
Refinement of blood sampling from the sublingual vein of rats

Walter Zeller, Heinz Weber, Basile Panoussis, Thomas Bürge & Reinhard Bergmann

Novartis Pharma Ltd, Basel, Switzerland

Summary

A refined method of repeated blood sampling is described: the tongue of the anaesthetized rat is pulled forward with the fingers and the sublingual vein is punctured with a 23 gauge hypodermic needle. Based on the requirement of a pharmacokinetic study, 0.5 or 1 ml of blood was collected 7 times at 0, 0.5, 1, 2, 4, 8 and 24 h. The degree of suffering was judged by determining the body weight and food and water consumption. All animals showed an increase in body weight already after 24 h and, therefore, the method of collecting blood from the sublingual vein can be recommended for repeated blood sampling. The haematological evaluation of groups of animals with differing body weight showed that sample volumes of up to 15% of the total blood volume lead to haematocrit values of approximately 40%. A remarkable initial drop in white blood cell counts followed by a marked rise 2 h after first sampling to values partly above the pre-test could not be directly related to the extracted blood volume.

Keywords Blood sampling; stress; maximal sampling volume; sublingual vein

Repeated blood samples of more than 0.5 ml can be obtained from the rat by puncturing the orbital sinus or the tail vein or by using an indwelling catheter in the femoral or jugular vein (Van Zutphen *et al.* 1993). Since it could be shown, that lesions in the orbital area could occur when the venous plexus was punctured (First Report of the BVA/FRAME/RSPCA/UFAW Joint Working Group on Refinement 1993) this technique was forbidden in certain cases or limited e.g. by setting minimal intervals between two samplings from the same orbita (Canadian Council on Animal Care 1980, Van Zutphen *et al.* 1993, Bundesamt für Veterinärwesen 1995). Repeated tail venepuncture requires restraint, often warming of the animal, considerable skill and there is a risk of inability

to obtain all the samples. The implantation of a catheter into a vein enables repeated blood sampling with minimal restraint of the animal, as well as replacement of the extracted blood volume. Over-the-needle catheters, whilst straightforward to use with practice, when left *in situ* in the tail vein for long periods are very susceptible to interference from the animal (Waynforth & Flecknell 1992). However if catheters are implanted surgically, the surgery involved may cause a number of other potential problems with discomfort for the animal (First Report of the BVA/FRAME/RSPCA/UFAW Joint Working Group on Refinement 1993).

Drawing blood from the sublingual vein could represent a possible alternative (Rauen 1964, Angelov *et al.* 1984): stretching the rat's head back and pulling the skin of the face backward causes a conscious animal to open its mouth and to press its tongue

Correspondence to: W. Zeller, Kantonales Veterinäramt, Postfach 264, CH-4025 Basel, Switzerland

against its palate. The right or left vena sublingualis is cut with eye scissors. After four weeks no significant differences in body weight, body weight gain or food consumption between rats bled from the venous plexus of the eye or bled from the vena sublingualis could be noticed (Angelov *et al.* 1984). Unfortunately in this study no short-term effects were measured and no control group used. Other authors, however, do not recommend the use of the sublingual vein and report swelling of the tongue and possible discomfort and pain to the animal (First Report of the BVA/FRAME/RSPCA/UFAW Joint Working Group on Refinement 1993).

Aim of the study

It was endeavoured to refine the technique of drawing blood from the sublingual vein as it was practised in some of our laboratories. The degree of pain and distress imposed on the animal by repeated puncturing of the veins was to be objectively determined by measuring body weight and food and water consumption. The haematological investigation of the blood samples should allow for an estimation of the maximal blood volume that could be extracted.

Materials and methods

Refinement of blood sampling from the sublingual veins

During the past years blood had been drawn from the sublingual veins from rats in toxicity tests in more than 1000 cases in the agro-toxicological department of SANDOZ Ltd, without any obvious negative consequences for the animals. The customary method was to extend the tongue of the anaesthetized animals in front with the aid of fine forceps and to incise the sublingual vein with small scissors. In order to refine this method, especially with a view to using it for repeated blood sampling at short intervals, we studied the structure and configuration of the oral cavity in dead and anaesthetized rats. Various methods of extending the tongue and puncturing the sublingual veins were tried. The following

procedure proved to be technically the simplest and least harmful for the animal.

Unfasted rats are anaesthetized with isoflurane in an inhalation chamber. An assistant holds the unconscious animal in a supine position and gathers up the loose skin at the nape of the neck in order to produce partial stasis in the venous return from the head. A second person extends the tongue in front with a cotton-tipped applicator stick and grasps it with thumb and forefinger. Seizing the tongue with forceps causes blunt trauma and should, therefore, be avoided.

The two sublingual veins are clearly visible at the base of the tongue situated left and right of the median line. One of the veins is punctured with a 23 gauge (23 G \times 1"; 0.60 \times 25 mm) hypodermic needle as far peripherally as possible (Fig 1). Preliminary experiments showed that this size of needle was necessary to allow sufficient blood flow. The vein should be punctured carefully to avoid sticking the needle completely through the vein. For further blood samples later, the sublingual veins can be punctured alternately and closer to the basis of the tongue.

After successful puncturing the rat is turned back into a prone position by the assistant and the blood allowed to drip into a tube which was held ready (Fig 2). As soon as the required volume of blood has been collected, the compression is ceased by releasing the scruff of the neck. The animal is



Fig 1 The sublingual vein of an anaesthetized rat held in supine position is being punctured by the person holding the animal's tongue

again placed in a supine position, in order to extend the tongue in front as described above. The stab wound is mopped with a cotton-tipped applicator stick dipped into a 50% solution of iron chloride (Ferrum sesquichloratum solutum, G. Streuli, Uznach, Switzerland). After the procedure the animal is returned to its cage for recovery.

Experimental procedure

The experiments were performed on 36 male Icolbm:OFA rats (breeder: Biological Research Laboratories, Füllinsdorf, Switzerland) weighing 240 to 570 g. The animals were housed in rooms with controlled environment (22°C, 50–70% relative humidity, light/darkness cycle 06:30/18:30) in groups in macrolon cages (dimensions in accordance with the Swiss Animal Welfare Legislation: floor area 1800 cm², height 19 cm) with wood granulate as bedding. They were fed a pelleted complete diet (NAFAG Nr. 850, Gossau, Switzerland) made available

solely through a netting cage top. Group housing is the normally used system for pharmacokinetic studies in our company. The used cages permitted group sizes of five animals in the lower weight range (see Table 1) and 2 or 3 animals in the higher weight range. The animals were grouped at least 2 weeks prior to the experiments.

Either 0.5 or 1 ml blood was collected from the sublingual veins of the animals 7 times within 24 h using the technique previously described. Sampling times were chosen based on the requirement of a typical pharmacokinetic study (0, $\frac{1}{2}$, 1, 2, 4, 8, 24 h). The first sample of the first animal of a given group was drawn at 08:00 h. Blood samples were collected in EDTA tubes and analysed with a Technikon H1-E Analyser (Bayer AG, GB Diagnostica, Zürich, Switzerland).

Five groups were formed with different body weight ranges and sample volumes (Table 1). The weight groups were chosen according to the approximate weight range of rats at the beginning and the end of toxicity trials, since pharmacokinetic tests are usually performed at these times. In the groups A and A control the individual body weights were determined every morning. Food and water consumption of the individual animals was calculated as the average of the total amount consumed by a group. Animals of a corresponding control group were submitted to anaesthesia at the same time points as the bled animals, but no blood was collected. Groups B–D served to investigate the effect of collecting different amounts of blood from animals of different weight on the haematological values. All rats were killed with an overdose of isoflurane at least one week after the last blood sample.

Statistical evaluation of the measured values was performed by repeated measures analysis to take into account the time course of the experimental data. Univariate and multivariate analyses were calculated.

Results

With two exceptions (one animal in group B and C each at 0 + 8 h) it was always possible to draw the required amount of blood. The tongue surface dried quickly after extension

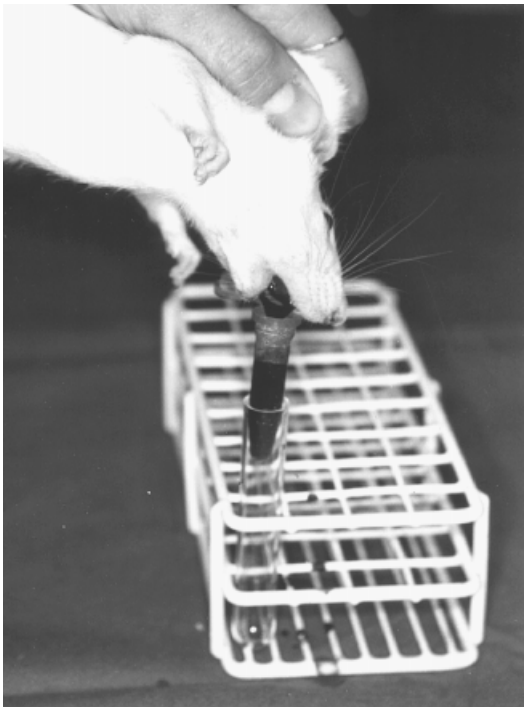


Fig 2 The blood of an anaesthetized rat is collected in a sampling tube immediately after the sublingual vein has been punctured. The scruff of the neck is released when sufficient blood has been collected

Table 1 Overview of experimental groups of rats submitted to consecutive collecting of seven blood samples within 24 h. Animals of group 'A control' were submitted to isoflurane anaesthesia alone, without blood collection

Group	Sample volume (ml)	Body weight (g)	No. of animals
A	0.5	270–310	10
A control	-	270–310	10
B	1	240–270	5
C	0.5	450–500	6
D	1	480–570	5

from the oral cavity. Small haematomas at the site of venepuncture were observed in about 50% of the rats, however, these did not hinder further blood samples from being taken. Mopping with iron chloride led to an immediate cessation of blood flow. Three animals died during anaesthesia (one animal each in groups A and C at +2 h and one in group D at +24 h). All other animals regained consciousness within minutes and no impairment was observed after the experiments. Any blood residues on the muzzle were licked off when the rats recovered from anaesthesia.

The results of the body weight determinations are given in Fig 3. All rats were still in the growth phase as is reflected in an increase of body weight during the 4 days

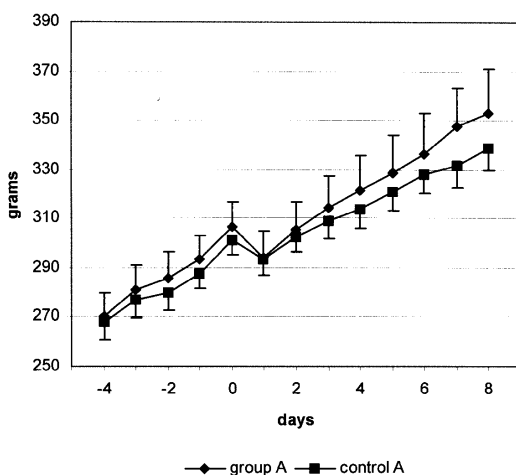


Fig 3 Body weight development (average \pm SEM) of rats of the lower weight range (270–310 g) before and after blood sampling (7×0.5 ml in group A) or anaesthesia only at the same time points (control A)

prior to the experiment. The day after the onset of blood sampling immediately before the 24 h sample was drawn, a decrease of the average body weight of 4% from 306.3 g (SEM 10.2) to 294.2 g (SEM 10.5) was observed in group A. In the control group in which the animals were only submitted to anaesthesia body weight dropped by 2.7% from 301.4 g (SEM 6.4) to 293.3 g (SEM 6.2). However, the univariate repeated measures analysis between subjects yielded no statistically significant differences between group A and control A with respect to whole time curve ($P=0.155$). Within the time course both curves showed significant growth ($P < 0.001$). The multivariate repeated measures analysis revealed the same results. One day after the last puncture the body weight of each individual animal had increased again.

Calculated median food consumption per animal (Fig 4) dropped from 28.0 g to 18.4 g (-34%) on the first day in the animals of group A that were subjected to blood sampling and from 24.5 g to 18.3 g (-25%) in the control group. On day 3 (2 days after the first blood sample) food consumption had returned to the initial levels. The average daily amount of water consumed 3 days before and after the blood sampling remained fairly constant between 30 and 34.5 ml per animal (Fig 4).

In the haematological investigations the erythrocyte counts of the rats with lower body weight (groups A and B) were significantly lower (Student's *t*-test ($P < 0.05$)) than those of the heavier animals (groups C and D) already at the beginning of the experiment. Nevertheless, a continuous decrease of the number of erythrocytes and

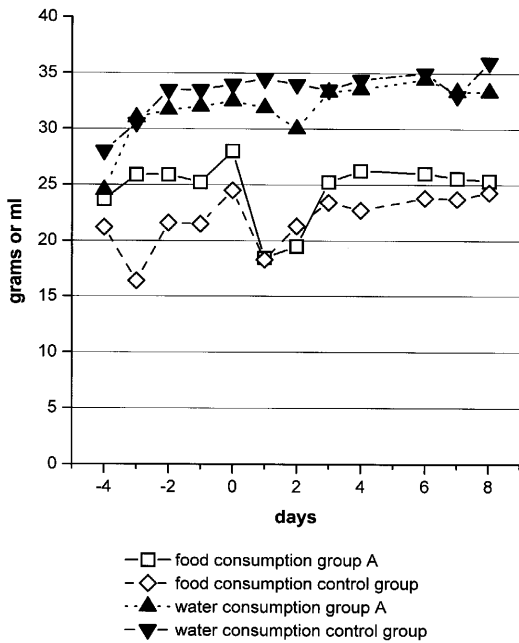


Fig 4 Median food and water consumption of rats of the lower weight range (270–310 g) before and after blood sampling (7 × 0.5 ml in group A) or anaesthesia only at the same time points (control A)

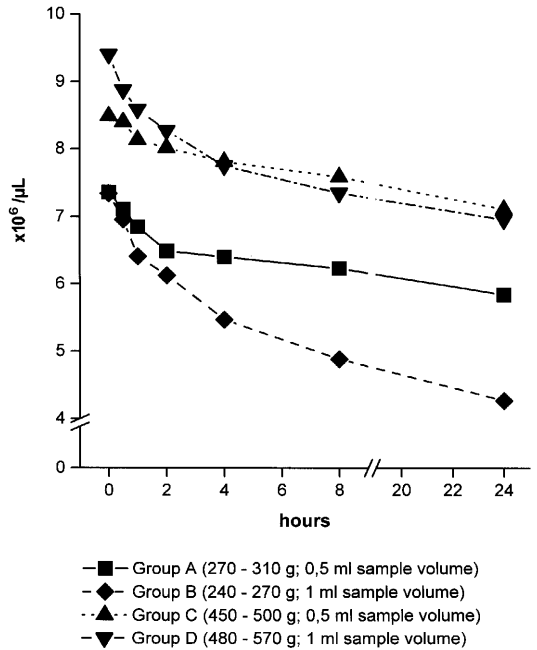


Fig 5 Red blood cell counts in four groups of rats of the lower (groups A and B) and higher (groups C and D) weight categories at the time points when volumes of either 0.5 or 1 ml blood were collected consecutively

the haematocrit values was obvious in all groups of both weight categories (Figs 5 and 6). The degree of reduction correlated with the amount of blood drawn and the body weight of the animals. Haematocrit values and number of red blood cells sank significantly ($P < 0.001$). The univariate repeated measures analysis yielded statistically significant differences between all groups ($P < 0.001$). No statistically significant differences existed in the higher weight category between groups C and D for haematocrit values and number of erythrocytes as well ($P = 0.393/0.260$). The multivariate repeated measures analysis confirmed these results.

White blood cell counts (Fig 7) dropped markedly initially. Minimal values were observed between 0 + 1 h (group B) and 0 + 2 h (other groups), then the values increased. In groups A and B significantly higher values than at the onset of the test were reached after 24 h (Student's *t*-test ($P < 0.05$)). The univariate repeated measures analysis proved that there are no statistically significant differences between the groups

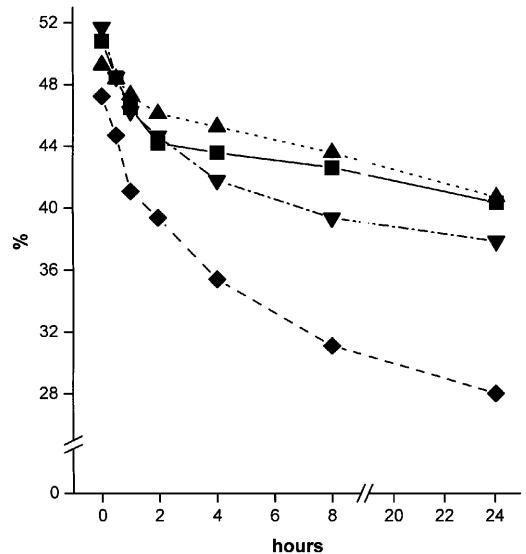


Fig 6 Haematocrit values in four groups of rats of the lower (groups A and B) and higher (groups C and D) weight categories at the time points when volumes of either 0.5 or 1 ml blood were collected consecutively. Refer to Fig 5 for key

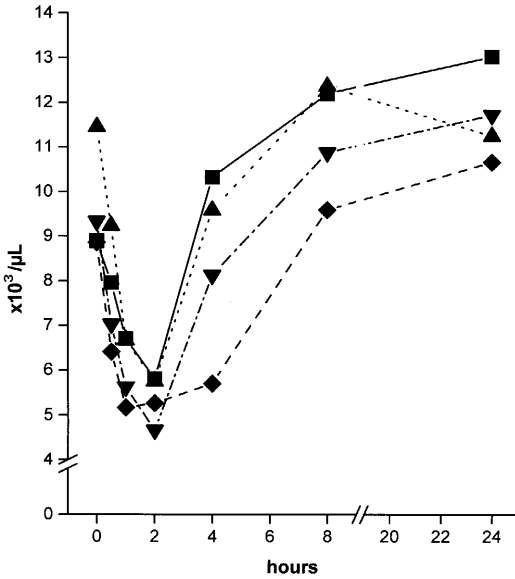


Fig 7 Leucocyte counts in four groups of rats of the lower (groups A and B) and higher (groups C and D) weight categories at the time points when volumes of either 0.5 or 1 ml blood were collected consecutively. Refer to Fig 5 for key

($P=0.414$). Comparison of single time points showed only a significant difference ($P=0.022$) for hour=4. Lymphocytes (Fig 8, no differences between groups ($P=0.083$),

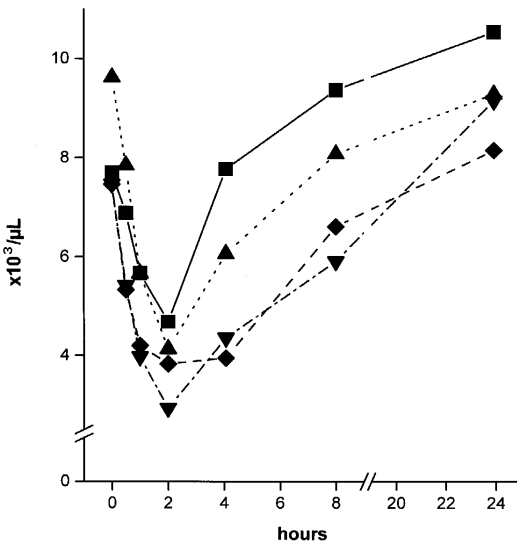


Fig 8 Lymphocyte counts in four groups of rats of the lower (groups A and B) and higher (groups C and D) weight categories at the time points when volumes of either 0.5 or 1 ml blood were collected consecutively. Refer to Fig 5 for key

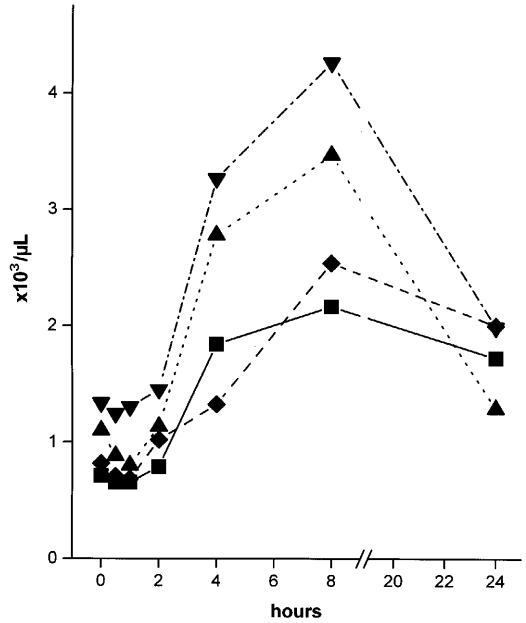


Fig 9 Number of neutrophil granulocytes in four groups of rats of the lower (groups A and B) and higher (groups C and D) weight categories at the time points when volumes of either 0.5 or 1 ml blood were collected consecutively. Refer to Fig 5 for key

significant difference on time points 1 ($P=0.031$), 2 ($P=0.022$) 4 ($P=0.000$) and 8 ($P=0.010$) h) basophils and eosinophils as well as the monocytes shared similar properties (data not shown).

The neutrophil granulocytes dropped in all groups slightly in the first hour but increased subsequently (Fig 9). The univariate repeated measures analysis proved that there are no statistically significant differences between the groups ($P=0.058$). Comparison of single time points showed only a significant difference ($P=0.020$) for hour=8. The multivariate repeated measures analysis confirmed these results ($P=0.061$).

Discussion

The method of Angelov *et al.* (1984) and Rauén (1964) could be refined by extending the tongue of the anesthetized animal without causing trauma and the targeted puncturing of the sublingual vein. The technique proved to be reliable and easy to learn, since the veins could be identified

visually. However, the three deaths during anaesthesia led us to recommend the use of a calibrated vaporizer instead of a simple inhalation chamber.

The determination of body weight and food and water intake permitted an unbiased estimation of the effects caused to the animals since pain in the oral cavity and the tongue would lead to reduced food and water consumption and consequently to a weight reduction. As expected, the induction of short anaesthesia seven times within 24 h did stress the animals. This was manifested by the reduction in food consumption and body weight seen in the control group to a similar extent. However, 24 h after the last of seven blood samples were collected, all the animals had gained weight again. We presume, therefore, that the animals were not severely distressed. We assume that all the techniques available today for repeated blood sampling over a short period of time cause some degree of discomfort to the animal. This is also true for the method of sublingual venepuncture under general anaesthesia which, however, does not seem to cause more discomfort than the other methods. The extreme adverse effects as described in the literature (First Report of the BVA/FRAME/RSPCA/UFAW Joint Working Group on Refinement 1993) were not observed with the method we applied.

The alternative method of puncturing the same orbital venous sinus is disapproved for repeated blood collection in short intervals (Canadian Council on Animal Care 1980, First Report of the BVA/FRAME/RSPCA/UFAW Joint Working Group on Refinement 1993) and not allowed by the Swiss Animal Welfare legislation (Bundesamt für Veterinärwesen 1995). As a consequence more animals would have to be used. With our improved method of collecting blood from the sublingual vein it is, therefore, possible to avoid increasing the number of animals required without causing unacceptable distress to the animals.

The implantation of an indwelling intravenous catheter would have the advantage over the method of blood sampling by venepuncture that the collected blood could be replaced. This would result (e.g. in pharma-

cokinetic studies) in more reliable data since some compounds may bind to erythrocytes or other blood components (Lemaire & Tillement 1982). However, the implantation of an indwelling catheter is often not possible—especially in toxicokinetic studies, in which the animals are experimented on for prolonged periods of time. In addition, it cannot be excluded that the surgical intervention and the reaction to the implanted catheter could have the stronger influence on the results of the toxicity test.

The total blood volume of the rat amounts to about 5–8% of its body weight (Van Zutphen *et al.* 1993, First Report of the BVA/FRAME/RSPCA/UFAW Joint Working Group on Refinement 1993). According to McGuill and Rowan (1989) up to 15% of the total blood volume may be drawn within a day. From a rat of about 300 g, therefore, 2.25–3.6 ml and from a rat of 500 g 3.75–6.0 ml of blood could be collected. In our experiments we collected 3.5 or 7 ml of blood from rats of both body weight groups of about 300 g and 500 g. The haematological data indicate that the withdrawal of 7 ml of blood in rats of 300 g body weight leads to unacceptably low haematocrit values. The withdrawal of 3.5 ml in animals of 300 g and of 7 ml in rats of 500 g, however, does not reduce the haematocrit values essentially below 40%. Our results correspond with the study of Scipioni *et al.* (1997). In addition they were able to show that the haematological parameters reached control values within approximately 14 days after blood sampling.

The effects on white blood cells differ from those in red blood cells. The changes do not coincide with the amount of blood withdrawn or with the changes in haematocrit or erythrocyte counts. The initial drop in lymphocyte counts is more marked than expected by blood loss and both lymphocytes and neutrophils increase again 2 h after the first sampling, in contrast to red blood cell values. The total neutrophil volume consists of the circulating neutrophils and the marginated tissue pool. The size of these pools is affected by stress (Handin *et al.* 1995). Glucocorticoids induce a rapid and reversible decrease in numbers and percentage of lymphocytes,

and an increase in numbers and percentage of neutrophils (Dhabhar *et al.* 1995). Some anaesthetics (Archer *et al.* 1981) or diurnal variations (Shatland *et al.* 1981) may also affect leukocyte values. Since these effects were also noticed with other methods (Dhabhar *et al.* 1995, Scipioni *et al.* 1997) they were considered not to be related to the blood sampling from the sublingual vein.

The refined method of collecting blood from the sublingual vein of rats has been accepted by the Swiss authorities for repeated sampling and is been routinely used by various research institutes. Studies to compare stress effects and clinical pathology parameters between the methods of blood collection from the sublingual vein and blood collection from the orbital plexus will be published separately (Mahl *et al.*, in preparation).

Acknowledgments The authors would like to thank Sandoz Pharma Ltd for support of this work and Gilles Sabo, Institute Dr. Viollier, Basel, for his help in interpreting the haematological data.

References

- Angelov O, Schroer RA, Heft S, James VC, Noble J (1984) A comparison of two methods of bleeding rats: the venous plexus of the eye versus the vena sublingualis. *Journal of Applied Toxicology* **4**, 258–60
- Archer RK, Riley J (1981) Standardized method for bleeding rats. *Laboratory Animals* **15**, 25–8
- Bundesamt für Veterinärwesen (1995) *Information Tierschutz 3.02: Blutentnahmen bei Labornagetieren und Kaninchen zu Versuchszwecken*. Liebefeld, Schweiz: Bundesmat für Veterinärwesen
- Canadian Council on Animal Care (1980) *Guide to the Care and Use of Experimental Animals*. Ottawa: Canadian Council on Animal Care
- Dhabhar FS, Miller AH, McEwen BS, Spencer RL (1995) Effects of stress on immune cell distribution. Dynamics and hormonal mechanisms. *Journal of Immunology* **154**, 5511–27
- First Report of the BVA/FRAME/RSPCA/UFAW Joint Working Group on Refinement (1993) Removal of blood from laboratory mammals and birds. *Laboratory Animals* **27**, 1–22
- Handin RI, Lux SE, Stossel TP (1995) *Blood—Principles & Practice of Hematology*. Philadelphia: JB Lippincott Company, pp 516–17
- Lemaire M, Tillement JP (1982) Role of lipoproteins and erythrocytes in the *in vitro* binding and distribution of cyclosporin A in the blood. *Journal of Pharmacy and Pharmacology* **34**, 715–18
- Mahl A, Heining P, Ulrich P, Jakubowski J, Bobadilla M, Zeller W, Singer T (1998) Comparison of clinical pathology parameters with two different blood sampling techniques in rats—retrobulbar plexus versus sublingual vein (in preparation)
- McGuill MW, Rowan AN (1989) Biological effects of blood loss: implications for sampling volumes and techniques. *Illar News* **31**, 5–18
- Rauen HM (1964) *Biochemisches Taschenbuch*. Berlin: Springer Verlag, p. 329
- Scipioni RL, Diters RW, Myers WR, Hart SM (1997) Clinical and clinicopathological assessment of serial phlebotomy in the Sprague Dawley rat. *Laboratory Animal Science* **47**, 293–9
- Shatland BE, Winkel P, Harris SC, Burdsall MJ, Saunders AM (1981) Evaluation of biological sources of variations of leukocyte counts and other hematologic quantities using very precise automated analyzers. *American Journal of Clinical Pathology* **69**, 48
- Van Zutphen LFM, Baumans V, Beynen AC (1993) *Principles of Laboratory Animals Science*. Amsterdam: Elsevier, p. 307