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PRACTICAL PHARMACOLOGY for the PHARMACEUTICAL SCIENCES

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Preface

It is a startling fact that it is 30 years since the last text book on pharmacology laboratory practicals (Kitchen, 1984) was published. An obvious assumption would be that there has been a drastic fall in demand. A common response is that laboratory practicals are redundant and have been replaced by computer-assisted learning (CAL) and simulated experiments (Hughes, 2003). This is due to the challenges of increasing student numbers, decline in staff numbers and the high cost of maintaining laboratories and animal facilities (Hughes, 2001). It has been claimed that CAL experiments and problem-based learning provide an equal or superior student learning experience (Hughes, 2001, 2002). Yet surveys of the curriculum of pharmacology courses in the United Kingdom (Dewhurst and Page, 1998), and currently using a world-wide Internet survey, quickly reveals that this is not the case. "Wet" laboratory practicals remain a central part of most courses, and clearly many universities and colleges are reluctant to abandon them completely, as employers in the pharmaceutical industry and academia expect hands-on experience of pharmacological techniques. In the United Kingdom, the British Pharmacology Society (www.bps.ac.uk/Education/University resources/Core curricula in pharmacology, (accessed June 2013)) currently recommends an undergraduate pharmacology core curriculum in which skills in pharmacological experimentation form an essential component (see www.bps.ac.uk/education/university resources/core curricula). Most courses now appear to rely on in-house schedules of variable quality. Meanwhile, the equipment available for the pharmacology laboratory has greatly improved mainly through the use of computers to control experiments and record data, which makes them easier to use and improve the quality of data obtained by novice students. It is a fear that some of the skills involved in real, wet experimentation may be lost

as new lecturers themselves have not been taught these methods. It is therefore timely to produce a book for use in pharmacology practical classes using state-of-the-art equipment and using modern nomenclature. Several books have recently described laboratory techniques and calculations for the biosciences in general. In contrast, this book specifically aims to introduce practical pharmacology to the pharmaceutical sciences undergraduate student.

The book opens with an outline of how to prepare to work in the pharmacology laboratory, and progresses to briefly describe some of the basic principles of pharmacology, which I believe are most clearly understood from a historical perspective. The central core includes experiments using *in vitro* tissues, isolated cells and cell-free biochemical systems, focusing on those that are unique to pharmacology. Some of these are classical experiments which were introduced some years ago, and form the basis of the discipline of pharmacology. However, it is important to note that they are still topical in that they are still being interpreted in new ways in the light of current research. Several techniques included in the BPS core curriculum, such as molecular biology, biochemistry, electrophysiology and tissue culture widely used in pharmacological research are only alluded to, as these have been well covered elsewhere. In conclusion, since no experiment is complete without communicating the results, there is a section on the presentation and interpretation of results and how to use and cite information sources. This book aspires to be useful for students in all pharmaceutical science courses that include pharmacology modules giving a real life experience in learning pharmacology.

Powerpoint slides to accompany this book can be downloaded from http://booksupport.wiley.com by entering the book name, author or isbn information.

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1

Before Entering the Pharmacology Laboratory

Before embarking on any new activity, it is wise to be familiar with the language, concepts and possible risks of the venture. So this book begins with a number of topics with which an experimenter must be familiar, such as health and safety, ethical and legal considerations and fundamental principles of experimental pharmacology. No experiment has much value unless a coherent design has been devised first. The design of an experiment is crucial if it is to yield meaningful results. Having obtained the experimental data, it is important to decide on the relevant statistical methods that will be employed to evaluate the results. Obvious as this may seem, it is shocking, even in professional research, how many experiments are wasted due to a lack of planning in design.

1.1 SAFETY AND RISK ASSESSMENT

All activities which involve the use of chemicals, from the factory floor to the research laboratory, are subject to the Health and Safety legislation. In the United Kingdom, this is done by the Health and Safety Executive (HSE), and of particular relevance in the laboratory is the Control of Substances Hazardous to Health Regulations (COSSH, 2002). In the United States, the body is the Occupational Safety and Health Administration (OSHA), who require a Chemical Hygiene Plan (CHP) for each

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experiment, whilst in the EC the relevant body is the European Agency for Safety and Health at Work (EU-OSHA).

In the United Kingdom, COSSH regulations apply to all places of work, and all workers must be conversant with all risks and safety procedures. A risk assessment of all procedures must be carried out and a documentation of how these risks are to be minimized during the procedure and safe procedures for disposal of chemicals must be displayed. Any accidents must be reported and logged for future reference.

The bioscience laboratory presents many hazards not encountered elsewhere, and COSSH regulations are especially important. All laboratory workers must be aware of the regulations governing all work in laboratories. Drinking, eating and smoking are banned in all laboratories. No chemicals should come into contact with the body – including the mouth, eyes and skin whilst inside a laboratory. Remember that in a pharmacology laboratory, there is exposure to many highly biologically potent chemicals. A protective coat (frequently white) must be worn at all times, and protective eye goggles and gloves worn when required. In addition, laboratory workers must be familiar with the international warning symbols for toxic, corrosive and inflammable chemicals and gases, cancer-causing and suspected cancer-causing chemicals, radioactive materials, biological hazards, and reproductive hazards. These are widely available and explained on the internet. If a student is unsure of the meaning of any symbols they should ask their supervisor. These are not only displayed at the entrance to laboratories, but also on individual chemicals and equipment.

For class laboratory exercises, it is the responsibility of the supervisor to identify all risks and display them in the laboratory. This is not just a piece of administration, but an important document all students must be familiar with before they start the experiment to ensure safe practice. Students must be familiar with all the chemicals to be used and if there are any special precautions that must be taken. Before a project is undertaken, a risk assessment must be carried out by the student with appropriate guidance. The information that must be sought is given as follows.

- What are the dangers of handling individual chemicals? These are shown on data sheets supplied by chemical distributors. It should be ascertained if there are any particular hazards associated with entry into the body of any of the chemicals; are any substances absorbed by the skin or inhaled through the nose? Precautions that might be necessary are the use of disposable gloves and/or goggles.

Volatile compounds should be handled in a fume cupboard, which is certified as conforming to legal requirements (such as those laid down by the HSE in the United Kingdom). Fume cupboards should not be used with the front open above the displayed marks to ensure the correct airflow.

- Are there any aspects of the use of equipment or procedures that expose laboratory workers to any hazards? There are the ubiquitous procedures, such as pipetting. This should never be done by mouth, and must be done using either an automatic pipette or a device that can be attached to the end of a plastic or glass pipette. The instructions for operating equipment must be adhered to. Examples are centrifuges, spectrophotometers and equipment containing lasers or radiation sources.
- A vital part of a risk assessment is to identify methods of disposal of hazardous chemicals and biological materials. Many water-soluble compounds can be disposed of in a sink, usually after appropriate dilution. Lipophilic compounds and solvents are disposed of in specially designated bottles. Biological waste is usually placed in yellow bag to await later incineration. Used plastic pipettes and tips are placed in special containers, as are sharp objects such as syringe needles.
- The procedures to be taken in event of an accident or emergency must be clear. Chemical spills are a common occurrence and different procedures are required depending on the nature of the chemical. Dilute solutions of water-soluble, non-toxic chemicals are easily cleaned up by use of absorbent materials such as paper towels. All other potential hazards must be assessed, such as flammability, reactivity to air or water, corrosion or high toxicity; the incident should be immediately reported. Special measures will have to be taken. Flammable chemicals are absorbed with sawdust or special pads and the laboratory is ventilated maximally. Acids and alkalis should be diluted and neutralized.

1.2 THE LABORATORY RECORD BOOK

The importance of keeping a laboratory notebook is often underrated. Evidence collected for any purpose will not be credible if a contemporaneous record of events is not available. This is no less true for laboratory evidence than it is for police and forensic records. A book must be kept where all procedures, calculations, observations and results, along with

the relevant health and safety forms, are kept. This should be a permanently bound book, and not a loose-leaf from which pages may be removed. Entries must be made contemporaneously in the laboratory at the time at which they occurred. This is frequently not appreciated by students who think that they will "write it up neatly" at some later time. This is unacceptable. For this reason, many hospital and research laboratories employ strategies such as forbidding record books to be removed from the laboratory, or insisting that duplicate records are kept and one copy left in the laboratory upon leaving at the end of the day. There are several essential pieces of information that must always be recorded.

- Entries must be done using a pen and not an erasable pencil. Corrections should be made by crossing out rather than deleted.
- Pages must be dated and the name(s) of experimenters be recorded. All entries of data on computers are date-stamped and not subject to later manipulation. Computer records should be backed up after each day to prevent loss.
- All details of methods, instruments and apparatus must be recorded. All details of chemicals and solutions (especially their concentrations) noted. Details of animals used must be available, including their species, age, weight and sex.
- Raw data must be carefully recorded and fully annotated. This includes any photographs or diagrams.
- All stages of calculations and dilutions must be written down so that any errors can later be unequivocally detected and corrected.
- Graphs and tables derived from the results should be drawn as soon as possible, preferably before leaving the laboratory. This enables an early interpretation of the results to be made, so that any adjustments in the protocol can be made before proceeding with further experimentation.

1.3 USE OF ANIMALS IN PRACTICAL PHARMACOLOGY

Even before enrolling on a pharmacology course, students must be aware that the use of living tissues and cells are integral to the discipline. Most universities post a caveat to this effect in their course descriptions. The anti-vivisectionist viewpoint is highly appreciated, and in all developed countries it is incorporated into the laws governing the use of animals in teaching and research. The use of living animals in teaching up to

graduate level is not advocated. At post-graduate level and beyond, there are strict laws that must be adhered to, and licenses that must be obtained before any work on living animals can proceed. In the United Kingdom, the laws governing animal experimentation are embodied in the Animals (Scientific Procedures) Act 1986 and Amendments (2012). In addition to the Codes of Practice relating to the general care, housing and treatment of animals, both a project and personal license must be obtained from the Home Office. It is stressed that these will not be granted unless the following criteria have been justified:

- that there are no non-animal alternatives,
- that the benefits expected from the programmes of work are judged to outweigh the likely adverse effects on the animals concerned,
- that the number of animals used and their suffering must be minimized. Any contravention of these regulations found by inspectors and others will lead to a ban of an institution and individuals from working with animals.

All the experiments in this book, which is targeted at graduate-level students, do not require a licence as no substance or treatment is ever administered to animals, the numbers of animals are minimized and the above criteria laid down by the United Kingdom. Codes of Practice of the Home Office are fulfilled. Most courses do not require the use of large numbers of animals, and institutions cannot justify the expense of maintaining an animal house and attendant technicians. An alternative is to have an animal holding room where animals are delivered from breeders and held for a matter of no more than a few days. Nevertheless, animals are housed in a quiet air-conditioned room, provided with a regular light–dark cycle. Before experimentation, animals are rapidly and humanely killed (usually by cervical dislocation) in a quiet location, and tissues removed and placed in an appropriate physiological buffer to maintain their viability.

1.4 EXPERIMENTAL DESIGN

Before designing an experiment, the answers to four basic questions must be clearly understood:

- What is the topic of the experiment?
- Why is the topic being addressed?

- How is the experiment going to be carried out?
- How is the data going to be analysed?

The first two questions are answered by doing sufficient background reading, both from review articles and more detailed reports. The question of how to economically perform a literature review is discussed in the Section 10.5. Having grasped an understanding of the topic, a hypothesis can be formulated, that can be tested which will allow an advance in the understanding of the subject. Even if it is intended to repeat some previously reported preliminary finding, a full understanding of the topic is essential if a valid experiment can be designed. Importantly, there must be critical eye for detail. It is much better to attempt to design an experiment to answer one well-defined question than to attempt to address several rather vaguer questions.

How the experiment is actually designed will depend on a number of factors, including available techniques and materials. There must be a realistic estimate of both cost and the time involved. Both of these frequently underestimated, so it is wise to allow a margin of error for both of these factors. If any of the techniques are new to the experimenter, it must be certain that help will be available from a person who has first-hand knowledge of carrying out the technique. It must be borne in mind that if time is to be allowed to learn a new technique, this must be factored into the time allowed. As a general rule, in carrying out experiments and projects which have been allocated a short period of time, the learning of new methodologies should be avoided.

The actual design of an experiment should include a full understanding of the following factors.

An experiment designed to attempt to disprove the hypothesis is more powerful than one from in which it is highly likely that the results will confirm it.

The inclusion of controls is vital if unequivocal conclusions are sought. These are frequently termed "positive" and "negative" controls. A positive control is one for which a positive response is expected. They may perform the function of being "quality" controls, or may merely confirm that the technique is functioning in a predictable manner. They can also give an indication of the sensitivity of the method, and that this is sufficient for the purposes of the experiment. A negative control is always included to ensure that the method is actually measuring changes in the dependent variable (response), and will exclude any interfering variables. A negative control is sometimes referred to as a "blank", and a high variable will indicate an interfering factor.

Another factor that will determine the design of the experiment is the type of statistical analysis that will be carried out on the final results. This will then determine the number of replicates that are necessary in order that a valid statistical test can be applied (see Section 1.6).

To arrive at a final experimental design, if usually necessary to perform some pilot studies ("proof of concept"), to ensure that all is working out as planned and that there is not some flaw that has been overlooked which will confound the experiment. Typically, these are factors such as the speed of response of measuring apparatus, instability of the preparation or poor replication of results. These must be resolved before a large-scale study can begin.

1.5 UNITS, DILUTIONS AND LOGARITHMS

It may seem banal to stress the importance of understanding the basic units of mass (this is essentially the same as weight, at least on Earth), volume and concentration. Whilst all units used in pharmacology are metric, there is some variation as to whether SI units (Le Système international d'unités), or units which are derived from the fundamental units of the SI system should be used. The base units of mass, length and force defined in the SI system are the kg, m, and N, respectively. In practice, the g and mL are used in the laboratory. Similarly, The SI system prescribes that units of concentration should be expressed as kg/dm^3, whereas g/mL are commonly used in the laboratory.

Since the magnitude of each of these parameters can fall over a vast range, or orders of magnitude (an order of magnitude is generally taken to be 10-fold), they are expressed as a single digit before the decimal point multiplied by 10 to the power of a number (e.g. 0.0004 g is 4×10^{-4} g). A more convenient nomenclature is to apply a prefix to the basic unit, so that 0.0004 g can also be expressed as 0.4 mg or 400 μg. The most common prefixes for base units are graded by a factor of a thousand.

Mega (M)	10^6
Kilo (K)	10^3
milli (m)	10^{-3}
micro (μ)	10^{-6}
nano (n)	10^{-9}
pico (p)	10^{-12}
femto (f)	10^{-15}

1.5.1 Units of Mass

The common unit of the mass is gram. However, it is more useful in many cases to express weight in *moles* (or for ions, *equivalents*). The reason for this is that 1 mol of any compound contains the same number of molecules, equal to Avogadro's number, 6.022×10^{23}. In pharmacology, and biological chemistry in general, it is more useful to work in moles than grams, since it is of more interest to know the number of molecules, rather than grams, participating in a reaction or competing for a binding site, such as a receptor. Obviously, the weights of different compounds that contain 1 mol will differ hugely. For example, 1 mol of acetylcholine chloride weighs 181.66 g, and 1 mol of human acetylcholinesterase weighs 67.796 kg, yet they both contain the same number of molecules. One mole of a compound contains the molar mass in grams. The molar mass is the molecular weight (MW) in grams. The MW is sometimes called the formula weight (FW) of the relative molecular mass (RMM), and can be expressed in daltons (Da). In the case of large MW compounds such as proteins or polynucleotides, the MW is expressed in kilodaltons (KDa). Strictly, 1 Da is 1/12 of the mass of carbon-12, but in practical terms this is the same as the weight of one hydrogen atom (or proton).

The *equivalent* is also a unit of weight and is a similar concept as a mole, but expresses the number of ions in solution. An equivalent of an ion is the molar mass divided by the valency. Thus, 1 mmol Na^+ = 1 mEq Na^+, but 1 mmol Ca^{2+} = 2 mEq Ca^{2+}. An electrochemically neutral solution must contain an equal number of equivalents of positive and negative ions.

1.5.2 Units, Concentrations and Logarithms

Concentration is expressed as weight/volume, or for liquids as vol/vol. Just as weight can be expressed in grams, moles or equivalents, concentration can be expressed in a variety of units. When working in a laboratory, it is invaluable to be able to rapidly convert these different units.

Weight/volume are commonly expressed as g/L (g L^{-1}) or mol/L (mol L^{-1}) = Molar (or M). Note that 1 μg/μL = 1 mg/mL = 1 g/L and 1 μmol/μL = 1 mmol/mL = 1 mol/L = 1 Molar or 1 M.

Occasionally, concentrations are expressed as weight%, which means weight per 100 mL, and vol% means volume per 100 mL.

It is important to distinguish the commonly used molar or molarity from molality. A 1 molal solution contains 1 mol per 100 kg of solvent.