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Dosing Limit Volumes: A European View

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Summary

The need to refine experiments, as laid down in European Law, and the role of the European Centre for the Validation of Alternative Methods (ECVAM) in this context are outlined. An initiative between the European Federation of Pharmaceutical Industries' Associations (EFPIA) and ECVAM to produce a best practice guide on the administration of substances in preclinical safety evaluation is described. This guide proposes both best practice dose volumes and limit volumes which, if exceeded, could result in scientific or welfare implications. To meet the increasing needs of the toxicologist, the guide also addresses continuous intravenous infusion and its use in embryotoxicity studies. A strategy for vehicle selection which both minimises adverse effects and avoids duplication of animal studies is proposed and discussed.

Introduction

The need to Replace, Reduce and Refine animal experiments is embodied within European legislation and has been adopted within the national laws of a number of Member States. The European Centre for the Validation of Alternative Methods (ECVAM), a European Union-funded research centre whose prime focus is the development of non-animal models, has broadened its remit to include the Refinement of animal experiments. To this aim it is collaborating with the European Federation of Pharmaceutical Industries' Associations to develop a number of best practice guides, including the administration of substances.

The objective of the best practice guide is to provide the researcher in the preclinical safety evaluation laboratory with an up-to-date, easy to use set of data sheets to aid in the study design process whilst affording maximum welfare considerations to the experimental animals. Although this publication is targeted at researchers in the European Pharmaceutical Industry it is considered that the principles underpinning the data sets and refinement proposals are equally applicable to all those who use these techniques, whether in Research Institutes, Universities or other sectors of Industry.

There are numerous publications dealing with the administration of test substances and, additionally, many laboratories also have their own 'in-house' guidelines which have been developed by custom and practice over many years. The aim of this publication is to bring together all sources of information and provide a consensus document based on current scientific evidence where this is available. The document also addresses the continuing need to refine the techniques associated with the administration of substances and suggests ways of doing so. One important consideration is the vehicle used to administer the test substances by the required route. The vehicles themselves should offer optimal exposure but should not influence the results obtained for the compound under investigation and, as such, should ideally be biologically inert, have no effect on the biophysical properties of the compound and have no toxic effects on the animals. Unfortunately, this situation does not always exist and so a strategy for vehicle selection is proposed in the document which aims to minimise adverse effects on the animal whilst offering the pharmaceutical scientist a way forward with compounds of limited aqueous solubility.

European Law and the Experimental Animal

Animal Welfare in Europe is under the authority of two bodies, the Council of Europe and the European Union. The roles and responsibilities of each group have been described by Lwoff & de Greeve (1998) and are summarised below:

Animal Welfare in Europe

Council of Europe (CoE)	European Union (EU)
* 40 States	* 15 Member States
* Strasbourg	* Brussels
* Multilateral Consultation	* Advisors to Commission
* Recommendations & Conventions	* Directives
* Moral Obligation	* Competent Authority

The Council of the European Union Directive 86/609EEC (EU, 1986) “on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes”, was adopted in November 1986. This marked the beginning of the EC’s legislative competence relating to animal experimentation. It also facilitated the adoption of such animal protection legislation for the first time in several of the Member States. Although not expressly stated in the text, the Directive effectively embraces the principles of the 3Rs approach - Reduction, Refinement and Replacement, which are widely understood and broadly accepted by scientists, regulatory and licensing authorities, industry and animal protection organisations.

In addressing refinement, Article 7(3) specifies that: “In a choice between experiments, those which use the minimum number of animals, involve animals with the lowest degree of neurophysiological sensitivity, cause the least pain, suffering, distress or lasting harm and which are most likely to provide satisfactory results shall be selected.”

Article 23 requires that the Commission and Member States should “.. encourage research into the development and validation of alternative techniques which could provide the same level of information as that obtained in experiments using animals but which involve fewer animals or which entail less painful procedures ..” Towards this end, at Community level, the European Centre for the Validation of Alternative Methods (ECVAM), was established in 1992 at the EC Joint Research Centre based in Ispra, Italy.

Although Council Directive 86/609 is the most significant piece of animal legislation in Europe, a number of other laws have impact on those working with laboratory animals. Each of these is administered by a controlling body (Directorate General), with the principle ones highlighted below:

Responsible Body	Area of Impact
DGIII:	Pharmaceuticals, cosmetics, ICH
DGIV:	Animal transport
DGXI:	Industry & Environment, 86/609, ECVAM
DGXII:	Science Research and Development
DGXXIV:	New scientific committees, eg health & welfare, cosmetics, non-food animals
Secretary General	Ethics & new technologies

The EFPIA/ECVAM Initiative

In an attempt to widen its area of impact, ECVAM is collaborating with The European Federation of Pharmaceutical Industries' Associations to produce Best Practice Guides for a number of technical procedures, such as administration of substances, removal of blood, the rodent protection test and production of monoclonal antibodies. The first of these, the administration of substances, has been developed through a Working Group with representatives of the pharmaceutical industry, academia and animal welfare. The remit of the group was to provide data sheets to aid study design whilst affording maximum welfare considerations to the experimental animal.

The Development of a Best Practice Guide

The objectives of the working group were:

- *to identify 'best practice' dose volumes*

to provide consensus values for routine use which represent best practice in terms of animal welfare and practicality. The species chosen should represent those most commonly used in preclinical safety evaluation. The routes of administration should be those most frequently used. Since continuous intravenous infusion is being increasingly widely used it should be addressed separately and include both repeat dose toxicity studies and embryotoxicity studies.

- *to identify 'limit doses'*

a 'limit dose' can be defined as one, which if exceeded, could result in scientific or welfare implications. Although not considered as absolute maxima the investigator should not exceed these values without reference to the responsible veterinary surgeon. Additional authority may be required through the local ethical committee or the national licensing body.

- *to propose refinement of techniques*

in addition to using the lowest dose volume practically possible, it is important to employ refinement techniques wherever possible. The guide should identify technical improvements in line with current best practice.

- *to address vehicle limitations*

one important consideration in the administration of test substance is the nature of the vehicle being used. Ideally, a vehicle should offer optimal exposure whilst not influencing the results for the compound under investigation. A strategy for the selection of vehicles should be proposed.

Factors Impacting on the Guide

In considering a best practice guide to the administration of substances, it is necessary to consider those factors which are impacting on preclinical safety evaluation. The first of these is the involvement of toxicology in the early research process to facilitate faster drug development. It is no longer acceptable for the R&D processes to be separate entities. There is much that the toxicologist can do to support the early phases of research, well before the nomination of a candidate drug. It is essential, therefore, that any *in vivo* work performed in toxicology has relevance to pharmacology screens. It is equally important that any animal work performed in pharmacology can be used to aid the safety evaluation process. To this end, the use of simple, common vehicles in pharmacology may obviate the need for dose ranging studies in toxicology. Duplication of studies must be avoided at all costs. One other significant change in the R&D process is the involvement of drug metabolism and pharmacokinetics early in the research phase. As early as lead generation, the pharmacokineticist is involved in optimising the delivery of the active substance to the site of action. Long before secondary pharmacology screens are performed it is essential to show that the intended drug is absorbed and bioavailable, thus avoiding future unpredictable animal studies. A further impact on the toxicologist is the increasing number of compounds of poor aqueous solubility which are being nominated as candidate drugs. Even those intended for oral use will, at some time, have to be administered intravenously to laboratory animals and therefore offer considerable challenge. The development of vehicle databases and sharing of

information should be encouraged to minimise the need to conduct additional toxicity studies to demonstrate 'safe' doses of co-solvent and complex pharmaceutical formulations.

Administration Volumes

Dosing of experimental animals is necessary for a variety of scientific investigations. The pharmaceutical industry, in particular, has investigated the level of dosing compatible with animal welfare and valid science (Hull, 1995).

In the preclinical stage of the safety evaluation of new drugs it is normal practice to use multiples of the 'effective dose' in order to attempt to establish the necessary safety margins. Where chemicals are of low toxicity or are only poorly soluble in acceptable formulations, a large volume may be required to be given to individual animals to satisfy both scientific and regulatory requirements. The intended clinical use may also impact on the acceptability of larger than usual dose volumes, eg imaging agents or plasma expanders for intravenous application.

Table 1 presents administration volumes for the commonly employed routes in the most frequently used species. They are consensus figures based on published literature and internal guidelines. The marmoset and minipig are now considered within this category since they are being used increasingly in Europe.

Table 1

**Limit Volumes Considered Best Practice
(and possible maximal dose volumes)**

Route and Volumes (ml/kg except * ml/site)								
Species	Oral	sc	ip	im	iv bolus	iv (slow inj)	id	
Mouse	10 (50)	10 (40)	20 (80)	0.05* (0.1)*	10 (25)		0.05*	
Rat	10 (40)	5 (10)	10 (20)	0.1* (0.2)*	5 (20)		0.1*	
Rabbit	10 (15)	1 (2)	5 (20)	0.25 (0.5)	2 (10)		0.1*	
Dog	5 (15)	1 (2)	1 (20)	0.25 (0.5)	2.5 (5)		0.1*	
Macaque	5 (15)	2 (5)	- (10)	0.25 (0.5)	2 (-)		0.1*	
Marmoset	10 (15)	2 (5)	- (20)	0.25 (0.5)	2.5 (10)		0.1*	
Minipig	5 (15)	1 (2)	1 (20)	0.25 (0.5)	2.5 (5)		0.1*	

Two sets of figures are shown in each column. Figures in the left side of columns are intended as a guideline for 'best practice' dose volumes for single or multiple dosing. The second bracketed set of figures, where given, are the possible maximal values. If exceeded, welfare or scientific implications may result and reference to the responsible veterinary surgeon should be made.

Some of these suggested maximum values have been obtained from recent literature, but appear high when compared with 'best practice' values. The need for careful attention to animal welfare, and formulation of material used at high dose volumes, is emphasised, particularly if repeat dosing is intended. Study duration could be restricted and scientific validity compromised by physiological reaction to high dose volumes. It is therefore essential from an ethical standpoint that these issues are fully considered before protocols are finalised and work commences. It is also strongly recommended for ethical as well as scientific reasons that physicochemical compatibility studies and small scale pilot studies are carried out on any new formulation before committing to larger scale studies.

Oral Route

On occasions, if large volumes are to be given (eg rat 20 ml/kg), it may be necessary to restrict the animals' food intake before dosing. The duration of fasting will depend upon the feeding pattern of the species, the starting time for food restriction, physiology of the species, length of time of dosing, diet

and light cycle. It is recommended that for accuracy of dosing, and to avoid dosing accidents, that liquids are administered by gavage. It is suggested that for non-aqueous liquids the dose volume should be reduced by 50%.

Parenteral Routes

For substances administered parenterally, the dose volume used, stability of the formulation before and after administration, the pH, viscosity, osmolality, buffering capacity, sterility and biocompatibility of the formulation are factors to consider. This is particularly important for multiple dose studies. These factors are reviewed in some detail by Claassen (1994).

a) **Subcutaneous**

This route is frequently used. The rate and extent of absorption depend on formulation.

b) **Intraperitoneal**

This route is used infrequently for multiple dose studies because of possible complications. There is a possibility of injecting into intestinal tract and irritant materials may cause peritonitis. Drug absorption from the peritoneal cavity after the administration of the compound as a suspension is dependent on the properties of the drug particles and the vehicle.

c) **Intramuscular**

Intramuscular injections may be painful, because muscle fibres are necessarily placed under tension by the injected material. Sites need to be chosen to minimise the possibility of nerve damage. Sites should be rotated for multiple dose studies. A distinction needs to be made between aqueous and oily formulations (speed of absorption, oily formulations likely to remain as a depot for > 24 hours). With multiple dose studies there is a need to consider the occurrence of inflammation and its sequelae.

d) **Intravenous administration**

For this route, distinctions are made between *bolus injection*, *slow intravenous injection*, and *intravenous infusion*. The values in Table 1 relate to *bolus injection* and *slow intravenous injection*.

Bolus injection: In most studies using the intravenous route the test substance is given over a short period, ... two minutes. Such relatively rapid injections require the test substance to be compatible with blood and not too viscous. When large volumes are required the injection material should be warmed to body temperature to minimise circulatory shock. The rate of injection is an important factor in intravenous administration. Total dose volumes greater than the maximum quoted in Table 1 for bolus injections can be given at a slower rate. No detectable changes in haematocrit or heart rate were observed in dogs following rapid intravenous injection of 6 ml/kg, but 20 ml/kg was associated with 15% haemodilution and a transient tachycardia (+46% over 1 min) (Zeoli *et al* 1998).

Slow intravenous injection or infusion. Because of the expected clinical application of the compound, or because of limiting factors such as solubility or irritancy, it may be necessary to consider administering substances by slow intravenous injection or by infusion. Typically, different techniques would be applied for slow injection or infusion to minimise the possibility of extravascular injection of material. For slow intravenous injection over the course of 5 – 10 minutes a standard or butterfly needle might be used, or possibly a cannula. Dependent on the duration of administration a cannula may be inserted and taped in place in a superficial vein (short term), or surgically placed some time prior to use (longer term, or multiple infusions).

It has been shown that rats may be given daily intravenous infusions of isotonic saline at dosages up to 80 ml/kg at 1 ml/min for 4 days without significant signs of distress or pulmonary lesions (Morton *et al*, 1997a). Pulmonary lesions increased in incidence and severity when the duration of treatment increased to 30 days and the infusion was administered at either 0.25, 0.5 or 1.0 ml/min (Morton *et al*, 1997b)

Careful consideration is needed if prolonged intravenous infusions are necessary. The volume and rate of administration will depend on the substance being given and take account of fluid therapy practice. As a guide, the volume administered on a single occasion will be less than 10% of the circulating blood volume over two hours. Information on circulating blood volumes has been published by Altman & Dittmer (1974). Minimal effective restraint of animals with least stress is a key factor to consider for prolonged infusions.

The total duration of an infusion is also a factor. Table 2 presents recommended dose rates and volumes for discontinuous (4 hour per day) and continuous (24 hour) infusion. Further data are required to complete this table.

Table 2

Repeated Intravenous Infusion - Dose Volumes/Rates

Daily Infusion Period	Mouse	Rat	Rabbit*	Dog	Macaque	Mini Pig
Total Daily Volumes (ml/kg)						
4 Hour	-	20	-	20	-	-
24 Hour	96	48	24	48	-	24
Rate (ml/kg/h)						
4 Hour	-	5	-	5	-	-
24 Hour	4	2	1	2	-	1

*based on teratology studies

Volumes and rates for the rabbit are based on data derived from embryotoxicity studies which showed no effects on the foetus but perivascular granular leukocyte cuffing and proliferative endocarditis in dams receiving 2 ml/kg/h and above (McKeon *et al*, 1998). Infusion rates in rats are typically in the range of 1 to 4 ml/kg/h (Loget *et al*, 1997; Cave *et al*, 1995; Barrow & Herities, 1995) but should not exceed 2 ml/kg/h in embryotoxicity studies. Values for the mouse (van Wijk, 1997), dog and minipig (unpublished data) are based on repeat dose studies of one month duration.

Other limits, indicating the importance of the vehicle formulation at high dose volumes are highlighted in four publications (Cornelius *et al*, 1978; Concannon *et al*, 1992; Manetti *et al*, 1992; Mann & Kinter, 1993). These data indicate that there are large differences in tolerated volume by iv infusion, dependent upon the vehicle used. As an example, the difference between Dextran 70 and saline can be used to illustrate the point. Dextran 70, a plasma volume expander, was shown to induce minimal haemostatic abnormalities when infused at 20 ml/kg over 30 or 60 minutes while saline infused at >1ml/kg/min for 60 minutes caused significant haemodilution and tachycardia in dogs.

e) Intradermal

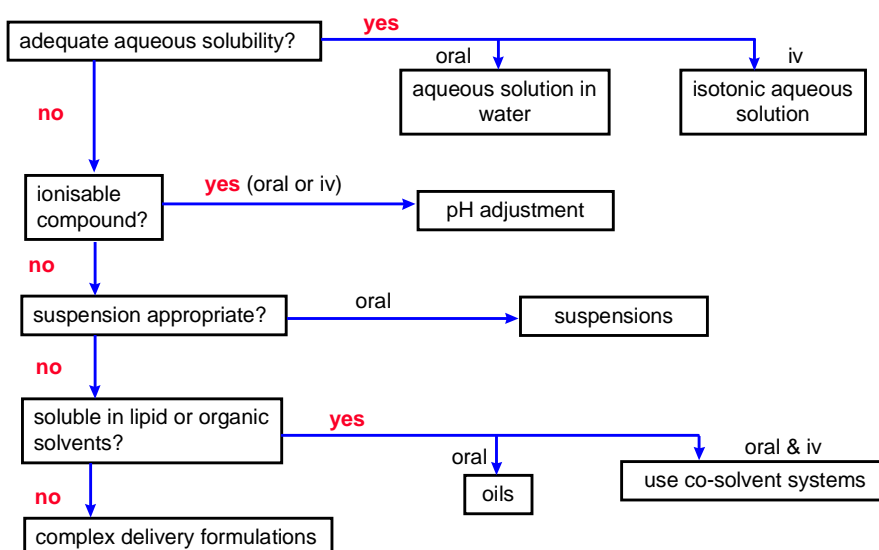
This site is typically used for assessment of immune, inflammation, or sensitisation response. Material may be formulated with an adjuvant.

A Strategy for Vehicle Selection

Vehicle selection is an important consideration in animal investigations. Vehicles themselves should offer optimal exposure but should not influence the results

obtained for the compound under investigation and, as such, they should ideally be biologically inert, have no effect on the biophysical properties of the compound and have no toxic effects on the animals. If a component of the vehicle has biological effects, the dose should be limited such that these effects are minimised or not produced. Simple vehicles used to administer compounds include aqueous isotonic solutions, buffered solutions, co-solvent systems, suspensions and oils. Laboratories are encouraged to develop decision trees to facilitate selection of the most appropriate vehicle based on the animal study being performed and the properties of the compound. A generic decision tree is shown below.

Vehicle Decision Tree



Within each category, a preferred list of vehicles can be prepared for each route and species under investigation. Two examples of preferred lists are shown below:

Examples of Preferred Vehicles

Single iv dose to rodent 1ml/kg co-solvent system

- PEG400:0.9% NaCl 50:50
- PEG400:Ethanol:0.9% NaCl 40:5:55
- Glycerol:Ethanol:0.9% NaCl 15:5:80
- PG:Ethanol:PEG400:0.9% NaCl 15:5:20:60
- DMA:PEG400:0.9% NaCl 40:40:20

Repeat oral dose to dog 5ml/kg suspension

- 1.0% Carboxymethylcellulose
- 1.0% Carboxymethylcellulose/
0.1% Tween 80
- 1.0% Methylcellulose
- Oils
- 0.5% Guar Gum
- 5.0% Gum Arabic (Acacia)

These preferred lists have been developed following discussion between the departments of pharmacology, DMPK, toxicology and pharmaceutical development and should enable transferable data to be generated, even in the very early animal studies. The development of preferred lists requires detailed knowledge on each proposed vehicle, including toxicity, under varying conditions of administration. Data sharing between laboratories should be encouraged to avoid duplication of animal work.

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