Refining procedures for the administration of substances

Report of the BVAAWF/FRAME/RSPCA/UFAW Joint Working Group on Refinement

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Preface

This workshop report is the fifth in the series produced by the Joint Working Group on Refinement organized by the British Veterinary Association Animal Welfare Foundation (BVAAWF), the Fund for the Replacement of Animals in Medical Experiments (FRAME), the Royal Society for the Prevention of Cruelty to Animals (RSPCA) and the Universities Federation for Animal Welfare (UFAW). The workshops are based on the important principle that whenever animals are used in laboratories, minimizing any pain and distress should be as important an objective as achieving the experimental results. This is essential for humanitarian concerns. It is also necessary in order to satisfy the broad legal principles in national and international legislation, and to produce reliable and reproducible scientific data.

The members of each Working Party are drawn from the scientific community, from industry, academia and animal welfare organizations. Some of the organizations participating (for example, the Royal Society for the Prevention of Cruelty to Animals) are opposed to the use of animals in experiments that may cause the animals pain, suffering, distress or lasting harm, and this particular workshop was a difficult one for anyone in such a position to be involved with because of the nature of the procedures discussed. (Note: participation in this Workshop does not indicate endorsement by the Society of research requiring the use of the procedures described.) However, the common aim is to reduce animal suffering wherever it occurs and the report is intended to help achieve this, particularly if read in conjunction with other recent reports on the recognition, measurement and alleviation of pain or distress in animals.

This particular workshop also proved extremely difficult to report, partly because of the breadth of the subject and the numbers of procedures we set out to cover. In addition, although many of the techniques are described briefly elsewhere, the sort of detailed information required regarding potential problems and solutions, and about ways of further refining techniques, had not previously been collated and were not available in the literature.

It is hoped that the report will be widely circulated in an international forum, and that the recommendations will be adopted as current good practice.

1 Introduction and aims of the report

The procedures involved in the administration of substances to animals can have a significant effect on the welfare of the animal and the scientific value of the results—if carried out incorrectly then both can be compromised. Refining administration procedures therefore provides opportunities for improving both welfare and science. The welfare benefits are widely recognized; the scientific benefits result from better quality data obtained from more carefully prepared experiments, using less stressed, more 'normal' animals (Manser 1992, Vogel 1993, Poole 1997).

The administration of substances is a very broad topic—many different kinds of substance are administered by many different techniques and routes and for a variety of purposes. The methods involved are described in a number of publications (e.g. Paget & Thomson 1979, Kirk & Bistner 1985, Poole 1987, Rollin & Kessel 1990, Waynforth & Flecknell 1992, Tuffery 1995, Wolfensohn & Lloyd 1998) and guidelines on good practice have recently been produced by the Laboratory Animal Science Association (1998). This report is intended to complement the existing literature by identifying potential problems with individual methods and procedures and focusing on how these can be refined to reduce adverse effects and develop best practice. The most commonly used routes in the common laboratory species are covered with notes on additional methods or other species where appropriate. The emphasis is on techniques carried out in biomedical research and testing but some of the principles also have clinical application in veterinary medicine.

The report should provide useful information for anyone planning or carrying out procedures, or who has to deal with their

consequences. General principles of 'good practice' when administering a substance by any route are set out in Section 2, together with recommendations for refinement with regard to the substance administered, equipment and techniques. Section 3 then describes specific refinements within individual routes and techniques. The report concludes with a section addressing special considerations when administering substances to wild animals.

The report is based on the published literature where this is available and on the considered expert opinion of the working group and their colleagues.

2 General principles of `good practice'

When administering a substance to an animal by any route the aim should be to achieve 'best practice', since mistakes at any stage can cause avoidable suffering and/or waste animals' lives. Best practice depends on minimizing or avoiding adverse effects, minimizing the number of animals used, and maximizing the quality and applicability of results.

2.1 Planning and preparation

The likelihood of difficulties arising is reduced by thorough preparation. Contingency plans should always be prepared in case problems do occur. A 'checklist' for planning procedures is given in Table 1.

2.1.1 Experimental aims

The scientific aims must be met by the route and administration regime selected, so it is important to check this first. The likely pattern of results should be considered, together with the way they relate to the study's goals and what will subsequently be done with the results and with the animals.

At this stage it is important to consider not only whether the experiment *can* be done but also whether, given the likely effects on the animals, it *should* be done.

2.1.2 The route

The choice of route is determined by the purpose of the experiment, the species of the animal, the possible effects of the dosing technique on the animal and the expected frequency of dosing. The choice of technique and, where relevant, the site selected are both also influenced by such factors.

Some routes and techniques are more stressful than others and the least severe consistent with the experimental aims should be selected (see Table 2).

2.1.3 The substance

There are constraints on the formulations that can be used for any particular route. Both the substance *and* the dosing vehicle must be appropriate for the route, the species and the purpose of the experiment. Detailed information on factors to be considered is available in the literature (see for example, Sanderson 1959, Spiegel & Noseworthy 1963, The Merck Index 1968, Claasen 1994, Waynforth 1995, Reynolds 1996) and the following brief summary is intended only as a starting point.

Physicochemical properties: The physicochemical properties of the substance and/or its vehicle may adversely affect animals. These include: the formulation, solubility, viscosity, pH, biocompatibility, purity, stability, standardization and microbial contamination. Background data on such properties together with any extraneous effects (e.g. irritancy), should always be investigated. The expected effect may be altered by concentration and dose volume so this should be checked too. A useful reference document is the *Dictionary of Substances and their Effects* (Richardson 1993, Claasen 1994).

Solutions and solvents: Where substances are dosed as solutions, water for injection or physiological saline are the commonest and simplest solvents. For water-insoluble compounds a suitable organic solvent may be employed. Ideally this should lack pharmacological effects, be stable under conditions of use, non-toxic, non-irritant and non-sensitizing. The viscosity should be suitable for ease of injection. The solvent must remain fluid at the temperatures at which it will be

Table 1 Checklist when planning procedures

Experimental aims	What are you trying to achieve scientifically? Will the administration regime selected meet the aims of the experiment? Consider not just whether it *can* be done but whether it *should* be done and whether there is a better way of doing it?
The route	Is the administration route suitable for the substance? Does the proposed route have a high severity rating? Would a less severe route achieve the same aim? Is it suitable for repetitive doses?
The substance	Are you certain you know what you are administering? Will the substance have any adverse effects on the animal and are there any background data on these? If so, have the necessary preparations been made? Could the nature of the formulation alter the expected effect? Does the substance need to be freshly prepared? Will the concentration and dose volume alter the expected effect? Are there any additional concerns regarding the physico-chemical properties of the substance or associated solvents, e.g. osmolarity? Can the volume be reduced? Can the frequency of administration be reduced? If the substance is toxic can the dose be reduced? Is the substance likely to be irritant? Are pilot studies needed, e.g. to ascertain a tolerated and/or effective dose?
The animal	Are there any problems with the particular individuals, species or strain? Is the animal easily stressed by handling? Is it the most appropriate for the study in terms of these factors? Can the animal be trained to cooperate with the procedures? Does the animal need time to acclimatize to the procedures? Is an anaesthetic, sedative or analgesic required. Would it reduce stress or confound the experiment? Have you done a pilot study for tolerated and effective dose levels in the strain used?
The technique	What are the scientific problems (e.g. first-pass metabolism in the liver after oral or i.p. dosing, degree or rate of absorption, local effects)? What are the technical problems (e.g. what is the correct way to hold an animal to allow insertion of a gavage tube with minimum distress)? Will the technique itself have any effect on the animals? Are the severity limits/humane end-points clearly defined? What refinements can be introduced to overcome any adverse effects? Is a pilot study necessary? Have you checked on references and sources of expertise in other organizations?
Staff	Do staff have the necessary licences? Are staff competent in the technique and trained to deal with any untoward effects? Who are the best members of staff to carry out the procedure, when considering both the handling of the animals and the procedure? Are enough staff available to restrain and dose the animals and to monitor them post-administration? Are staff aware of the severity limits and have the delegated authority and skill to kill animals if the severity limits are exceeded?

used and ideally have a sufficiently high boiling point to allow heat sterilization if required. Unfortunately no ideal solvent exists—many are suitable only for particular routes and those available must be evaluated to decide which is most appropriate.

Insoluble solids or immiscible chemicals may be dosed as suspensions or emulsions in suspending agents such as gum tragacanth or methyl cellulose, Lissapol™, Sorpol™ or Tween™. (Note that material such as Tween™ and Cremophor™ can cause anaphylactic-like

reactions when administered intravenously to dogs.)

Formulations should be used as soon after preparation as possible, or within the period defined as satisfactory for maintenance of the stability and quality of the formulation.

Rate of absorption: The expected pharmacology or toxicity of a substance may be influenced by its rate of absorption which is in turn influenced by: (a) its physical properties—such as molecular conformation, charge, particle size, crystalline form, lipid solubility, salt form, rate of disintegration of the solid dosage form, the degree to which the chemical is ionized at the absorbing membrane; and (b) the route of administration (see Waynforth 1995 for more details). Pilot pharmocokinetics/metabolism studies may be valuable (or essential) before long-term administration to confirm the dose is reaching the target organs.

Extraneous factors: Extraneous factors can also have an effect on the expected pharmacology or toxicity of a substance. These include variations in the environmental temperature, the number and/or density of animals housed together, their dietary status and the time of dosing in relation to a diurnal rhythm usually controlled by the light/dark cycle (Friedman & Walker 1972, Weihe 1973, Rao 1986, Wollnik 1989, Waynforth 1995).

2.1.4 *The animal*

Species and strain: When selecting the species and strain of animal, welfare factors should be considered as well as the need to satisfy scientific goals. Important points to consider are whether there are any contra-indications about using any individual species or strain of animal, whether the animal is appropriate for the route and the dosing compound, whether satisfactory husbandry can be provided and whether the animal is easy to handle. Some strains of common laboratory species are more docile and easily handled and it may be beneficial to select such a strain to reduce unnecessary stress.

Sex: Some studies may require only males or females and there may be significant wastage of the unwanted sex as a result. The justification—scientific, economic or traditional—for using a single sex should always be questioned to try to reduce such wastage. The motivation may initially have been the welfare implications, for example the ease of group housing female animals such as mice. However, it may be possible to adapt the husbandry system so that male animals can also be group housed (Jennings *et al*. 1998).

Habituation and training: Many laboratory animals will have had little or no experience of being handled or restrained before arriving at a research establishment so on the first occasion they are picked up they are likely to be apprehensive and frightened. This not only makes the procedure more distressing for the animal and more difficult for the doser, but is also likely to increase any stress-related background variance in the results. The first stage therefore should be to get the animals accustomed to being handled in their new surroundings. Many species become familiar with individual people, so it is helpful for the staff who will be carrying out the dosing to develop this familiarity beforehand. The importance of this for dogs and primates is recognized but other species such as rats and mice will also benefit. During handling animals can then be accustomed to being held in the position for dosing so that any stress is minimized when dosing does take place.

Where substances are administered infrequently but on a long-term basis (e.g. dietary toxicology in rodents) handling the animals during routine daily husbandry will help reduce stress when subsequently dosing them. In general, staff should be encouraged to handle animals as much as possible. There are exceptions to this general rule, e.g. with neonates, or if handling is likely to compound the effects of procedures.

Some species of animal can be trained to cooperate with the handler during handling and dosing and this can further reduce distress. Training-by-reward (positive reinforcement) can encourage animals to participate in the administration procedure, e.g. pri-

Table 2 Welfare impact of dosing routes

Route	No. of doses	Restraint	Comments	Welfare impact
Intra-articular	S	Anaesthesia	Can damage the joint; sterility essential; use once only	●●●
Intracerebral	S	Anaesthesia	Technically difficult in neonates. Death can result from poor technique, volume or nature of material, or from rejection by dam	●●●
Intradermal	R	M	Good technique essential to ensure injection is not subcutaneous	●●
Intramuscular	R	M	Irritant effects can be a serious problem. Possible damage to nerves. Avoid injecting into fascial planes or blood vessels. Effects of large-dose volume and tissue damage are hidden. Care with adjuvants. Consider injecting into different sites for successive administrations	●●
Intranasal	R	M	Not easy to ensure complete dose is in the nostril. Adverse effects unlikely, but care with fluids	●
Intraperitoneal	R	M	Irritant substances cause severe problems. Easy to misplace dose into an organ but not easy to know it has happened. Not recommended for animals larger than rodents	●●
Intratracheal	S	Anaesthesia	Can result in death if substance is administered wrongly or material is irritant	●●●
Intravaginal	R	M	Can be difficult to retain substance in vagina	●
Intravenous, non-rodent	R	M	Bolus effects on central nervous system (and probably heart) are relatively common	●●
Intravenous, rodent	R	M	Might need to warm animal to dilate vein; this should be done with care. Rapid injection can result in a bolus reaching the central nervous system or other organ and being fatal	●●
Intravenous	R	Tethers, jackets	Aseptic technique critical. Surgery may be required. An expanding field; with new infusion techniques being developed	●●●
Oral:				
In food/water	R	None	Dose may vary with food/water intake. Dosing in food causes little stress but unpalatibility can restrict intake; possible distress from thirst. Knowledge of feeding behaviour important	●
Capsule	R	M	Easy administration in dogs and primates and failure is very unusual. May be used to aid administration of tablets	●
Gavage	R	M	Accurate placing of tube essential. Failure should be rare but death can occur in rodents if tube misplaced. Restraint can be stressful for primates	●●
Osmotic pumps	Continuous	Anaesthesia, then none	Avoids multiple injection	●●

Respiratory:				
Whole body exposure	R	None	Difficult to measure dose, but failures or technical/welfare problems are rare	•
Inhalation nose only (rodent)	R	Tubes	Stressful because of tube restraint. Training and habituation of animal essential	••
Inhalation mask (dog or primate)	R	Slings or chairs	Restraint stressful. Training and habituation of animal essential	••
Subcutaneous	R	M	Change site for successive doses; care with adjuvants	•
Topical dermal	R	Dressings, collars, jackets	Removal of adhesive dressings can be painful. Animals need to be trained to accept collars or other restraints. Irritant substances a problem	••
Topical ocular	R	M	Technique easy but eye damage can result from irritant substances and be very painful	•

M = manual restraint during a brief period of dosing; R = repeat dosing; S = single dose
• Least impact: e.g. not painful, minimal restraint, quick or non-invasive
••• Most impact: e.g. anaesthesia may be required (with attendant risks), death or serious injury could result from incorrect technique
Notes: The scoring system refers to the impact of the route, *not* the substance, and assumes that the technique is carried out by trained and competent staff with appropriate resources. Scoring is also based on human experience of the procedure. The severity of any particular technique may vary with the species being dosed

mates will present an arm (Reinhardt 1991, 1997).

On rare occasions, for example one-off dosing when there is not time to accustom the animal to handling or to the procedure, it may be best to consider sedating the animal before dosing. However, since this in itself will require both restraint and a dosing procedure, the advantages and disadvantages of sedation must be carefully weighed.

2.1.5 The technique

There may be practical difficulties with individual techniques which could compromise the scientific goals or the welfare of the animals and these need to be recognized in advance, and avoided. For example, there may be a limit on the maximum dose volume or degree of absorption of a substance, or a procedure (such as oral gavage) may demand considerable technical skill.

It is important to study a technique before carrying it out and to obtain information from the literature and/or from others expert in the technique. Particular factors to consider are whether: the technique, as opposed to the substance, will have any effect on the animal; the equipment to be used is the most appropriate; the frequency of administration could be reduced; and the end-points are clearly defined and humane.

At this stage, consider whether there is any way the techniques or procedures could be further refined.

2.1.6 Staff and training

The best way to ensure minimal discomfort for animals during procedures is to carry these out competently and efficiently—the severity of even a simple procedure is increased if it is poorly performed. It is the responsibility of all facilities to ensure that animals are only held, restrained, dosed and monitored by thoroughly trained and competent staff. There should be enough competent staff available at all stages.

The selection of staff to carry out procedures should take into account their skill in handling the animals competently and sympathetically, as well as in performing the technique. Staff training and adequate

supervision in the learning stages is very important. The essential components include:

Animal handling: Training in the handling of the species to be dosed is important since sensitive and competent handling and restraint minimizes animal distress. It also helps ensure confidence and dexterity in carrying out the procedure which further reduces animal distress.

Use of training aids: Videos and educational computer software provide a useful resource (see Zinko *et al*. 1997), preferably watched or worked through with an appropriately experienced person. Preserved or recently killed animals and animal models, such as the Koken rat and rabbit, can be used to assist study of anatomy and the practice of technique. Particularly useful are models developed by Moredun Isolators Ltd (Pentland Science Park, Bush Loan, Penicuik, Scotland EH26 OPZ).

Practice with inanimate objects: Objects such as fruits can be used to help gain familiarity in handling and using needles, syringes and other equipment, e.g. try satsumas for subcutaneous injections.

Observation and assistance: The trainee should observe and assist in the relevant techniques as much as possible prior to carrying out a procedure. This is an essential part of training, and its importance cannot be over-emphasized. It may also be necessary and helpful to visit establishments with expertise in a particular technique.

Supervision: Once authorized to carry out a procedure the 'trainee' should initially assist and then carry it out under the direct supervision of an experienced member of staff until sufficient expertise has been gained to avoid mistakes. Supervisors should explain fully what they are doing and point out how and where things can go wrong to help the trainee avoid problems. They should encourage the trainee to ask questions. Even when the trainee has become familiar with the technique, the supervisor should always be available to help and answer questions if necessary. (In the UK, supervision is generally required under the personal licencing system and should be discussed with the local Home Office Inspector.)

Maintaining competence: All staff should maintain their own level of competence or allocate the work to a person who is competent and uses the technique regularly. This is particularly applicable to those who only perform a procedure occasionally or on a small number of animals. Consistent high standards can be ensured by procedures always being carried out by experienced animal house staff rather than by scientists who are less skilled in such techniques. Where this is not an option, re-training may be necessary and videos and models are particularly useful for this purpose. There may also be a need for further supervision.

In the UK attendance on an accredited training course, together with supervision until competent, is a necessity, with additional training for surgical and other specialized techniques. Practicing techniques on living animals protected under UK law is not allowed.

Awareness: Staff should have a detailed knowledge of what is being done to animals in their charge and when it is being done. They should know the end-points and severity limits of the project and be able to recognize if these are exceeded. They should also have the delegated authority and skill to kill animals if necessary. Information on who to go to for advice (experienced laboratory animal scientists, animal technologists, the veterinarian attached to the establishment) should be easily available to all staff who need it.

2.2 Technical preparation and aftercare

Before commencing dosing: Before substances are administered to animals for the first time, a pilot study with a small number of animals should be considered if there is any risk of adverse effects. Always:

- Make sure you are doing what you think you are doing, i.e. check that both the

substance and its vehicle are stable and pure; check the labels on the substance and the cages or animals to ensure the correct substance is given to the correct animal at the correct dose.
- Ensure the necessary equipment is to hand, clean or sterile if necessary, and working properly.
- Confirm the time plan and experimental protocol to ascertain the numbering of animals in each dose group and the timing of any additional investigations related to dosing (e.g. urine collection or blood sampling) and have the necessary receptacle(s) ready and pre-labelled.
- Check the sex, age, weight and condition of each animal.

Animals should always be approached and handled firmly, quietly and sympathetically, and reassured during the procedure. Only the minimum restraint necessary should be used.

Consider whether an anaesthetic, sedative or analgesic is required and whether other procedures (e.g. weighing or palpation) can be carried out at the same time. In general animals should be weighed before dosing so that the correct dose can be calculated for the weight, but if handling them twice is likely to be stressful they should be weighed and dosed at the same time.

After dosing: Animals must be closely monitored after dosing. Staff must always be prepared to deal with both expected and unexpected adverse effects. There must be adequate arrangements to cover the possibility of animals becoming distressed overnight or at weekends. If animals die as a result of the procedure a post-mortem examination should be carried out to ascertain the cause of death and to avoid the situation recurring.

Repeat dosing: Repeat dosing should be carried out at approximately the same time each day to avoid variability associated with circadian rhythms.

2.3 General refinement for all routes

There are some opportunities for refinement with most procedures (e.g. with respect to the dosing substance, equipment and technique) that can have significant effects on the welfare of the animal and the quality of scientific data and these are identified below.

The substance

(i) *Irritancy/pH*: The substance may cause irritation and/or ulceration of skin, mucous membranes or muscles, or destroy tissue locally (e.g. the endothelium of blood vessels). This may not be easily visible depending on the route of administration, but may cause serious welfare problems. Irritation may be a particular problem with the intraperitoneal, ocular, inhalation and intratracheal routes.

- Always check background data on the substance and its effects. Therapeutic data may help assess the local effect of a substance prior to dosing.
- Solutions for injection should ideally be close to neutral pH since high or low extremes are not tolerated by tissues. Waynforth and Flecknell (1992) recommend a working range of pH 4.5–8.0. The order of degree of tolerance of pH for different dosing routes is: oral > intravenous > intramuscular > subcutaneous. Note that the pH alone is an insufficient guide to irritant potential since irritancy also depends on the concentration and ionization point (pK) of the compound.
- Some effects, e.g. acute skin or endothelial damage, will be apparent even if the substance is applied to recently killed animals so check this first, then consider assessing the effect on an anaesthetized animal.

(ii) *Solubility*: Particulate matter may be present in the material being injected or the substance may fall out of solution resulting in formation of large particles. If administration is intramuscular this can be very painful and can prevent absorption.

- Ideally use biocompatible stable solutions.
- Before dosing, check whether the formulation is aqueous or oil based and whether it is stable or likely to fall out of solution. If there is any chance of particulate matter being present or of the substance coming out of solution then a filter (e.g. micropore) can be included in the injection line. However, note that test material can be filtered out or adsorbed onto the filter and the dose reduced as a result.

(iii) *Biocompatibility*: Substances that are not biocompatible may cause tissue damage.

- Use *in vitro* techniques to identify adverse effects, e.g. haemolytic and cytopathic effects, if appropriate.

(iv) *Viscosity*: Highly viscous liquids may cause discomfort and are difficult to inject requiring a larger needle size.

- Try not to use viscous substances.

(v) *Sterility*: Contaminated substances can cause infection and cause irritation at the site of injection. This can lead to self-mutilation and, at worst, the death of the animal.

- All substances for injection should be sterile—autoclaving and microfiltration are usually the most practicable ways of achieving this. Use aseptic techniques throughout in order to minimize the risk of introducing pathogens into the body at the time of dosing.

(vi) *Quality, standardization and stability of the substance*: All of these factors can affect experimental results.

- Check the compound is pure. Avoid contamination with other substances or microorganisms and avoid variation either in batch constituents or in the physical and chemical form of the substance (e.g. differing salts). Note the batch number for future reference.
- Stability of substances can be improved by microencapsulation. This can also be used to separate incompatible or reactive substances, to provide controlled and sustained release, or to mask bitter tastes (Melnick *et al*. 1987).

(vii) *Temperature*: Injection of cold substances, e.g. straight from the refrigerator, can cause discomfort and shock.

- Warm substances to room, or better still, body temperature immediately before administration.

Use of antiseptic solutions

(i) A thick hair coat prevents contact of the antiseptic with the skin. Excessive application of antiseptic solutions to the skin may cause loss of body heat through evaporation, especially in small mammals.

- Part the hair, or it may be necessary to clip the hair, from the injection site to allow antiseptic preparations to work effectively. Use these preparations with care.

Injections

Needle-free injectors are becoming more widely available and their use for administering substances to animals should be encouraged.

(i) *Needles*: Insertion of large needles can be painful and cause unnecessary and excessive tissue damage.

- A skin anaesthetic, e.g. EMLA (Astra Pharmaceuticals Ltd, Home Park, King's Langley, Hertfordshire, UK) cream (Flecknell *et al*. 1990) can reduce the pain of needle insertion. EMLA needs to be applied 30–45 min prior to injection to have full effect.
- It is important to balance the size of needle necessary for ease of injection of the substance with that which will cause minimal pain, i.e. use sharp needles of the narrowest gauge consistent with efficient accurate dosing

Table 3 Needle sizes for administration of substances

Species	Intradermal	Subcutaneous	Intramuscular	Intravenous	Intraperitoneal
Mouse	27G	25G	27G	26–28G	25–27G
Rat	27G	25G	25G	25–27G	23–25G
Guineapig	25G	23–25G	25G	25–27G	23–25G
Hamster	25G	25G	25G	25–27G	23–25G
Rabbit	25G	21–25G	25G	23–25G	21–23G
Ferret	25G	21–23G	23–25G	21–25G	21–23G
Dog	25G	21–23G	21–23G	21–25G	21–23G
Cat	25G	21–23G	23G	21–25G	21–23G
Large primate	25G	21–25G	23–25G	21–25G	21–23G
Small primate	25G	23–25G	23–25G	21–25G	21–25G
Sheep	25G	19–23G	21G	19–21G	19–21G

Adapted from Wolfensohn and Lloyd (1998)

(see Table 3). The gauge depends on the toughness of the tissues to be penetrated (especially skin) which in turn depends on the species and the site. The needle bore is determined by the viscosity of the formulation and the speed of administration; the needle length depends on the depth of injection.

- Change needles between animals to avoid transfer of infection. Ensure a sharp needle is always used.
- Consider catheterizing the vein if repeat dosing over a short period.

(ii) *Accurate positioning and injection technique*: Severe adverse effects can be caused if needles or gavage tubes are incorrectly placed, e.g. an intramuscular dose injected intravenously can be fatal. Tissues can be damaged if injections are made clumsily.

- It is essential to study the anatomy of the animal, e.g. the joints for intra-articular dosing; the length of the oesophagus for gavage, the position of nerves for intramuscular dosing. Accurate measurement of depth of insertion can be made using stops or marks on needles/tubes.
- Insert needles/tubes firmly but gently and depress plunger gently. Once a needle has been inserted withdraw the syringe plunger to ensure the needle has not entered a blood vessel unintentionally. If blood has entered the syringe, withdraw the needle a short distance and redirect it. The pain receptors are in the skin and so this is less painful than withdrawing the needle and re-injecting. After injection withdraw the needle slowly but firmly.

(iii) *Leakage*: Fluid may leak from the site of injection reducing accuracy of dosing.

- It may be better to inject at a distance from the entry site using a longer needle and to push the skin to one side before entry so that a skin seal is formed. It helps to massage the site after injection to disperse the substrate, and also to apply pressure to the site after injection.

(iv) *Bleeding after injection*: Sites of injection may bleed especially after intravenous dosing.

- Apply gentle but firm pressure with a swab until the bleeding stops. Wipe traces of blood away to prevent excessive licking or gnawing at the injection site.

Volumes/Sizes of capsules

(i) *Large volumes*: Administration of large volumes can be difficult in practice and can cause adverse physiological effects, compromising animal well-being.

Table 4 Guidelines: maximum volumes for common routes of administration in the common laboratory species

Routes and volumes					
Oral (ml/kg)	ip	iv[a]	im (ml/kg/site)	sc[b]	id[c] (ml/site)
10	10	5	0.05	2–5	0.05–0.1

The essential requirement when determining dosing volumes is that the volume given should minimize any discomfort or welfare problem and should not cause any physiological or pathological change which compromises the experiment. As far as an animal is concerned the lower the dose volume the better

Most current guidance gives different volumes for different species but in most cases there are no physiological reasons for doing so. This was borne in mind when drawing up this table such that a single guideline volume was considered to be applicable to most species for any given route. When determining volumes for less commonly used species and other routes the anatomical and physiological characteristics of the species must always be considered

Notes: sc = subcutaneous; ip = intraperitoneal; iv = intravenous; id = intradermal; im = intramuscular iv[a] = Bolus injection carried out over a relatively short period of time (approximately one minute); sc[b] = Volume depends on the looseness of the skin of the animal (and therefore the potential subcutaneous space). Multiple sites can be considered for administration of greater volumes, although when repeated daily administration is intended, four sites should normally be considered the maximum. Voume does *not* include Freund's adjuvant administration. Freund's limited to 0.1 ml CFA/site; id[c] = Volume depends on thickness of skin which varies with site and species. Maximum number of sites = 6; im = This is the volume for a single site. The use of more than one site in more than one limb may cause multiple limb lameness; ip = This is not a common route in dogs, birds and primates

- The maximum dose level in terms of fluid volume and sizes of capsules and tablets varies with the route and the species. The essential requirement is to ensure the volume given causes minimum discomfort and does not result in physiological or pathological change which would compromise the experiment. As far as the animal is concerned, the lower the dose volume the better (see Table 4).

Use of adjuvants to enhance antibody production

(i) Many adjuvants can be noxious to animals and produce significant pathological lesions. Most information on the efficacy of different adjuvants is anecdotal so it can be difficult to select the most appropriate (Jackson & Fox 1995, Animal Welfare Information Center 1997, Leenars *et al*. 1997, Palmer *et al*. 1997). The following recommendations are reproduced from Palmer *et al*. (1997):

- Choose the least harmful adjuvant.
- Never use Freund's complete adjuvant (FCA) twice in the same animal. In the UK, use of FCA requires justification to the Home Office.
- Inject the smallest possible volume of adjuvant no more than 100 µl per site (use concentrated antigen).
- Choose an injection site where local pathological responses cause least pain, can be readily observed and treated if necessary (e.g. subcutaneous).
- Use long booster intervals (at least 4 weeks).
- Take advantage of the antibody memory response.
- Minimize animal numbers by sequential rather than simultaneous immunizations of animals.
- Choose animal species that yield large amounts of blood (e.g. sheep and goats).
- Plan ahead and be patient; successful production of a good antiserum can take 6 months or more. However, note that animals used to raise antibodies should not be kept for indefinite periods of time without the justification of an authorized scientific need.

Neonatal/Young animals

(i) *Rejection*: Neonatal/young animals may be rejected or cannibalized by the mother after handling.

- Be familiar with the maternal behaviour of the species.
- Use gloves when handling young animals to mask odours and avoid mis-mothering. Handle all the young in a litter so that the mother cannot discriminate between them.
- If appropriate to the species, remove the mother before returning the young. Put them in the front of the cage and mix with bedding to mask odd scents, then return the mother.
- Minimize disturbance when observing animals.

Restraint

(i) Restraint can be as, or even more, stressful than the procedure itself, particularly for animals that are not used to being handled. Periods of restraint in tubes or stocks are liable to cause stress. There are particular problems with inhalation restraint tubes; if these are not the correct size and shape for the animals they can partly turn around, and are likely to become distressed and may die.

- Use the restraint procedure most appropriate, i.e. least distressful, to the animal and of minimum duration (Poole 1987, van Zutphen *et al*. 1993, Tuffery 1995).
- Habituation and/or training of the animal may help reduce stress (see Section 2.1.4).
- Ensure staff are skilled at getting animals to relax when being held; restraint should be firm, yet sensitive.
- Ensure equipment such as restraint tubes are the correct size and shape for the animal.
- Never leave restrained animals unattended.

3 Refinement for individual routes and procedures

Recommendations for refinement are provided for each of the common routes taking into account potential problems or difficulties relating to the substance, the technique and the procedure as a whole.

A brief summary of each technique, sufficient to identify the procedures described, is given with full descriptions provided only where such information is not easily available in other publications (see Paget & Thomson 1979, Kirk & Bistner 1985, Poole 1987, Rollin & Kessel 1990, Waynforth & Flecknell 1992, Tuffery 1995, Wolfensohn & Lloyd 1998).

3.1 Intra-articular

Use of the technique

This method is used for local dosing into the joint space (normally the stifle joint) either to check the efficacy of drugs intended for clinical treatment of joint disease, or to model joint diseases. The technique has a clinical application in veterinary therapeutics (Kirk 1980).

Summary of the protocol

The technique requires the animals to remain very still. Short-term anaesthesia for restraint is preferred in all species and is essential for rodents, rabbits or dogs. In larger species, injections are done using local anaesthesia at the site of needle insertion, together with adequate restraint or sedation to stop the animal moving at a critical moment.

The hair over the joint should be clipped, the stifle joint palpated to estimate the position of the joint space and the needle (without the syringe attached) inserted through the anterior aspect of the joint, i.e. through or adjacent to the patellar ligament. The needle is then connected to the syringe and the substance injected. Location within the space is felt as a sudden forward movement of the needle after it has penetrated the skin and the capsule of the knee joint. In larger animals, synovial fluid should flow back along the needle.

Potential problems and refinement

The technique can be very painful and can cause damage to articular surfaces if not carried out with great care. However, proper attention to anatomy and aseptic technique

should minimize the incidence of adverse effects.

Incorrect placement of the needle may damage the surrounding tissues and cause pain. Even when the needle is correctly inserted damage can occur.

- It is essential to have a good knowledge of the anatomy of the joint.
- Use a needle size as small and fine as possible with respect to the species and the joint, e.g. 26G × 3/8" for rats. It is unlikely to be necessary to use needles greater than 21G even in larger species such as the dog.

If the animal moves, the needle will damage sensitive tissues in and around the joint. This will be painful and may cause lameness.

- Ensure the animal is immobile when carrying out the procedure. Use general anaesthesia. If this is not appropriate, accustom the animal to the restraint required so that it relaxes and remains immobile during injection.
- Monitor the animal for lameness after completing the procedure. Give analgesics unless this will seriously compromise the aims of the study.

Failure to apply good aseptic technique will result in a septic arthritis. This can cause severe lameness, heat and swelling of the joint and is extremely painful.

- Sterile technique is essential: swab the skin with antiseptic, wear sterile gloves and use sterile syringes and needles.

Injected substances may distend the joint capsule causing pain.

- Avoid over-distension of the joint with excessive volumes of substance—where possible, remove an equal volume of synovial fluid before injecting. The maximum volume that can be administered (assuming aspiration of an equivalent volume of synovial fluid) is 15 μl for rats (Waynforth & Flecknell 1992); 200 μl for rabbits; 1 ml for dogs.

The more times the joint is penetrated by repeat dosing the greater the risk of introducing an infection and of trauma.

- As a scientific procedure on an individual animal, this technique should be used once only and on a single joint.

3.2 Intracerebral (intracerebro-ventricular)

Use of the technique

This technique is used to deliver pharmacological agents to the central nervous system either where the blood-brain barrier must be crossed, or to avoid direct systemic effects. Intracerebral injection is also used to isolate or assay microorganisms.

Summary of the protocol

The route is used in neonatal and adult rodents and neonatal chicks. In adult rodents a stereotaxic frame is used for accuracy and to minimize tissue damage.

Neonatal rodents and chicks

Use a small bore, low volume (less than 0.5 ml) plastic syringe with a 27G needle. Draw the inoculum into the syringe and check smooth operation of the plunger (avoiding aerosol generation) before picking up the animal.

Mice, rats and chicks should be no more than 3 days of age. Hold the animal gently on an absorbent towel/pad on a firm surface with the dorsal surface of the animal held uppermost between the gloved thumb and forefinger.

Insert the tip of the needle into the brain, usually from the front, at an angle of 45°, in the area of the anterior cranial fontanelle (top, midline) and gently depress the plunger of the syringe to expel the dose. Return the animal to its mother, check for adverse effects after one hour (to avoid too much immediate disturbance) and monitor closely at least once per day post-injection for adverse effects of the technique or the inoculum.

There is debate over whether the use of anaesthesia for animals of this age would be appropriate, since this in itself can cause distress which has to be balanced against the stress of intracerebral injection. However, modern anaesthetics can be given safely by competent personnel and topical anaesthetics should not be a problem. The per-

ception of pain in neonatal animals has been the subject of considerable investigation and advance in the last few years. In some species, including rats and humans, the descending inhibitory fibres which mitigate the feeling of pain and raise the pain threshold do not develop until 2–3 weeks after birth. Some neuroscientists now believe that neonatal animals with immature nervous systems are more likely to feel pain than older animals. The current non-use of anaesthesia in very young animals is part of outdated wisdom based on 'small things do not feel pain'. It may also result from using methods of restraint which are relatively so powerful that we either do not realize the animal is struggling, or put this down to a response to the restraint alone.

Adult rodents

A stereotaxic frame is needed to ensure that there is no lateral movement of the needle which would cause trauma. Anaesthesia is essential for this as is aseptic technique and sterile material. Injection volumes should not exceed 2% of brain volume and should always be given slowly enough to avoid increasing cerebrospinal fluid pressure. Penetration of the ventricle is readily assessed by the sudden reduction of back pressure in the injection line. Chronic placement of cannulae allows injections, or continuous intraventricular infusion, without need for repeated anaesthesia.

Potential problems and refinements

Alternative methods of obtaining the results required should always be considered before using this route. The procedure is not easy to perform and the most common adverse effects result from clumsy technique.

Expelling small volumes of inoculum gently from the syringe is difficult. The skull of neonatal animals is very thin and fragile and can easily be damaged, as can the brain, with haemorrhage occuring at the site of inoculation. Movement of the animal during dosing will result in brain damage.

- Neonatal animals require very careful, gentle handling and it is essential to gain experience in handling them *before* starting procedures. To perform the procedure hold the animal gently but firmly. It is easiest to do this by resting the animal on an absorbent towel on a firm surface.
- Practise minimal depression of syringe plungers using fragile inanimate objects (such as artificial sponges) which mimic the texture and the pressure needed to expel fluids.
- Keep volumes of inoculum in the syringe small. Fine control is not then reduced by stretching the finger to reach the plunger.

Incorrect placement of the needle, (e.g. insertion from the rear of the brain, from the wrong angle, or too deeply) can kill the animal.

- Know precisely where to inject the animal and to what depth. It may be possible to put stops on the needle or mark the needle in some way to assess depth.

Needle sizes greater than 26G cause excessive damage to the brain of neonatal animals, and may kill them.

- Do not use needles greater than 27G. Avoid inoculating viscous substances which would require larger needles.

Injection of an excessive volume can distend the brain and skull.

- Do not inject more than 20 μl in neonatal rodents.

Substances with high protein content may cause the death of the animal.

- Always check the protein content of the substance to be administered and avoid the intra-cerebral route if the protein content is high.

Occasionally the mother rejects the young when they are returned to her. If there is blood leaking from the injection site she may eat them.

- Minimize disturbance of the litter after inoculation. Check after half an hour, one hour, and daily thereafter. Any animal showing unexpected neural deficits, e.g. ataxis or paralysis, within

the first 24–36 h should be humanely killed as this is most likely to be due to faulty technique. See also Section 2.3 (Neonatal/Young animals).

3.3 Intradermal

Use of the technique

Intradermal injection is a technique commonly used in studies of inflammation, sensitization and cutaneous blood-flow diagnostics, and in immunology. Often the objective is to administer a putative antigen or inflammatory mediator and look for a reaction (oedema, swelling or redness) which might occur rapidly or after a period of time from minutes to days.

Summary of the protocol

The injection is made into the outer layers of the skin, usually on the back. The dorsolateral skin should be closely shaved with electric clippers (avoiding any skin damage) at least one hour before injection. The needle should be held almost parallel to the skin surface and advanced carefully a few millimetres into the skin. If there is a sudden loss of resistance to the passage of the needle, this is usually due to it passing subcutaneously. It must be withdrawn and re-introduced. Rotating the needle in the injection site just after insertion and immediately prior to withdrawal helps minimize leakage of injected fluid. A successful injection will result in the formation of a bleb.

Potential problems and refinements

Sympathetic restraint is very important. Good technique is essential to ensure intradermal rather than subcutaneous administration.

The volume to be injected may be large and there is no natural space to contain it.

- Injection volume should not normally exceed 100 μl per site; 50 μl may be preferable in some instances.

It is easy to inject subcutaneously in error instead of intradermally.

- Good training is essential to ensure accurate injection. If in doubt clip or shave sites carefully to aid visibility. Depress the syringe plunger as gently as possible. Confirm correct location by the bleb that results from the fluid injection parting the cutaneous layers.

The density of the skin may make needle insertion difficult.

- Use the smallest and sharpest needle that will penetrate the skin (27G for rodents; 25G for dogs and primates).

An intradermal injection, even without inflammation, can cause distress if not performed correctly.

- Consider the use of a local anaesthetic cream (e.g. EMLA cream).
- Minimize adverse reactions by splitting the dose over a few sites (but see below).
- Do not inject an individual site more than once.

The response from multiple injection sites may coalesce.

- The maximum number of sites should not normally exceed 6.
- Ensure sites are sufficiently far apart to avoid coalescence. Consider using general anaesthesia, particularly if more than 6 sites are required.
- When carrying out multiple injections use a statistically designed Latin square system to account for regional variations in skin thickness.

3.4 Intramuscular

Use of the technique

In general, intramuscular injection is used as a route of systemic administration. It is sometimes used in slow release studies where implants or oily formulations are employed to provide a depot from which a drug is gradually absorbed. It is also used in testing vaccines and to administer anaesthetics, particularly to wild animals via a dart gun.

Summary of the protocol

Two people may be required, one to hold the animal, the other to inject the material. The injection should be made into a suitable large muscle mass, appropriate to the purpose of the procedure and away from major blood vessels and nerves to avoid damaging them.

This will normally be into the lateral and cranial thigh muscles (the vastus group) rather than the hamstring muscles in the hind limb. The injection site in birds is the thigh or pectoral muscles; in fish the dorsal musculature is used.

The animal's leg should be held firmly and the needle inserted carefully and deliberately. The syringe plunger should be withdrawn slightly prior to injecting to ensure the needle is not in a blood vessel. The contents of the syringe should then be expelled slowly, the needle withdrawn and the site gently massaged.

Potential problems and refinements

Intramuscular injections appear to cause more pain than injections at other sites, both at the time and afterwards, as evidenced by temporary limping. There is also a greater chance that major nerve trunks or minor intramuscular nerves may inadvertently be injured, as well as injury to muscle tissue caused by the distension in a closed fascial compartment.

The route should only be used if a less painful alternative is impossible, or if it is required clinically. The potential irritancy of a substance if injected subcutaneously or intraperitoneally should not be a reason for choosing the intramuscular route where it is equally likely to be irritant. Before using this technique carefully consider what you are trying to achieve, the likely damage, whether there is an alternative, whether the technique is a one-off, and whether repeat doses will be required.

If irritant substances are administered intramuscularly the resulting tissue necrosis at the injection site is likely to be painful since muscles are continually used to maintain posture. Use of adjuvants can compound the problem as they provide an ongoing irritant focus (see Section 2.3).

- Do not use irritant substances.
- Clip the injection site so that any local reactions can be seen. In birds, the feathers can be dampened with an alcohol solution and parted—do not pluck feathers (see also Hawkins *et al.* 2001). This is particularly important if there is any danger of the substances being irritant.

The injection itself may cause the animal serious distress.

- In such instances, complete the injection as quickly as possible or, if the pain reaction becomes violent, withdraw the needle to avoid unnecessary pain or mechanical damage. The injection should be completed at another site when the pain reaction has subsided and the animal has been calmed.
- Re-assess the necessity of using this route and formulation.

Nerves (e.g. the sciatic nerve) can be damaged by incorrect placement of the needle, or by a reaction to the substance administered. Trauma near to the sciatic nerve can lead to self-mutilation in rabbits. The substance may be inadvertently injected into a blood vessel within the muscle mass, and there is also a risk of injecting the material into the fascial tissue rather than the muscle mass itself. This will introduce experimental variation.

Movement of the point of the needle whilst in the muscle can also cause damage, e.g. when withdrawing the plunger to check for penetration of a blood vessel.

- Keep the animal very still—sensitive restraint is essential, as is familiarity with anatomy, particularly of the muscle masses and routes of nerves.
- Take great care to avoid nerves and blood vessels when placing the needle. The sciatic nerve is situated deep in the posterior (caudal) femoral muscle mass and along the line of the bone, so for preference inject into the muscle mass on the anterior of the thigh. Do not insert the needle too deeply (beyond 1.5 cm in adult cats and 2.5 cm in adult dogs).

The volume injected can physically distend the muscle causing swelling at the injection site with associated damage and pain. This is a particular problem for small mammals.

- The volume that can be comfortably accommodated depends on the species

and the shape and size of muscles. Do not use the technique for small animals (e.g. rats, mice or hamsters) unless there are exceptional circumstances when it should be used with extreme care.
- It may be better to divide larger volumes between, for example, hind and front legs, or to have 4 sites and rotate around them. However, this must be balanced against the potential to cause multiple limb lameness. Sites should be separated by at least 4 cm.
- Disperse aqueous substances after injection by gentle massage.

With good restraint and a familiar handler, large mammals such as sheep and cattle, will normally remain still for injection but pigs will not.
- Using a butterfly needle with a length of polythene tubing will make injection easier as the operator can follow the pig round the pen and inject at the same time.
- Feeding the animal with some nuts simultaneously will also help.

In fish, injection can dislodge scales.
- It may be necessary to construct a restrainer from a block of foam rubber or sponge. A 'V' shape large enough to hold the fish is cut in the surface of the block. The 'V' is lined with wet tissues and the whole block saturated with water (see also Brattelid & Smith 2000).

3.5 Intranasal

Use of the technique
Intranasal administration is used when investigating the respiratory tract as a route of infection and when studying pharmaceuticals absorbed from the mucosa.

Summary of the protocol
The test material is administered by allowing small droplets from an automatic pipette or spray pump to fall into one or both nostrils. The technique can only be used to administer small volumes (e.g. 50 μl to rodents and rabbits, 500 μl to dogs).

Small animals such as rodents are restrained by scruffing. They must be still and relaxed with the nostrils pointing vertically upwards. The orifice of the adaptor should be placed about 1 mm above the nostril. The pipette plunger is then depressed so that a droplet falls into the nostril. If the droplet fails to separate from the pipette it can be touched gently against the nasal orifice so that it drains into the nostril.

Two people are required to dose dogs. One holds the animal in a sitting position, the other (the doser) angles the dog's head slightly (about 30°) above the horizontal. For application use a syringe or a spray pump held in the doser's other hand and positioned 2–3 mm inside the nostril, but not touching the mucosa. The spray pump is activated, usually once or twice, and then removed. The dog's head is held slightly upwards for about 30 s to allow the test material to drain into the nasal passages. If a very small volume is administered this may not be necessary.

An equivalent procedure is used for primates, although for large Old World primates, three people are required, two to hold the animal and one to dose.

Potential problems and refinements
The technique imposes little stress if the animal is well trained. If the first attempt fails the technique can be repeated provided that the animal remains relaxed and unstressed.

Dosing is nearly always inaccurate since the animals often sneeze when the dose is administered. Sneezing can contaminate other animals or staff with test materials.
- Dose animals away from others that could be contaminated and allow for potential inaccuracies when calculating the dose.

If the animal moves some of the material may miss the nasal orifice or the pipette may jolt the nose and hurt the animal.
- Train animals to accept restraint and dosing (e.g. with saline) before commencing the study. Hold the animal very still, with the administration device very close to (i.e. within 1 mm), or within, the nostril. Brief parenteral anaesthesia/sedation may be preferable

when administering a single dose or when dosing small rodents.
- A very steady hand is needed for this technique. Practise the technique by dosing into a small container and weighing the dose delivered.

3.6 Intraperitoneal

Use of the technique
Intraperitoneal injection is used to administer relatively large volumes of soluble substances, such as anaesthetics, to small animals where these need to be rapidly absorbed and where the intravenous or oral route is inappropriate. This is a common route for administering substances to fish.

Summary of the protocol
The technique involves injection through the abdominal wall into the peritoneal cavity. It is described in detail for rats in Waynforth and Flecknell (1992).

For fish, intraperitoneal injections are best given antero-laterally to the anus with the depth of penetration gauged by maintaining a slight pressure on the syringe. Penetration should cease as soon as the fluid injected starts to flow freely indicating the needle's presence in the peritoneal cavity.

Potential problems and refinements
This route should not be used routinely (except for administration of certain anaesthetics) as other routes are conveniently accessible and usually preferred for research purposes.

The technique is not recommended for animals larger than rodents, nor for pregnant animals since the needle can puncture the gravid uterus. Never use this technique with birds because the substance may go into the air sacs.

Substances which are irritant can be life threatening when administered by this route. There can be appreciable reaction in the peritoneal cavity including pain, formation of fibrous tissue and adhesions. Many non-aqueous solvents cause swelling of edges of the liver lobes.
- Consider the pH, irritancy, toxicity and compatibility of the substance very carefully (see Section 2.3). Pilot studies are advisable.

This is a difficult technique to perform correctly, particularly in small rodents (Lewis *et al*. 1966, Claasen 1994), because it is difficult to ensure the dose is administered into the peritoneal cavity rather than into the intestine, gut, urinary bladder, muscle or other organ. Occasionally blood vessels may be damaged resulting in haemorrhage.
- Good staff training is essential. To avoid puncturing the abdominal viscera, introduce the needle rapidly at an angle of 30°, slightly left of the midline umbilicus, about halfway between the pubic symphysis and the xiphisternum (see Waynforth & Flecknell 1992). For rodents, the technique may best be performed by holding the animals with the head tilted downwards. Note that with this technique withdrawal of the plunger will not usually be helpful as gut contents are too viscous to be drawn into the needle.

Injection of too large a volume of substance will distend the abdomen and cause discomfort.
- The volume of injection depends on the size of the animal and what it can accommodate. Use 10 ml/kg as a guide.

Repeated use of this technique can cause serious stress because of the restraint required, the cumulative irritant effect, needle damage and the potential for injecting into abdominal organs.
- Do not inject individual animals more than once per day. The number of subsequent injections needs rigorous justification. Brief anaesthesia can make the technique more reliable and less stressful.
- Where repeated/continuous administration is required (for example, where this is used to imitate ambulatory peritoneal dialysis in humans), consider whether it would be less stressful to implant animals with a minipump dose delivery system (see Section 3.11).

3.7 Intratracheal

Use of the technique
Intratracheal administration is used to study the effects of poorly soluble substances (e.g. dust and fibres) or poorly soluble drugs in the airways of laboratory animals.

Summary of the protocol
The technique is usually used with hamsters, rats, guineapigs and rabbits. It involves administration into the trachea via the oral cavity, or into the lungs by tracheotomy to allow for repeat dosing. There are a number of references describing the technique in rats and rabbits (see Sedgwick 1988, Waynforth & Flecknell 1992, Cambron *et al*. 1995, Davies *et al*. 1996) and primates (Morris *et al*. 1997). Tracheotomy is not covered in this report.

The animal should be anaesthetized and placed supine on a padded table. Intubation should be carried out with an appropriately sized tube (e.g. orogastric tube), the uninflated cuffed end being guided into the trachea via a laryngoscope. It is important to check correct placement by the movement of inspired and expired air through the tube. The test substance is drawn into a syringe which is then attached to an intravenous catheter. This is then placed inside the endotracheal tube (which acts as a guide) and inserted to the point of bifurcation of the airways. This can be felt quite easily.

An alternative technique which can be used for rats, is to place the anaesthetized animal in sternal recumbency with the bottom jaw anchored behind the incisors and the top jaw raised by an elastic band placed behind the incisors. This maintains a wide gape to allow access to and visualization of the larynx by means of an adapted canine otoscope (Sedgwick 1988). A flexible catheter can then be passed an appropriate distance through the larynx for material to be instilled. Shining a fibreoptic light onto the neck at the level of the larynx helps to transilluminate the pharynx.

Potential problems and refinements
Intratracheal administration involves general anaesthesia so before using this route think very carefully about whether it is really necessary and whether it would be satisfactory to administer the substance intranasally or by inhalation instead.

The technique has the potential to damage the respiratory tract and is not appropriate for repeated administration. Tracheotomy should be considered if this is absolutely necessary.

Putting any foreign material directly into the respiratory tract can cause an adverse reaction. Irritant materials can cause serious problems.

- Be aware of pH and other factors that may affect irritancy. Avoid using irritant materials (see Section 2.3).

The physical presence of the substance particularly as large volumes or amounts of solid can damage the pharynx or cause local lung irritation.

- Do not exceed maximum volumes of 500 µl for rabbits and 40 µl for rats. The maximum particle size of solids administered should be dependent on their not impeding the airways or acting as a mechanical irritant.

The tongue can be damaged if not handled carefully. The pharynx/larynx can be damaged by the passage of the tube and this can cause laryngospasm.

- Take great care when handling the tongue and when passing tubes. A good understanding of the anatomy of the animal is essential as is appropriate training (e.g. using recently killed animals).
- Lubricate the tube with a water soluble sterile lubricant (Davies *et al*. 1996). A local anaesthetic spray can reduce the chance of inducing laryngospasm.
- Monitor the animal closely during the recovery period and for up to 3 h thereafter.

3.8 Intravaginal

Use of the technique
This technique has limited application since it is used to imitate the route of exposure to a pathogen (e.g. herpes, thrush) to pharmaceuticals or other products such as IUDs, tampons or pessaries.

Summary of the protocol

Viscous fluids and creams can be administered to rodents, rabbits, dogs and primates using a sterile syringe inserted gently between the lips of the vulva and advanced upwards and forwards into the lumen of the vagina. An oral dosing catheter cut down to approximately 10 cm can be used instead of a syringe for rabbits and rodents.

Where a vaginal suppository is to be administered, this is held between the forefinger and thumb, and inserted gently between the lips of the vulva and thence into the vagina. Deeper insertion is then accomplished by pushing the pessary or suppository forward into the vagina using the index finger or other suitable probe, such as a smooth plastic rod.

Potential problems and refinements

Substances may be absorbed through the vaginal mucosa and this may result in unintended systemic toxicity.

- Before dosing check available data on absorption rates to assess potential adverse effects.

It may be difficult, particularly with smaller animals, to gain entry into the vagina and the urethral orifice may be entered in error. There is also a risk of penetrating the vaginal wall with a syringe or rod, if the depth or angle of insertion is incorrect.

- Take great care when inserting anything into the vagina. It is very important to have a thorough knowledge of the anatomy of the animal and in particular the length of the vagina and the position of the urethral orifice. During insertion, stay on the upper (dorsal) surface so as to avoid the urethral opening. It is important to appreciate the relative position of the cervix within the pelvic cavity, and to advance the syringe sufficiently far forward (e.g. 8.5 cm in 6–15 kg bitches) for the substance to be retained in the vagina.
- Check the substance is not in the urethra by pulling back the plunger slightly to check for the presence of urine. A wider catheter is less likely to enter the urethra.
- It may be useful to dilate the vagina with a suitably lubricated (and warm!, e.g. plastic) speculum. This should be inserted gently between the lips of the vulva and thence into the vagina. Gentle closure of the handles of the speculum will cause dilation of the vagina allowing easy administration of the dose by finger, tampon or tube.

The animal may push the suppository back out or incorrect placement may result in it falling out.

- Check the animal at intervals after returning her to her cage to ensure this has not happened (but remember, the ejected suppository may be swallowed).

The animal may not retain the whole dose if the volume is large, rendering the dosage inaccurate.

- Keep volumes small—less than 3 ml in dogs, less than 500 µl for rabbits, less than 50 µl for rodents.

3.9 Intravenous and intra-arterial

Use of the technique

The intravenous route is often used in pharmaco-toxicological experiments to mimic the route of exposure to drug formulations, blood-replacement products, nutrient solutions, infectious and diagnostic agents. It ensures that maximum plasma exposure is achieved as rapidly as possible and avoids the possibility of pre-enterohepatic metabolism and elimination.

The intra-arterial route is used infrequently, except to assess the possible consequences of accidental intra-arterial injection of a formulation intended for intravenous administration.

Summary of protocols

Intravenous dosing: A detailed description of this route for rats is given in Waynforth and Flecknell (1992) with additional information on other common laboratory species in Paget and Thomson (1979) and Tuffery (1995). Further information on methods as they are updated and refined is published in journals such as *Laboratory Animals*, *The Toxicologist* and *Human & Experimental*

Table 5 Intravenous injection (sites of injection, needle sizes and maximum volumes)

Species	Typical catheter/needle size	Usual (occasional) vein for injection
Mouse 20–25 g	25–27G × 5/8"	Tail
Rat 200 g	23G × 1"	Tail
Rabbit 2.5 kg	21G × 1"	Marginal ear
Dog 6 kg	21–25G up to 1"	Cephalic (saphenous)
Primate	21–25G up to 1"	Cephalic (saphenous)
Bird	21–25G × 1"	Brachial (jugular)
Waterfowl/pigeon	23–26G × 1"	Tarsal

The choice of infusion device is partly determined by the volume to be infused, the rate of administration and the species. Larger bore needles can be used for the heavier animals within a species group
Maximum volumes should typically be 4% and no more than 5% of circulating blood volume
Infusion volumes should be based on kidney function data; generally around 5% circulating blood volumes per hour, or 4 ml/kg/h

Toxicology. The principles of refinement are similar to those that apply when taking blood samples and therefore another useful reference document is the BVA(AWF)/FRAME/RSPCA/UFAW report on removal of blood (Morton *et al*. 1993).

In the majority of cases of intravenous administration, substances are introduced by a single injection into a suitable peripheral vein. Table 5 gives recommended sites and volumes for different species. Some degree of physical restraint is always required.

Intra-arterial administration: Recommendations regarding sampling of arterial blood (see above) may also be relevant to administration of substances by this route.

Rabbits and dogs are used and restraint is always necessary. For rabbits a sedative or neuroleptoanalgesic may be better than physical restraint; dogs must be anaesthetized. The usual sites are the central artery of the ear in rabbits and the femoral artery for dogs. Needle sizes and administration volumes are similar to those recommended for intravenous dosing. Injections should be made under aseptic conditions in the same direction as the blood flow and administered fairly slowly over approximately 30 s.

Infusion techniques: Infusion techniques (either intermittent or continuous) may be used as alternatives to repeated injections, but this option should be considered very carefully, since there are inherent welfare problems relating to surgery—implantation of indwelling catheter may require a surgical procedure which must be performed under anaesthesia. Other welfare issues relate to restraint by tethering systems or the use of jackets, restricted cage environments and single housing.

Infusion techniques are an advancing field which is outside the scope of this document. There are detailed descriptions of the practical aspects of infusion in the literature (see Gregory 1995, van Wijk 1997, Laboratory Animal Science Association 1998, Healing & Smith 2000).

Potential problems and refinements

With this route there are many factors which can compromise the experimental objectives and increase the severity of the procedure.

The substance

The buffering capacity of the blood allows slow administration of solutions with a wide range of pH (between 2–11) provided that contact with blood vessel walls is not prolonged. Formulations for injection must be miscible with blood without precipitation. Substances must not cause haemolysis or coagulation, and not elicit degenerative or inflammatory reactions in blood vessel walls or surrounding perivascular tissues.

Some materials can damage blood vessels at the point of injection.

- Consider altering the formulation (e.g. increasing the dilution) and/or altering the vehicle. If this is not possible then consider flushing the equipment and blood vessel with saline before and after administration of the

test substances using a T-connector with two syringes attached.

The consequences of intra-arterial administration of tissue irritating chemicals and arterial-constricting substances are potentially catastrophic.

- Never administer such materials by this route. Compatibility testing with blood is essential prior to dosing. Take care to ensure that there is no risk of subsequent arterial haemorrhage on completion of the procedure.
- Always dilute substances well, using a suitable formulation and flush the artery with saline after injection.

There may be unanticipated reactions in some species. For example, some components of formulations (e.g. Tween 80, polyvinylpyrrolidine, some liposomes) cause anaphylactic-like reactions if administered i.v. to dogs, but do not cause such reactions in rodents and man.

- Check the potential effect of the substance and its vehicle with background data in a range of species.

Particulate matter may be present in the material being injected or unsuitable material may precipitate out between preparation and injection.

- Filter the material, e.g. by including a filter in the injection line between syringe and blood vessel (*Note*: filters can adsorb and retain test material.)

If the substance comes out of solution when injected into the tail vein, the tail may blacken due to thrombosis. Lung oedema and/or embolism may also occur.

- Ensure the substance is adequately dissolved in an appropriate solvent and check its compatibility with blood before starting the procedure.

The effect of a compound can be influenced by the rate of administration. Injecting intravenously and intra-arterially over a few seconds will result in transiently high levels of the substance in the blood with possibly toxic effects occurring very rapidly.

- It is preferable to inject substances more slowly over a period of minutes. This may require additional restraint procedures rather than manual restraint so weigh the disadvantages of this against the benefits of the slower injection.

Volumes

Adding large volumes of fluid to the blood stream causes haemodilution, increased central venous pressure, alterations in acid-base equilibrium and diuresis. It may also cause pulmonary oedema. There will be additional problems of toxicity and haemolysis with non-aqueous solutions.

- Be aware of blood volume values for different species. To minimize adverse effects, avoid increasing the blood-volume by more than 4% for rapid bolus injections (see Table 5) or infusing at greater than 4 ml/kg/h.

Injection technique

If the animal moves during the course of the injection thus dislodging the needle from the blood vessel, part of the test liquid may be injected perivascularly and/or subcutaneously producing a swelling of the skin adjacent to the vein/artery.

- Before carrying out the procedure, always check that the animal is adequately restrained and that the needle is firmly affixed to the syringe. Use of an over-the-needle catheter or a butterfly needle with a length of connecting tubing will reduce the risk of the needle dislodging from the syringe. If the animal moves check the position of the needle. If it has come out of the vein withdraw it immediately and repeat the injection at a different site.
- Use a local anaesthetic cream, e.g. EMLA (Flecknell *et al.* 1990), so that the animal does not feel any pain, thus reducing the risk of it moving.
- If a perivascular injection occurs and if the solution is irritant, counteract the effect, e.g. by injecting buffered saline

and local anaesthetic at the site as a diluent.

Bad injection technique may damage the vessel making subsequent injections difficult.

- When using the ear vein make the first injection as near the tip as possible so that the vein remains patent. In rodents, give injections near the tip of the tail rather than at the base since this is usually easier and therefore more accurate.
- Rotate the injection sites for repeat doses.

Intravascular injection can result in emboli (e.g. skin fragments) depositing in the lung. This is rarely of clinical significance but can affect the histopathology.

- Use a fine needle and inject with minimal trauma.

Locating the vein/warming the animal

The vein may be difficult to see.

- Try dilating the vessel to render it more visible by rubbing the surface, warming the ear or tail (e.g. in a warming box), or using certain sedatives, e.g. Hypnorm™ (Janssen Pharmaceutica, Turnhoutsebaan 30, 2340 Beerse, Belgium) in rabbits. Carefully removing the hair may also improve visibility.

The animal will overheat if intense heat is used for too long.

- Any warming technique must ensure a carefully controlled maximum temperature. Warming the tail alone is less likely to cause a problem, although locally applied warmth does not work as well as whole-body warming.
- Do not use hair driers or heat lamps as they can overheat animals and cause skin burns.

Where a warming box is used, the thermostat on the box can fail. The animals may overheat leading to unnecessary stress and, in extreme cases, death.

- The chamber temperature should not exceed 37°C. Calibrate and monitor the temperature of the box carefully and make sure it has very good thermostatic control. Observe the animals constantly for signs of distress. The maximum warming period for mice is 5 min and for rats 15 min. As a guide, no more than five animals should be placed in the box at one time.

Infusion techniques

Since this involves surgery, all the refinements that apply to surgical techniques, e.g. appropriate use of anaesthesia, asepsis, appropriate wound closure methods and dressings, and recovery environment, and post-operative analgesia should be implemented as appropriate. Some potential problems and suggestions for refinement are given below, but refer to the literature for further details.

Surgical techniques for implantation of catheters or infusion devices can traumatize the animal and introduce infection.

- Ensure staff are competent in the anaesthesia, surgery and after-care required, including aseptic technique.
- Reduce post-operative trauma by acclimatizing animals to jackets for several days before surgery.
- Think about how the animal's behaviour will be affected by the catheterization and exteriorization site before going ahead.

Constant or frequent contact of an implanted catheter with the blood vessel wall can cause thrombo-embolism.

- Accurate placement of the catheter is essential, as is the involvement of an experienced veterinary surgeon. Make sure staff have appropriate training and are fully competent in the procedures.
- Use of biocompatible materials is essential.

Technical problems may occur with infusion equipment, e.g. if small volumes are given intermittently, then pumps may be inaccurate.

- Ensure pumps are meshed in/warmed up every time they are used.

- Ensure adequate quality control procedures are in place so that the selected device delivers the formulation at the correct volume and rate of flow. Sophisticated computer-controlled systems are now available allowing remote control of pre-programmable pumping devices.
- Do not use mechanical pumping devices for mice as the flow rate for such small animals cannot be adequately controlled.

The use of tethering systems or portable jacket systems can be uncomfortable, restrict the animals' movements or limit the possibilities for social housing.

- Techniques which allow the pump and reservoir of dosing solution to be carried by the animal are preferred since tethering is avoided. Use jackets which allow social housing of social species or redesign jackets where no suitable ones exist.
- Check the fit of jackets and tethering attachments daily.
- Use subcutaneous ports for discontinuous infusion, so that no restraint is necessary when the animal is not being infused.

3.10 Oral routes

Use of the technique

The oral route is commonly used when systemic exposure is required and it is known that there is good absorption from the gastrointestinal tract and there is little first-pass metabolism in the liver. In toxicological investigations it is commonly used regardless of first-pass metabolism. It is also used when studying a local effect in the gastrointestinal tract.

Substances can be introduced into an animal's mouth or stomach by:

(i) Inclusion in food or water—this method most closely mimics the ingestion of substances in humans and is particularly appropriate where lifetime administration of the compound is required, e.g. in carcinogenicity testing.
(ii) Oropharyngeal administration of capsules, pills or fluids—this method is used when a material is unpalatable or when more accurate dosing is required.
(iii) Oral gavage—this technique also avoids palatibility problems and is the most accurate method for administration of substances into the gastrointestinal tract.

The method of choice will depend on the species, the nutritional requirements and physiological state of the animals, the purpose of the experiment, and the accuracy of dosing required.

3.10.1 Inclusion in an animal's food or water

Summary of the protocol

The substance is incorporated in the animal's food or water. Highly palatable substances are readily consumed when offered in this way, or will be licked from the end of a dosing device such as a syringe. Where animals are fastidious eaters, it is essential to understand their feeding behaviour in order to determine the best way of incorporating the substance into the diet. A pilot study to test the substances acceptability, measuring food consumption and body weight may be necessary.

Potential problems and refinements

This is the least stressful method of administering a substance, but it can also be the least accurate. There may be problems relating to the physico-chemical properties of the substance, its palatability and the feeding behaviour of the species. However, the ease of administration, together with the low level of stress on the animal, may outweigh many of the disadvantages.

Properties of the substance and its palatability

The stability of the substance or its homogeneity in the diet may cause a variety of practical and scientific problems and alter its pharmocokinetics.

The substance may: bind to ingredients in the diet; be altered by the heat of the process

if pelleted; react with other dietary components—for example, with essential vitamins or amino acids, rendering them unavailable to the animal; break down to more or less toxic derivatives (see Sections 2.1.3 and 2.3).

Irritant substances may damage the mucosa of the oesophagus and stomach.

- A detailed knowledge of the properties of the substance in relation to the dosing route is essential.
- Always check background data for indicators of likely adverse effects on the species to be used.
- Do not use irritant materials.

The substance may be unpalatable. If it imparts an unpleasant flavour to the food or water, the animals' consumption will be reduced. If animals refuse to eat, it may be necessary to withold the normal diet.

- Careful preparation of the compound, e.g. by micro-encapsulation, may remove many of the palatability problems.
- Mask unpleasant flavours in water by adding sugar (water bottles containing sugar solutions should be changed daily in order to minimize microbial growth). Another option is to use flavoured (e.g. blackcurrant, orange) gelatin which rats and other animals appear to like. Gelatin has a melting point of around 60°C but a solidification point of around 25°C. It can therefore be used to dissolve water soluble substances (or substances dissolved in a miscible solution) which are temperature sensitive. The stock solution of a compound can be added to give a final concentration that the animal will comfortably and reliably eat.
- Do not withold normal food for long periods and always consider the metabolic rate, age and condition of the animals before doing so.

The substance may cause dental problems, e.g. feeding wet mash to dogs for prolonged periods can lead to accumulation of tartar on teeth. In rodents, too coarse a ground diet can cause gingivitis, tooth decay and pharyngeal penetration.

- Do not feed pellets that are too hard.
- Check the animals' teeth regularly to ensure there are no dental problems.

The substance may have limited nutritional value but still constitute a large proportion of food. This upsets the normal dietary balance (e.g. vitamin, protein levels). In rodent studies, up to 10% non-nutritional substances may be added to the diet.

- Be aware of the nutritional requirements and physiological state of the animals (e.g. growing, pregnant or lactating animals). Monitor them closely for signs of loss of condition or weight loss.

Species fastidiousness

Some species (e.g. non-human primates) readily detect dietary inclusions. Cats are also fastidious feeders and it may be difficult to hide substances in their food. Pigs have a highly developed sense of taste and smell so similar problems arise. Rats and mice may also refuse to eat novel substances.

- It is essential to be familiar with the feeding habits and preferences of individual species.
- Primates can be trained in operant feeding techniques. They readily drink fruit juices so substances can be dissolved in the juice and given directly. Liquids can also be injected into a grape, an orange slice or banana. Powdered solids can be thoroughly mixed with a favourite titbit; such offerings should be small enough so that the animals will consume them quickly without taking several bites (Schrier 1997).
- Dogs eat food quickly so they can be dosed before feeding and the food then acts as a reward.

Accuracy of dosing

The dose level and duration of dosing cannot always be accurately controlled when the compound is simply included in the daily diet. The amount of substance consumed will vary with the individual animal's food

and water intake. Water intake is particularly variable and difficult to measure.

There are particular problems with the feeding habits of certain species. Old World primates will store food in their mouth pouches for as long as 30 min, so it is difficult to say when it has been swallowed. Hamsters will store food in cheek pouches (and in their cage) making accurate dosage difficult. Rats eat over several hours whereas dogs consume their food very quickly. This creates problems in assessing duration of dosing. Coprophagic animals present particular problems, e.g. recycling unabsorbed material or its metabolites. (This will always be a problem whichever route is chosen.)

In most species the dietary intake per unit body-weight reduces with age.

- Try presenting smaller amounts of food at regular intervals to ensure accurate doses are consumed. Use highly palatable food making sure animals are used to the new food and recognize it as a treat.
- Vary the concentration in diet/water to achieve a steady intake as 'mg' or 'ml/kg'. Doses should be expressed in terms of the weight of the base (not of the salt) given per unit body weight or body surface area.
- Use special containers which enable the amount of diet consumed to be more accurately measured. These have been described for feeding mice, rats, guinea-pigs and hamsters (Poole 1987).

There may be cross-contamination of diet from spillage and/or dust and this can affect accuracy.

- Animals may need to be separated for feeding; careful ventilation/airflow control may be necessary.

Other welfare problems

Animals may have to be singly housed to assess dietary intake more precisely.

- Always question the necessity for precise measurement of dietary intake and thus for single housing. It may be relatively unimportant scientifically but cause unnecessary stress by depriving animals of social interaction. Balance the requirement to standardize between treatment groups against the desirability of providing social/environmental enrichment.

Animals are handled less frequently because there is no need for restraint. This may make the conduct of other procedures more difficult.

- Overcome any problems resulting from infrequency of handling animals by asking for daily body weight measurement as part of the technique or by routinely handling the animals during husbandry procedures.

3.10.2 Dosing directly into the pharynx

Summary of the protocol

The technique is commonly used only in cats, dogs, ferrets, farm animals and birds, although rabbits can also be dosed in this way (see Tuffery 1995, Wolfensohn & Lloyd 1998). A suspended, encapsulated, or solid amount of material is placed onto the back of the tongue and a swallowing reflex induced by stroking the throat. Fluids can be instilled onto the tongue or poured in the labial/cheek pouch at the angle of the jaw with the muzzle held upwards. To administer a capsule to a primate or rat, put the capsule on the end of a gavage tube and 'blow' it out (Lax *et al.* 1983). Rats need to be trained to accept the gavage tube first.

Neonates can be dosed by allowing them to suck the test compound as an emulsion from PE50 plastic tubing. Volumes of 40–50 μl can be given to 10-day-old mice, body weight approximately 4–6 g. The technique is more stressful than oral dosing in food or water because restraint is necessary.

Potential problems and refinements

The same problems occur as in 3.10.1 above. In addition:

If the substance has an unpleasant taste, the animal may develop a conditioned response and vomit before being dosed.

- Try making the substance more palatable by flavouring it or by encapsulation—choose the correct size of capsule

for the species. Treats can be given as a reward after dosing.

Dosing may be inaccurate if substances are spilled, spat out (immediately or as a delayed response) or regurgitated.

- Encapsulation overcomes this problem but be aware that capsules themselves may break, releasing the compound in the wrong part of the gut.

Dosing accidents, such as choking, can occur.

- Avoid choking by giving fluid in small aliquots (5 ml for dogs, 250–500 μl for rabbits), allowing the animal to swallow between aliquots.

Repeat dosing may be more stressful for the animal and the opportunity for error is increased.

- Limit the number of repeated doses given per day to between 2 to 4. This often mimics typical clinical usage and may thus be a more accurate and appropriate method of dosing.
- Reduce stress by training the animal to cooperate in the procedure.

3.10.3 Oral gavage

Summary of the protocol

The technique has been described in detail for all the common laboratory species in a number of texts (Paget & Thomson 1979, Poole 1987, Waynforth & Flecknell 1992, Tuffery 1995). In summary, a feeding tube or gun is passed into the oesophagus/stomach and the substance to be dosed is gently expelled at a regular controlled rate slow enough to deter regurgitation. The tube is withdrawn gently to avoid reflex vomiting or regurgitation.

Potential problems and refinements

This is the most stressful, and technically difficult, method of oral administration, although an experienced and skilled technician can make it seem deceptively easy.

The substance

Substances may be irritant. The effects may be apparent e.g. wheezing if droplets from the end of the gavage tube enter the lungs, or inapparent, e.g. ulceration in the stomach.

- Do not administer substances which cause gastric irritation.
- Avoid direct contact of the tube with the oesophageal or stomach wall by ensuring the correct length of gavage tube.
- Avoid generation of droplets which may be inhaled and cause severe reactions. It is important to make sure there is no droplet on the end of the tube as it is pushed down the oesophagus. Blot or wipe it with a tissue and flush the tube with a small volume of water after dosing so no irritant drops are inadvertently released as the tube is removed.

Compounds may froth and block the trachea or cause a foreign body pneumonia if administered into the lungs by mistake.

- Take particular care to ensure the gavage tube is in the stomach. Consider maximum volumes carefully for compounds that can froth.

The technique

If the tube is incorrectly placed, if undue force is used, or if the animal moves, the tube may penetrate the trachea or pass through the oesophagus or stomach wall into the thoracic or peritoneal cavity. Subcutaneous abscesses may result from infection tracking out from the mediastinum, e.g. swellings in the axillary region. Repeated insertion of the tube for frequent dosing may cause inflammation and ulceration of the oesophagus.

When a rigid gavage tube attached to a syringe is being used, negative pressure may draw in air, rather than create a vacuum, if the tube has inadvertently been inserted into the lungs rather than the oesophagus or stomach.

- Keep the animal still and ensure the best angle of the head and body to facilitate dosing. This is very important and requires correct and sensitive restraint.

- Be familiar with the anatomical relationships of the oropharynx and develop the necessary high degree of skill before commencing dosing to ensure accurate placement of the gavage tube.
- Always use the correct size (length and width) of feeding tube to ensure the dose enters the stomach and not the oesophagus. For example, in dogs the correct length corresponds to the distance from the nose via the acromion of the shoulder to the tenth costochondral junction—correct placement is confirmed by the smell of the gut contents. In mice the tube should extend from the tip of the nose to the last rib. Mark the length in advance. Make allowance for young or small individual animals.
- Check for condensation which may be visible if the tube has inadvertently been placed in the trachea.
- Use tubes with a small smooth knob on the end to prevent penetration of the gut wall. Flexible catheters are preferable to rigid tubes and plastic can be used instead of metal (but consider possible reactions between the compound and the tube).
- Consider lubricating tubes with petroleum jelly or medical paraffin to ease passage.
- If any resistance is felt during insertion of the needle, or if there is any sign of choking or distress, withdraw the tube and re-start the procedure.
- Always monitor the animal for a short period after returning it to its cage to check for any adverse reaction to the procedure and to check whether regurgitation or vomiting occurs. If animals die following oral dosing perform a necropsy examination to ascertain the cause of death and eliminate poor technique as a factor.
- If you suspect you have dosed into the lungs hold the animal's chest close to your ear and listen for 'gurgling' or 'rasping' sounds. If the dose has gone into the lungs kill the animal to prevent further suffering.

Gags can cause discomfort.

- Only use gags if they make the technique easier to perform and less stressful for the animal. Make sure any gags are designed to be comfortable for the animal. Tapering one end makes it easier to put into the mouth.

The animal may regurgitate the dose or vomit. This is a particular problem with ferrets.

- Always expel the contents of the gavage tube at a controlled rate and withdraw the tube carefully. Monitor the animal carefully once it has been returned to the cage.

Withholding food

Food is often withheld overnight, for as long as 18 h, in order to empty the animal's stomach before dosing. This is a long time, particularly for rats or other animals whose normal feeding behaviour is 'little and often' and whose feeding time is at night.

- Think carefully about the reasons for witholding food. It is rarely justified—a recent study by Vermeulen *et al.* (1997) demonstrated that the stomach of male rats was empty after 6 h without food—and requires a good scientific reason (i.e. if the absorption of the compound varies depending on whether the animal is fed or fasted). If food is withdrawn, water should always be offered. Authority may be required from licencing authorities or Animal Care and Use Committees for food withdrawal together with precise instructions for time of next feed.

Young animals

Extreme care should be taken with young animals. There are special considerations with respect to their size, disturbance of litters and time of dosing after ingesting milk. Dosing pre-weaned animals is difficult and should be avoided if possible. Gavaging rats less than 6 days old should be avoided.

Primates
Oral gavage of Old World primates should only be considered in cases where it is essential to ensure that the full dose of a drug reaches the stomach at the same time. It is a straightforward procedure *provided* staff are highly skilled and experienced in carrying it out. The stress of the technique is compounded by the stress of the restraint required and staff *must* be well trained and competent in this too. Lubrication of the tube will ease its passage.

If at all possible, oral gavage should be avoided and substances should be administered orally by other means.

3.11 Osmotic minipumps

Use of the technique
Osmotic minipumps can be used to allow continuous drug delivery over long periods (Theeuwes & Yum 1976, Nau 1985, Collins 1987) and to avoid the stress of repeat dosing.

Summary of the protocol
Commercially available pumps are designed to deliver different durations of dosing. A steady outflow of test material can be produced for up to 2 weeks. Minipumps are commonly used in rats, mice, guineapigs, hamsters and rabbits, although they can be implanted in virtually any species. In mice, minipumps are large relative to the body size of the animal, but are tolerated well, provided they are sited in a position which does not interfere with the animal's movement. The device is inserted subcutaneously, or occasionally intraperitoneally, with a single surgical procedure. Aseptic technique, anaesthesia and analgesia are necessary.

Analgesia should be administered as soon as the animal is anaesthetized. It will have then become effective by the time the animal regains consciousness. The hair should be gently removed from the dorsal area with fine electric clippers and the skin cleaned with an antiseptic skin preparation. A skin incision, about 1 cm long, should be made on the dorsum of the animal midway between the head and base of the tail. The pump should be placed along the axis of the body to one side of the backbone. The incision should be positioned about 1–2 cm caudal to the estimated caudal end of the minipump when this is in place. A pair of haemostats (straight artery forceps) is inserted through the incision under the skin in a cranial direction and the jaws on the haemostats opened to make a subcutaneous pocket for the minipump. The minipump should be inserted, delivery end first, into the subcutaneous pocket. The skin wound is closed with wound staples (not nickel) or sutures. Appropriate peri-operative monitoring and care must be provided.

Some protocols may require the removal or replacement of the minipump after a specified period. This should also be done under general anaesthesia using aseptic technique and with analgesia. The original incision is opened, the pump removed with forceps, and the wound sutured.

Some manufacturers/distributers of minipumps (ALZA Corporation 1990) provide a video which demonstrates the basic technique for subcutaneous implantation plus other applications, such as intraperitoneal implantation and connection to catheters for intravenous infusion. These provide information on practical surgery but do not address welfare issues.

It is possible to supply the material for dosing to an establishment and then buy the animals already implanted. However, note that the transport of surgically prepared and dosed animals also has welfare implications which must be taken into account.

Potential problems and refinements
The method can have significant animal welfare benefits over repeated dosing by other routes where restraint and infusion or injection may be required, but this must be balanced against the stress of surgery. The most likely adverse effects of the technique are those due to toxicity of the compound or drug contained in the minipump.

A reaction to the minipump may be seen if its surface is abraded. Weight loss may occur.

- Handle pumps very carefully. When monitoring body weight in small animals, remember to take account of the

weight of the minipump and the compound. Any weight loss should not be expected to exceed that seen after anaesthesia alone.

The technique necessitates anaesthesia and surgery both of which are potentially traumatic procedures. Most wounds should heal well but wound breakdowns may occasionally occur.

- This technique should only be carried out by experienced and competent staff. Training in anaesthesia, aseptic surgical technique, and peri-operative care, including post-operative analgesia, is essential.
- Monitor the animal carefully after implantation. It may be necessary to house the animal on its own for 24 h for the skin to seal adequately.

Only small volumes can be administered so the formulation is often very concentrated. It may precipitate in contact with body fluids and the pump can become blocked as a result.

- Check (*in vitro*) that precipitation will not occur.

3.12 Respiratory routes

Use of the technique

The respiratory route is used frequently in toxicology and is selected when it is the route of human exposure for the test substance. It is also used to administer certain vaccines and medicaments particularly when effects on the respiratory tract are required or when very rapid systemic absorption is needed. The range of experimental exposure techniques in animals reflects the variation of human exposure. This can vary from a few seconds (some vaccines and medicaments) to virtually continuous exposure, 24 h per day 7 days per week (for airborne environmental pollutants). Exposure may be:

(i) Whole body
(ii) Nose only
(iii) Mask

The potential adverse effects of the substance on the respiratory tract are common to all, and factors such as pH and irritancy should be considered in each case (see Section 2.3). Pilot studies are advisable to assess and avoid potential adverse effects. Inhalation administration of substances is described in detail by Phalen (1984), (see also Kennedy *et al.* 1989, Waynforth & Flecknell 1992).

3.12.1 Whole body exposure

Summary of the protocol

Animals are exposed to the substance either in a specially designed inhalation chamber, or where exposure is for many hours per day (i.e. close to continuous exposure), in cages with similar dimensions to their normal housing. The cages may be made of mesh (to ensure circulation of the atmosphere) and there may be other minor modifications.

Potential problems and refinements

There should be little or no more distress from the technique than occurs for stock animals in holding rooms, provided standard cages are used. However, any restrictions on available space, food or water or on the nature of the physical and social environment may cause distress, as may the noise from ventilation or dosing equipment.

It may not be easy to regularly monitor animals in inhalation chambers.

- Use clear polycarbonate cages to facilitate observation of animals and/or place cages in optimal relation to lighting and windows. Monitor the animals' behaviour very closely for signs of distress, possibly using video equipment.

Some inhalation chamber designs have no litter trays so as not to impede air circulation. The rats in lower levels may then receive the faeces and urine from rats housed higher in the chamber.

- This design of cage is unacceptable and should not be used.

Inhalation chambers are designed for the purpose of the procedure rather than the comfort of the animals. The floor space may be reduced and the opportunity for environmental enrichment is restricted.

- Look for ways of improving the comfort of the cage and enriching the environ-

ment. Return animals to conventional cages between exposures.

Where exposures last for several hours (e.g. 1–6 h) per day, food and water are generally withheld.

- Ideally, do not withhold food and especially not water for more than 6–8 h/day depending on the species, particularly if exposure is over several days. Water should be continuously available.

The generation of test atmospheres usually uses clean, filtered, dried air. In the longer term, this can result in a low relative humidity in the inhalation chamber and thus the risk of ringtail in young rodents.

- Ensure humidity is adequately controlled ($>40\%$) and that the air supply cannot fail.

Animals are sometimes washed and dried or otherwise cleaned to remove test material deposited on the fur after completion of exposure. This adds to their overall stress.

- Ascertain whether deposition of test material is really a problem in terms of exposure and do not wash animals if it is not necessary. If animals do have to be cleaned the cleaning agent should be carefully selected so as not to cause adverse effects.

Food and water can be contaminated with the substance.

- Design food and water containers to minimize contamination and/or measure the content of test material in food/water. There may be no need to expose animals with their food/water present if exposure is only for short periods.

The apparatus used to generate test atmospheres may be noisy, either in the audible or ultrasound range.

- Be alert to this possibility when assessing animals' behaviour. Measure ultrasound as well as normal frequencies and do not use noisy equipment more than once in 24 h. Habituate the animal to the chamber to avoid stress of a novel environment.

3.12.2 Nose only/Snout only exposure

Use of the technique

This technique is a more precise method of dosing than whole-body exposure. It is normally only used with rats, mice and hamsters.

Summary of the protocol

Animals are placed in clear polycarbonate conical tubes so that the snout protrudes from a hole at the point of the cone. Tubes are made in a range of sizes to match the sizes of rodent to be exposed. The Batelle type tube includes a backstop with a hole through which the rodent's tail is passed. This prevents the animal reversing out of the tube. Vents in the side of the tube allow the animal to remain cool and reduces the build-up of water vapour.

Potential problems and refinements

This technique is inevitably stressful because of the tube restraint. Deaths can and will occur if this is not carried out competently.

Tubes that are not the correct size and shape for the animal may allow the animal to turn partly around. They will then become distressed and may die or inhalation exposure will fail.

- The shape of tubes is obviously critical, so the construction of 'in-house' devices should not be attempted without a thorough appreciation of requirements and preliminary tests not involving animals.
- Ensure the fit of the animal in the cone is such that it cannot turn round. Always monitor the animals continuously whilst in the tubes to identify and help any that are in distress.

Placing a rat or mouse in a tube may seem simple and require little training. However, poor technique can result in unnecessary stress and even the death of animals.

- Ensure all staff are trained and competent in the procedure.

Animals may be restrained for long periods each day for many months in some toxicological studies.

- Habituate animals to the tubes to minimize stress. The period of restraint should be built up over several days before exposures to test material begin; however, even if the animals become used to the tubes, the first day of actual dosing can be very stressful.
- Only keep animals in tubes for the minimum time consistent with scientific requirements. This should not exceed 4 h. Reduce time in the tube by ensuring the animals that are first in, are also first out. Try to have more than one skilled person to load and unload animals thereby reducing the overall restraint time.

Faeces and urine accumulate in the tubes during exposure.

- During long-term exposures monitor and remove faeces and urine. Make sure tubes are washed well after use.

3.12.3 Mask exposure

Use of the technique

Face masks are used frequently for inhalation exposure in dogs and primates. The technique is broadly (but not entirely) equivalent to snout only exposure of rodents.

Summary of the protocol

Exposure can be once or several times daily (up to 5 exposures per day) and might be for a matter of seconds per occasion or up to one hour per day in a single session for several weeks or months.

Animals must be restrained and if exposure is brief (seconds or a few minutes) it is best to do this manually. Dogs require two people, one to hold, one to dose, although if animals are trained a single technician can suffice. Large primates may require three people. For longer exposures dogs may either be held on the lap of an animal technician or may be held in slings whilst wearing the masks. Primates can be restrained in chairs. Animals in slings or chairs must NEVER be left unattended, although one person may be able to supervise several well trained animals at a time.

Face masks generally have a rubber sleeve to provide an airtight fit around the mouth and nostrils. A tube goes through the mask and into the animal's mouth over the tongue. The positioning of the tube relative to the tongue can be checked by looking down the tube. The remainder of the equipment includes a one-way inhalation valve; a pressure sensor (this may be necessary to detect inspiration so that a pressure pack, i.e. a dosing pack with air pressure delivery, can be 'activated' coincident with inspiration); and a one-way exhalation valve. The precise nature of the dosing, air supply and venting equipment will vary with the test material (i.e. liquid, solid, gas), pressure pack, accuracy of dosing and the need to expose the nasal passages.

Note: For metered dose inhaler (MDI) dosing for short-term exposure, an oropharyngeal tube may be used instead of a mask. This rests on top of the larynx enabling the respiration to be monitored.

Potential problems and refinements

The restraint required can make this technique potentially highly stressful, especially when it is for long periods.

Wearing a mask will be unpleasant for the animal. It can cause discomfort and/or may leak.

- Make sure the masks are designed for comfort and acceptability. Check them regularly to ensure they fit each individual animal comfortably and securely. Where oropharyngeal tubes are incorporated, smaller dogs will require shorter and narrower tubes.
- Train animals to accept the restraint and then the face mask before the study starts. This may take up to 3 weeks and adequate time must be allowed for this. Individual animals that do not adapt (i.e. that show distress) should not be put on study.

The test material, e.g. dry powders, can cause drying of mucosal surfaces and result in discomfort to the animals. In primates this can lead to breath holding and fainting.

- Inspect the animals' oral cavity before each exposure and reduce the dose and period of inhalation exposure. Formulate in aqueous if at all possible.

Mechanical equipment can fail.

- Check equipment regularly. Always ensure the animal will be able to breathe in the event of an equipment failure.

3.13 Subcutaneous

Use of the technique

Subcutaneous injection is commonly used for the parenteral administration of many substances. The route provides for slow release avoiding first-pass liver metabolism and may be used to imitate the route of administration of a pharmaceutical.

Summary of the protocol

The site for a single injection for rodents and rabbits is normally the scapular region. In dogs and cats the preferred site is behind the neck or occasionally in the sub-lumbar fossa on the flank, or behind the neck. In primates the injection is made under a fold of skin on the animal's back. In birds, subcutaneous injections can be given into the medial aspect of the thigh where there is usually ample space and where the position of the needle can be clearly seen if the feathers are dampened with alcohol. There should be no need to pluck feathers (Hawkins *et al*. 2001). See Waynforth and Flecknell (1992), Tuffery (1995) and Wolfensohn and Lloyd (1998) for a description of the technique.

Potential problems and refinements

This technique is *very* painful if the pH or osmolarity is wrong, or if the material is irritant or cytotoxic. Tissue necrosis can occur. If osmolarity is incorrect, tissue fluids may diffuse into the site.

- Where the effect of test substances is unknown good pilot studies are essential. Adverse reactions must be fully resolved before repeat dosing.
- Select the site with care. If adverse effects may occur clip the hair/fur around the site to enable early detection of problems so that they will be easier to resolve.
- Ensure the solution is sterile and that pH and osmolarity are correct. Reactions to adjuvants, particularly Freunds complete adjuvant, can have a compounding effect on the injection. For example, such a reaction in the skin over the respiratory muscles in the flank of rabbits can make breathing painful.
- Carefully consider the nature of the adjuvant and the protocol with reference to literature such as Jackson and Fox (1995), Animal Welfare Information Center (1997) and Palmer *et al*. (1997). See Section 2.3 for detailed recommendations.

Using skin antiseptics to try to 'sterilize' the skin may cause unnecessary damage or disturbance of the skin's commensal organisms.

- Normally there is no need to clean the site, except perhaps in farm animals. Ensure substances for injection are sterile (and warm) and use a new needle for each animal.

Incorrect or fumbled insertion of the needle can hurt the animal, or puncture a blood vessel, or deliver the substance incorrectly. If the animal moves, an unwanted intramuscular injection or puncture of a blood vessel may occur. This is a particular problem with dogs.

- Hold the animal very still. Introduce the needle point into the subcutaneous space with a firm but rapid movement. Point the needle towards the side of the neck with the bevel upwards. Pull the plunger back to make sure the needle has not entered a blood vessel.

The volume of fluid that can be accommodated varies with the size of animal, the site, looseness of the skin and the physicochemical nature of the substance.

- Choose a site where there is most loose skin to accommodate the volume and associated skin movement (see Table 4). Multiple sites can be considered for administration of greater volumes although, when repeated daily administration is intended, four sites should normally be considered the maximum.

When carrying out repeat dose studies, the substance may accumulate at a single site.

- Use smaller volumes for repeat dose than for single dose studies. Massage the skin overlying the injection site to disperse any fluid.
- Change sites for repeat dose studies.

3.14 Topical—dermal

Use of the technique

The dermal route is used to investigate both local and systemic effects following dermal absorption and metabolism. It may be used in testing potential therapeutic agents for skin disease, or in checking the potential to cause skin irritation or sensitization/allergenicity.

Summary of the protocol

Almost without exception regulatory toxicology guidelines now only call for the conduct of dermal toxicity tests on the intact skin.

The most commonly used species are the rabbit, rat, mouse and guineapig. Adequate restraint of conscious animals is required. Liquids may be applied diluted or undiluted. Solid test materials should be pulverized and moistened to a paste with saline or an appropriate solvent which will not 'degrease' the skin. The application site may or may not be occluded or semi-occluded depending on the most likely pattern of exposure.

Occlusion or semi-occlusion permits use of greater dose volumes, and in general also increases absorption. At the end of the exposure period the dressings should be carefully removed and the site is usually washed clean with warm water and gently dried.

If the site is not occluded animals can be prevented from ingesting material, either by locating the application close to the head or by application of an Elizabethan collar.

Potential problems with refinements

Irritant substances can cause serious adverse effects.

- A step-wise approach is essential (Home Office 1994). First check physico-chemical properties for irritant potential, then assess this further in cell cultures if appropriate, then test one animal first.
- In general, do not place irritant substances on the skin, unless checking the irritancy potential is the reason for the test. In such cases monitor the animal particularly carefully and be prepared to remove the animal from the study if it becomes distressed.
- It may be possible to quantify the erythema and identify an earlier endpoint by an intravenous injection of methylene blue.
- Apply test substances uniformly covering no more than 10% of the body surface (e.g. 5 cm × 5 cm for rats, 12 cm × 14 cm for rabbits and 7 cm × 10 cm for guineapigs). The area of application for highly toxic substances should be much less. Avoid eyes and genital areas.

Unintended skin abrasion may occur when clipping or shaving the hair. This can increase potential irritancy for the animal and introduce experimental error by increasing rate of absorption. Oil or grease from clippers can effect the response to the test substance.

- Only use animals with clean, intact and ungrazed skin. Take great care when clipping or shaving the animal, and make sure clippers are clean. Prepare the site 24 h previously to allow the animal time to recover.

For some tests the skin is deliberately abraded before applying the test substance.

- Always question the necessity to conduct this more severe procedure and make specific referral to the Regulatory Authority that demands it. If there is no alternative then check whether application and removal of sellotape provides sufficient abrasion (but note that this can also cause discomfort). In any case it is normally sufficient to remove the epidermis, e.g. by light scratching with a needle not sufficient to cause bleeding.

Volumes larger than about 500 μl tend to run off the back of smaller animals, defeating the object of the experiment.

- The maximum dose of a test material that is required by regulatory authorities for toxicological studies is 2 g/kg. Administer this in volumes of less than 500 µl.

Dressings can affect movement. Overtight dressings can cause excessive abdominal pressure which may lead to internal damage that is not apparent.

- Ensure dressings are not too tight and do not restrict the movement of the animal or cause excessive abdominal pressure. The maximum time of occlusion should be 24 h. *Note*: The standard Magnusson and Kligman test requires 48 h occlusion (Organization of Economic Co-operation and Development 1992).

Removal of adhesive dressings can pull the animals' hair or skin which can cause pain.

- Do not apply highly adhesive materials to the hair or skin if they are to be removed before the animal is killed.
- Determine the least painful method of removing dressings by practising on yourself before applying a dressing to the animal. Consider whether light anaesthesia or sedation would be beneficial.

The use of collars in rats appears to be particularly stressful.

- Avoid using collars for rats by applying the substance to a small area of skin close to the head.
- All animals need to be trained to accept a collar.

3.15 Topical—ocular

Use of the technique

Substances are applied to the eye in order to assess potential irritant effects. Application to the conjunctival mucous membranes and the corneal epithelium is also used to achieve high local concentrations of drugs for ocular therapy. The rabbit is universally used for eye irritation studies, even though there are shortcomings and exceptions in predictability. Dogs and primates are sometimes used for investigation of pharmaceuticals intended for long-term use in the human eye.

Summary of the protocol

Very useful guidance on the conduct of eye irritation studies have been produced by the UK Home Office (1994) and there is a requirement in the UK to follow these guidelines. The principles are universally applicable.

Substances can be applied to the eye by two methods: direct application into the conjunctival sac, or directly on to the cornea. The conjunctival exposure method has been commonly adopted because of ease of application. However, the current thinking is that conjunctival exposure is inappropriate under many circumstances, especially when the test material is a powder which may become entrapped in the conjunctival sac resulting in mechanical damage to the eye. The corneal exposure method more closely mimics accidental human exposure as occurs for example in chemical accidents.

A stepwise tier system approach (Home Office 1994) should always be followed where there is any chance of irritation or other damage. Do not proceed to using animals until initial physico-chemical characterization, *in vitro* cytotoxicity or other studies, and dermal tolerance assays have been conducted. An initial study in one animal using one eye only should always be carried out.

Various applicators for corneal exposure have been developed but for routine purposes the eyelids are gently retracted and the test formulation is applied directly to the cornea. The treated eye may be irrigated with water 20–30 s after application of the test substance. Animals must then be observed very closely with at least 24 h allowed for damage to become apparent.

Potential problems and refinements

This test has the potential to cause animals serious distress. Substances which are severe skin irritants must never be tested in the eye. Animals should be closely monitored for adverse effects throughout the procedure. If a severe adverse reaction occurs the animal

should be killed and no further animals should be used.

Substances may cause opacity, serious irritation, pain, inflammation or ulceration. The main area of concern is related to effects on the cornea, conjunctiva and iris although if the applied material can penetrate deeper into the eye other structures may also be affected.

- Do not apply known corrosive substances, those with a high oxidation potential, detergents, or known irritants, to the eye (Home Office 1994). Always check the physico-chemical properties for evidence of irritancy. The pH limits are 2–11.5 but neutral pH is best (see Section 2.3).
- The eye can be anaesthetized the first time that the substance is administered by application of a topical anaesthetic so that if any unexpected irritation does occur the animal will not feel it (Seabaugh *et al*. 1993). If the anaesthetic is incompatible with the compound or there are more general doubts, use a general anaesthetic for the first test.
- Be aware of the scale of likely responses and be able to recognize and respond rapidly and appropriately to the adverse effects as set out in the Home Office 1994 guidelines.

Insoluble, hard substances may cause mechanical damage when applied to the conjunctiva.

- Do not place rough, hard, insoluble substances in animals' eyes. Crystals should be micronized.

Irrigation may affect the outcome of the test and, if this has not been taken into account in the experimental protocol, then the test may have to be repeated using more animals.

- The effect of irrigation on the test (as opposed to the effect on the animal) is dependent on the chemical, the concentration, the time lag between exposure and initiation of the irrigation, and on the volume of irrigation. Always weigh these factors carefully *vis à vis* the legal requirement for the test before carrying out the procedure.

Volumes in excess of 50 µl may overestimate human sensitivity according to some scientific opinion and may simply overflow from the eye.

- Anatomic and physiological considerations suggest that the maximum practicable volume which can be instilled into the rabbit conjunctival sac is 30–50 µl (*Note*: 100 µl is required in OECD guidelines), so question the requirement for larger volumes. A low volume eye test can be considered using 10 µl.

Animals dosed and then held to assess recovery may suffer continuing distress.

- The necessity for assessment of recovery should always be questioned and established with the regulatory authority. Monitor animals very carefully and consider giving systemic analgesics (Fielder *et al*. 1987).

3.16 Footpad

This route has a high disturbance index (Barclay *et al*. 1988), and it is strongly recommended that it is not used unless proven to be the *only* effective one for achieving a specific objective.

Footpads were used as a site of injection for antibody production, popliteal lymph node assays, adjuvant arthritis, assessment of analgesic agents and investigation of mechanisms of pain and inflammation, and mycobacterial research, but alternative routes for virtually all these applications are now considered satisfactory (Bennett *et al*. 1992, Leenars *et al*. 1997). For example, for antibody production, other subcutaneous sites and careful selection of adjuvant almost always allows adequate titres to be obtained and the use of footpad injections for this purpose is considered unnecessary. For popliteal lymph node assays, most investigators now consider that injection into any tissue which drains into the lymph node will give good results. Alternative assays for skin sensitization have been developed, such as skin painting, local lymph node assay and

mouse ear swelling test. To induce adjuvant arthritis, intradermal and subcutaneous routes are now considered effective enough to replace the footpad.

It is difficult to find a practical way of avoiding the footpad route of injection for assessment of analgesic agents and investigations of mechanisms of pain and inflammation. Such experiments are short term and may involve injection of hyperalgesic or inflammatory agents (such as prostaglandins or carageenan) into the footpad which sets up an inflammatory reaction, or mimics it, and so sensitizes the animal to a subsequent painful stimulus, such as paw pressure. Paw swelling and paw withdrawal time can then be measured.

In the case of *Mycobacterium leprae*, infection has to be in the footpads because this is the only site which permits growth of the organism.

Summary of the protocol for Mycobacterium leprae

Mycobacterium leprae is inoculated subcutaneously into the hind footpads. The bacteria are contained in a saline suspension and a maximum of 10 μl is injected. The resulting infection is limited to the footpads and can be easily quantified. No macroscopic lesions develop. In the case of nude mice, infection develops to a higher level and some footpad swelling may occur.

- Injecting into both footpads interferes with locomotion, therefore never inject more than one foot.
- Monitor animals closely. Any animal showing swelling of the footpad accompanied by weight loss, or decreased fluid intake, or subdued behaviour patterns and/or impairment of normal mobility should be humanely killed.
- Animals *must* be given soft bedding. Grid floors should not be used. Food and water should be placed adjacent to the bed to avoid them having to walk far if the foot is painful (Wolfensohn & Lloyd 1998).
- Limit the frequency and severity of adverse effects by limiting the dose volume and ensuring that the material is injected into the footpad, and not into other tissues in the foot.

3.17 Uncommon routes

There are a number of routes that have limited use for specific scientific purposes (e.g. intra-anal; intrabursal; intrahepatic; intraneural; intra-ocular; intra-oesophageal/intragastric; intrapenile; intrarenal; intraspinal; intrathecal; intravesicular; peri-, epi- and subdural; retrovulvar; and rumen fistula). The general principles of best practice detailed in Section 2 of this report apply to these, as do many of the problems and refinements described in the rest of the report.

The Working Group would like to hear from anyone carrying out these techniques with a view to publishing an addendum to this report.

4 Special considerations for wild animals

An increasing number of publications address the use of wild animals for research, both in the laboratory and field. Information is available with regard to their capture and transport, the ethics of wild animal use and the ecological effect of removing animals (Bekoff 1995, Putman 1995, Tribe & Spielman 1996, Gaunt & Oving 1999, Hawkins *et al*. 2001). Information on the administration of substances to wild animals is, however, sparse and mainly relates to anaesthetic regimens. The same principles regarding the substance and technique apply as when using laboratory animals. In addition, the following points are important.

- When administering substances to wild animals that are to remain in, or be returned to, the wild, their contact with people should be kept to a minimum. If, however, the animal is to be retained in captivity and repeatedly dosed, then it becomes less stressful if the animal is familiarized and accustomed to people. Food rewards can be very helpful in familiarization and training.
- Familiarity with the natural behaviour and habitat of the species is essential in

order to decide the least stressful way of handling them. Wild mice, for example, often panic and leap about trying to escape if handled in open areas in bright light, whilst those handled under dim or red lighting and allowed to stay in shadow are much calmer. This finding is common to many nocturnal species.

- Anaesthesia or sedation of wild species prior to administration of substances is desirable to minimize the stress of handling associated with such procedures. After sedation the animal should be left quietly alone with little stimulation until the drug has taken effect. This can often be achieved simply by covering the cage or container with a blanket. Full recovery from sedation must be ensured before release otherwise the animals' chance of survival will be reduced.
- Many species of mammal will willingly go into a dark space such as a bag or box and use should be made of this behaviour to reduce overall stress of a technique. The animals may then be injected through the bag or within the box if it is equipped with a restraining mechanism. For example, wild rabbits may be held in a black cloth bag with just their ears protruding and the marginal ear vein can then be injected. Wild rats will run into black bags or tubes and can then be transported into ventilated fume chambers to be anaesthetized by inhalation. Squirrels often retreat into a nest box when approached and can be transferred from this into a ventilated chamber.
- Some birds enter a state of tonic immobility (TI) while being handled, or during procedures, which may facilitate the administration of a substance. TI is generally regarded as an acute fear response and is not a state of 'hypnosis', so it should never be deliberately induced to keep birds still as this will cause avoidable stress. Birds can also recover very rapidly from TI so restraint should be maintained continuously until the procedure is complete.

The least stressful way to dose wild animals is via the oral route by simply adding the substance to their normal diet (see Section 3.10.1) or smearing it onto their fur where they are likely to lick themselves when grooming. For predatory species it is often possible to present the substance in dead prey. For example, a solution or suspension of the substance in a 5% (bovine spongiform encephalopathy free) gelatin solution can be injected into the abdomen of a dead, pre-chilled, chick. The gelatin will set inside the chick with no leakage and this can then be fed to the animal. When an injection has to be used then the subcutaneous route is usually the least painful and least stressful.

The most stressful procedure is intraperitoneal administration and this should be avoided. Intramuscular injection is generally quick and less stressful but may involve persistent localized pain or necrosis, which in some species may lead to self-mutilation and inability to escape a predator should the animal subsequently be released to the wild.

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Note: During the preparation of this report it came to the Working Group's attention that EFPIA (European Federation of Pharmaceutical Industries Associations) and ECVAM (European Centre for the Validation of Alternative Methods) were also producing 'A Good Practice Guide to the Administration of Substances and Removal of Blood, including Routes and Volumes', to be published in the *Journal of Applied Toxicology*. Both reports are aimed at ensuring good, if not best, practice.

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