Animals, materials and methods

Animals

Seventy-six outbred mice (Rivm:N:NIH), males and females in equal numbers at a

weight range of 10–14 g, were obtained from the specific pathogen-free (SPF) breeding facility in our institute. Animals were randomly distributed amongst the experimental groups, using equal numbers of male and female animals per group. The mice were housed under SPF conditions in polycarbonate boxes (22 × 16 × 16 cm) with sawdust bedding, four animals per cage. Food pellets (SRM-A, Hope Farms B.V. Woerden, The Netherlands) and drinking water were given *ad libitum*. Room temperature and relative humidity were kept at 20–23°C and 43–58%, respectively. Room air was changed approximately 10 times per hour. Illumination was restricted to 12 h, from 06:00 to 18:00 h.

Experiments were performed according to the rules of the 'Dutch Animal Experimentation Act' (1997) and 'Council Directive 86/609/EEC' (1986). The experimental protocol was approved by the Institutional Animal Care and Use Committee of the RIVM in its session of 19 February 1997.

Immunization and challenge

Animals were immunized and challenged according to the protocol as specified in *Ph.Eur.* monograph. In short, animals were intraperitoneally injected with 0.5 ml whole cell pertussis vaccine (pertussis reference preparation Kh 85/1) or with sterile phosphate-buffered saline (PBS) (the diluent) respectively. Fourteen days after immunization, animals were intracerebrally challenged with 10 µl of a virulent, freshly prepared suspension of 10⁵ viable *Bordetella pertussis* microorganisms in casamino acid, or with 10 µl of the vehicle (casamino acid), respectively. Challenge was performed under light anaesthesia with halothane/N₂O/O₂, using a versatile, semi-automatic, precision liquid processor (Hamilton Microlab[®] 500 system) for intracerebral administration of the virulent *Bordetella pertussis*. Starting 4 days before challenge (day 10), body temperature, body weight and clinical signs were recorded twice a day until the end of the observation period (day 28). The following experimental groups were included in the study:

- (A) Day 0: 0.5 ml sterile PBS; day 14: challenge with 10 μ l *Bordetella pertussis* ($n = 8$)
- (B) Day 0: 0.5 ml vaccine dilution (62.5 μ l Kh 85/1); day 14: challenge with 10 μ l *Bordetella pertussis* ($n = 16$)
- (C) Day 0: 0.5 ml vaccine dilution (12.5 μ l Kh 85/1); day 14: challenge with 10 μ l *Bordetella pertussis* ($n = 16$)
- (D) Day 0: 0.5 ml vaccine dilution (2.5 μ l Kh 85/1); day 14: challenge with 10 μ l *Bordetella pertussis* ($n = 16$)
- (E) Day 0: 0.5 ml vaccine dilution (0.5 μ l Kh 85/1); day 14: challenge with 10 μ l *Bordetella pertussis* ($n = 16$)
- (F) Day 0: 0.5 ml sterile PBS; day 14: i.c. injection with 10 μ l casamino acid ($n = 4$)

Clinical observations

Prior to the study, animals in routine experiments were videotaped in order to make an inventory of the clinical signs commonly seen in a pertussis infection, to identify the cardinal signs and to relate these signs to disease progress. Pertussis challenge in mice generally follows a fixed pattern—animals show reduced activity, lack of grooming, ruffled fur, crouching, loss of appetite and reduced weight, dehydration and sunken eyes, apathy, loss of locomotor control, and finally convulsions, ultimately leading to death. The first signs of pertussis generally occur within a few days after challenge. Most animals died within 6 to 8 days after challenge (day 20–22), although one of the animals died at the end of the observation period. Based on the data of the videotapes, cardinal signs were grouped according to their sequence into five clinical levels (Table 1). The levels were coded from 0 to 4.

In the experiment, animals were observed twice a day (at 08:30 and 16:00 h) and for each individual animal disease progress was expressed as a score, referring to the clinical level.

Body weight

From day 10 after immunization, animals were weighed twice a day, immediately after

Table 1 Clinical signs pertussis infection

Stage 0	No abnormalities
Stage 1	<i>Cardinal clinical signs:</i> Less alert, fur becomes dull Pilo-erection, especially around the neck <i>Clinical signs that might occasionally occur:</i> Vocalization, poor grooming, nose and eye discharge
Stage 2	Inactivity, hunched back posture, pilo-erection over the entire body, social isolation, no food and water intake, dehydration, signs of emaciation
Stage 3	<i>Cardinal clinical signs:</i> Same as 2, but animals also show disturbed locomotor activity. Animals fall easily after being gently pushed, but are still able to get up again <i>Clinical signs that might occur:</i> Hind legs paralysed
Stage 4	Tonic-clonic convulsions, comatose

clinical observation and temperature recording. A semi-automatic Mettler balance was used. The reduction in body weight is presented as the percentage of the body weight at day 14 after immunization (just before challenge).

Body temperature

In order to minimize stress on measuring body temperature, all animals received a temperature-sensitive microchip transponder (IPTT-100 transponders: PLEXX B.V., Elst, The Netherlands). The transponder was implanted subcutaneously between the scapula of each mice. Body temperature was recorded twice a day (08:30 and 16:00 h) by a specific reader (DAS-5002, PLEXX B.V.). The system used for data acquisition and storage was the portable system ELAMSTTM (Electronic Laboratory Animal Monitoring System, BioMedic Data System, Inc. Seaford DE, USA). All data, including the unique code of the animal, the body temperature and the clinical observation can be stored in the memory and can be transmitted to other data systems (Kort *et al.* 1999). Before use, transponders were calibrated in a water bath. Transponders having a deviation of $> 0.1^{\circ}\text{C}$ were excluded from further use.

Table 2a Clinical signs, body weight and body temperature of mouse 2.1 which died following challenge on day 14

Day (a.m.)	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
Clinical code					1	2	4								
Weight (%)	100	95	88	79	72	65	60								
Temperature (°C)	37.8	37.8	37.9	37.4	35.2	33.3	27.6								

Table 2b Clinical signs, body weight and body temperature of mouse 2.2 following challenge on day 14

Day (a.m.)	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
Clinical code					1	1	2	2	2	2	2	1	1	1	1
Weight (%)	100	97	89	81	75	70	70	66	67	70	67	73	71	73	73
Temperature (°C)	37.3	37.1	37.8	37.2	36.3	35.2	34.8	35.0	35.1	36.3	36.6	36.8	36.92	36.5	36.6

Results

From each individual animal, information was obtained on clinical signs, body weight and body temperature. As an example, data from two animals immunized with 12.5 µl Kh85/1 (one died, the other survived) are shown in Tables 2a and 2b.

Clinical signs

Thirty of the 76 animals died during the observation period. They were the animals receiving the lowest vaccine doses (Groups D and E) and the PBS control (Group A), although deaths also occurred in Group C, but not in Group B or Group F (casamino acid). No animals died as a result of the challenge procedure itself (death within 4 days after challenge). Table 3 shows the distribution of deaths in the observation period. As can be seen, most animals died from pertussis infection in 6 (day 20) to 8 days (day 22). In general, the time from the onset of clinical signs to death varied from 2 days to 9 days with a mean of about 4 days. There was no relationship between the clinical score (from 1 to 4) and its duration in time.

The theoretical reduction in 'days of suffering' of the animals that was possible to achieve when a clinical score of 4 was used as the endpoint is demonstrated in Table 4a; similarly, in Table 4b, if clinical score 3 was applied. Only data of the morning observation are shown. It can be seen from these tables that using clinical score 4, animals

Table 3 Distribution of animals (n=30) that died* during the observation period

Day	19	20	21	22	23	24	25	26	27	28	No. of animals surviving after 28 days
	2	11	11	5	-	-	-	1	-	-	46

*Observation in the afternoon

could have been killed 0–3 days before their natural death; whereas using clinical score 3, the reduction in suffering would have been 1–2 days with a range from 0 to 5 days. The total reduction in suffering for all animals that died in numbers of days would have been 7–37 when using score 4, and 23–53 days when using score 3 as the humane endpoint.

Body temperature

Following a pertussis challenge a decrease in body temperature was seen in non-protected or partly protected animals. The drop in body temperature generally started about 4 days after challenge. Body temperature could fall to levels below 34°C, even in animals that were not in a moribund state.

The theoretical reduction in days of suffering was also analysed for body temperature. Four cut-off points of body temperature were used: <35.5°C, <35.0°C, <34.5°C and <34.0°C. If body temperature of <34.5°C had been used as a humane endpoint, this would have reduced suffering for

Table 4a Reduction of suffering (days) using clinical score 4 as the endpoint. The number of days is given that the animal would have survived without euthanasia

Reduction (days)	0/1	1/2	2/3	3/4	4/5
Day of euthanasia after immunization					
14					
15					
16					
17					
18		1			
19	8	2	2		
20	8				
21	2				
22	5				
23	1				
24					
25					
26					
27	1				
Total animals	25	3	2		
Total suffering days (min)	0	3	4	=	7 days
Total suffering days (max)	25	6	6	=	37 days

Table 4b Reduction of suffering (days) using clinical score 3 as the endpoint. The number of days is given that the animal would have survived without euthanasia

Reduction (days)	0/1	1/2	2/3	3/4	4/5	
Day of euthanasia after immunization						
14						
15						
16						
17						
18		2	1			
19	7	4	3			
20	7	2	1			
21	1					
22	1				1	
23						
24						
25						
26						
27						
Total animals	16	8	4	1	1	
Total suffering days (min)	0	8	8	3	4	= 23 days
Total suffering days (max)	16	16	12	4	5	= 53 days

Table 5 Reduction of suffering (days) using body temperature < 34.5°C as an endpoint. The number of days is given that the animal would have survived without euthanasia

Reduction (days)	0/1	1/2	2/3	3/4	4/5	5/6
Day of euthanasia after immunization						
14						
15						
16						
17						
18		3	1			
19	5	6	2			
20	5	5				
21	1	1			1	
22						
23						
24						
25						
26						
27						
Total days	11	15	3		1	
Total suffering days (min)	0	15	6		5	= 26 days
Total suffering days (max)	11	30	9		6	= 56 days

1–2 days with a range of 1–7 days (Table 5). The total reduction in suffering in numbers of days would have been between 26 and 56 days when using a body temperature of < 34.5°C as the humane endpoint.

Body weight

In most animals the loss of body weight was the first indication of a pertussis infection. It preceded the first clinical signs and the decrease in body temperature (see also Table 2). Body weight could fall dramatically: within a few days animals could lose 20–30% of their initial weight and even a reduction of about 50% was seen. This is due to the fact that clinically infected animals stop their food and water intake. Cachexia and dehydration are prominent clinical signs. In a previous study it was shown that providing agar to facilitate liquid uptake did not improve the situation.

Validation of humane endpoints

As death of the animal is specified in the requirements on pertussis vaccine potency

testing, a precondition of any alternative (humane) endpoint is that it predicts correctly the impending death of the animal. A high percentage of false-positive predictions, but also false-negative predictions, would disqualify any humane endpoint. A false-positive result is euthanasia of the animal based on the humane endpoint criterion, when that animal would have survived the observation period. A false-negative result is when an animal dies without having reached the humane endpoint. Table 6 shows the results of the analysis of data. For the purpose of the analysis, arbitrary cut-off values for body weight and body temperature were taken.

From this table it can be seen from the morning observation that when using a body temperature of $< 35.5^{\circ}\text{C}$ as the humane endpoint (a.m. observation) 9 animals in fact would have survived the observation period. When using a decrease of body weight of 40%–50% of the initial weight, only one animal would have survived. However, in the right-hand column it can be seen that the number of false-negative predictions (25) would have been substantial. Overall, it can be seen from Table 6 that body weight is not a valid parameter for euthanasia. However, body temperature $< 34.5^{\circ}\text{C}$ and clinical score

3 would be quite acceptable endpoints as these produce very few false-positive or false-negative predictions.

Discussion

Current guidelines on the use of laboratory animals such as the Council Directive 86/609/EEC (1986) of the European Union and the European Convention (1986) of the Council of Europe require that experiments be designed so as not to cause avoidable distress, pain and suffering to laboratory animals. However, the same regulatory bodies are also responsible for guidelines on animal tests that include lethal endpoints: viz., the *Ph.Eur.*, being under the authority of the Council of Europe, specifies lethality as the test parameter in a number of monographs on the quality control of human and veterinary vaccines. From the results of an informal inquiry held amongst vaccine manufacturers and national control laboratories it appeared that almost all laboratories strictly adhere to death as the endpoint as indicated in the monographs. It was striking to note that even in the case of the tetanus vaccine potency test, for which the *Ph.Eur.* offers a choice between lethality and paralysis, most laboratories prefer lethality as an

Table 6 Number of false-positive and false-negative results

Humane endpoint	False positive (a.m.)	(n = 46) ¹ (p.m.)	% (a.m.)	% (p.m.)	False negative (a.m.)	(n = 30) ² (p.m.)	% (a.m.)	% (p.m.)
Temperature								
35.5–35.0°C	9	4	19.5	8.7	0	0	0	0
35.0–34.5°C	2	3	4.3	6.5	2	0	6.7	0
34.5–34.0°C	0	1	0	2.2	2	0	6.7	0
≤34.0°C	0	0	0	0	4	0	13.3	0
Body weight loss								
20–30%	21	17	45.6	37.0	0	0	0	0
30–40%	10	7	21.7	15.2	6	2	20	6.7
40–50%	1	0	2.2	0	25	15	83.3	50
> 50%	0	0	0	0	30	28	100	93.3
Clinical score								
1	22	37	47.8	80.4	0	0	0	0
2	11	13	23.9	28.3	0	0	0	0
3	1	2	2.17	4.3	21	6.7	2.2	
4	1 ³	1	0 ³	2.2	9	7	30	15.2

¹ 46 animals survived the observation period

² 30 animals died in the observation period

³ Most probably invalid data

endpoint. Apparently death as an endpoint is attractive to many, as it is the ultimate objective sign, and not time-consuming to determine.

There are various categories of parameters having the potential to replace death as an endpoint of a test and so reduce the suffering of animals. These include, amongst others, clinical signs, biochemical and immunological indicators and pathophysiological effects. Endpoints have to be related to the type of test, not only from a scientific point of view but also from a logistic viewpoint. So, while biochemical parameters might be a very effective endpoint in a vaccine potency test, it certainly would not be a very practical endpoint. Vaccine potency tests are characterized by the large numbers of animals required, and by their short experimental period. Therefore, a humane endpoint will only be accepted if it is easy to monitor and not labour-intensive. In addition, endpoints should have a high predictive power for impending death and reduce suffering both in time and intensity. In vaccine quality control much effort is being directed to replacing the challenge test by a sero-logical approach, where the induced antibody titre is used as an estimate of the potency of a new batch of vaccines. However, in the case of the whole cell pertussis vaccine, the development—and especially the validation—of an immunoassay is a tedious job (van der Ark *et al.* 1996) and no major modifications in the lethal challenge procedure are expected in the next few years.

In our study we evaluated the use of humane endpoints in the whole cell pertussis vaccine potency test, using the following parameters: clinical signs, body temperature and body weight. Animals were monitored twice a day and the parameters were scored in the order as given above. A decrease in body weight ($< 10\%$) is the first indication of a pertussis infection and is seen a few days after challenge. No effects on body weight, body temperature or clinical signs were seen in the control animals receiving only an intracerebral injection of the vehicle (casamino acid). In infected animals the decrease in body weight ($> 10\%$) preceded the first clinical signs as well as the change in body

temperature by about 2 days. However, we found that a single decrease in body weight was not a very useful indicator for a lethal outcome of pertussis infection. A relatively large percentage (21.7%, morning inspection) of the animals with a substantial weight loss (30–40%) survived, while when using a decrease of body weight ($> 40\%$), a relatively large number of animals would already have died (83.3%) before reaching this state (Table 6). This is in line with the observation by Kort *et al.* (1998) studying *Klebsiella pneumoniae* in rats and mice. It should be noted that in other reports a change of body weight of 20% is proposed as an indicator for euthanasia (e.g. Spinelli 1991), but this could never be acceptable in vaccine quality control.

The use of implantable microchip transponders is of great help when frequent monitoring of body temperature in undisturbed animals is needed. In order to facilitate easy reading of temperatures, transponders were placed subcutaneously between the scapulae, and temperatures taken here correctly reflect core temperatures (Kort *et al.* 1998, 1999). In accord with previous reports, only decreases in body temperature were seen (Soothill *et al.* 1992, Thuring & Schonbaum 1994, Kort *et al.* 1998, 1999). This might seem contradictory to the observation that animals prefer to lie separately to keep cool, but such behaviour would increase heat loss. Watkinson and Gordon (1993) suggested that hypothermia induced by autonomic and behavioural thermoregulatory mechanisms might be beneficial for survival and while they made this statement with respect to rats, it might also be true for mice. We could demonstrate in our study that the optimum body temperature to predict correctly a lethal outcome of a pertussis infection was hypothermia below 34.5°C. When using this endpoint, suffering of the animals could be reduced by 1–2 days. This body temperature is 0.5°C higher than the cut-off point suggested by Soothill *et al.* (1992). The main advantage of body temperature as the humane endpoint is its objectivity and ease of measurement when remote sensing is used. On the other hand, additional costs are required for transponders (although re-usable) and reader.

A straightforward and accurate parameter for early euthanasia of animals suffering from pertussis challenge and infection appeared to be the loss of muscle coordination. Almost all animals showing this clinical sign died within 1–3 days and, consequently, killing these animals at the onset of this clinical sign would have reduced suffering substantially. A potential drawback of clinical signs can be their semi-objective character. However, a loss of muscle coordination is quite easy to detect and observer variation can be reduced by additional training at the site.

Conclusion

It is concluded that critical evaluation of the clinical signs accompanying a disease, e.g. after pertussis challenge, can lead to parameters being identified that could be used as surrogate endpoints to replace the natural and protracted death of the animal—that is, these parameters would be used to indicate humane killing before an animal died naturally.

References

- Acred P, Hennessey TD, MacArthur-Clark JA, *et al.* (1994) Guidelines for the welfare of animals in rodent protection tests. *Laboratory Animals* **28**, 13–18
- Brown F, Cussler K, Hendriksen C, eds (1996) *Replacement, Reduction and Refinement of Animal Experiments in the Development and Control of Biological Products*. Dev.Biol.Stand, Vol. 86. Basel: Karger
- Council Directive 86/609/EEC (1986) on the Approximation of Laws, Regulations and Administrative Provisions of the Member States Regarding the Protection of Animals Used for Experimental and other Scientific Purposes. *Official Journal of the European Communities*, Series L, No. 358, pp 1–28
- Council of Europe (1986) *European Convention for the Protection of Vertebrate Animals Used for Experimental and Scientific Purposes*. Strasbourg
- Hamm TE (1995) Proposed Institutional Animal Care and Use Committee Guidelines for death as an endpoint in rodent studies. *Contemporary Topics* **34**, 69–71
- Kort WJ, Hekking-Weijma IM, TenKate MT, Sorm V, van Strik R (1998) A microchip implant system as a method to determine body temperature of terminally ill rats and mice. *Laboratory Animals* **32**, 260–9
- Kort WJ, Hekking-Weijma IM, TenKate MT, Sorm V, van Strik R (1999) Determining body temperature using a microchip implant system. In: *Humane Endpoints in Animal Experiments for Biomedical Research* (Hendriksen CFM, Morton DB, eds). London: Royal Society of Medicine Press, pp 122–6
- Morton DB (1995) Advances in refinement in animal experimentation over the past 25 years. *Alternatives to Laboratory Animals* **23**, 812–22
- European Pharmacopoeia Monograph (1997) *Vaccinum Pertussis (Adsorbatum)*, 1305/1306, 3rd edn
- Soothill JS, Morton DB, Ahmad A (1992) The HID⁵⁰ (hypothermia-inducing dose 50): an alternative to the LD⁵⁰ for measurement of bacterial virulence. *International Journal of Experimental Pathology* **73**, 95–8
- Spinelli JS (1991) Preventing suffering in laboratory animals. *Scandinavian Journal of Laboratory Animal Science* **4**, 159–64
- Svendsen O, Sandoe P, Thorn NA (1997) Laboratory animal science, welfare and ethics in pharmacology and toxicology. *Pharmacology and Toxicology* **80**, 3–5
- Thuring JWGM, Schonbaum E (1994) Hypothermia and social behaviour in mice. *Advances in Pharmacological Sciences* 497–501
- van der Ark A, van Straaten-van de Kapelle I, Hendriksen C (1996) Pertussis serological potency test as an alternative to the intracerebral mouse protection test. *Developments in Biological Standardization* **86**, 271–83
- Watkinson WP, Gordon CJ (1993) Caveats regarding the use of the laboratory rat as a model for acute toxicological studies: modulation of toxic response via physiological and behavioral mechanisms. *Toxicology* **81**, 15–31