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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 (COSHH, 2002). In the United States, the body is the Occupational Safety and Health Administration (OSHA), who require a Chemical Hygiene Plan (CHP) for each
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 postgraduate 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 $4 \times 10^{-4}$ 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 $\mu$g. The most common prefixes for base units are graded by a factor of a thousand.

<table>
<thead>
<tr>
<th>Prefix</th>
<th>Multiplier</th>
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</thead>
<tbody>
<tr>
<td>Mega (M)</td>
<td>$10^6$</td>
</tr>
<tr>
<td>Kilo (K)</td>
<td>$10^3$</td>
</tr>
<tr>
<td>milli (m)</td>
<td>$10^{-3}$</td>
</tr>
<tr>
<td>micro (μ)</td>
<td>$10^{-6}$</td>
</tr>
<tr>
<td>nano (n)</td>
<td>$10^{-9}$</td>
</tr>
<tr>
<td>pico (p)</td>
<td>$10^{-12}$</td>
</tr>
<tr>
<td>femto (f)</td>
<td>$10^{-15}$</td>
</tr>
</tbody>
</table>
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, $6.022 \times 10^{23}$. 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.
The molarity of a solution changes with temperature, since the volume of the liquid will expand, whereas molality does not. Many drugs that are used in pharmacology experiments are expensive, indeed some pharmaceuticals are among the most expensive on Earth and can only be bought in μg quantities. Solutions of expensive compounds have to be made in small volumes. As an example, if 10 mg of tubocurarine is purchased, and a 10 mM stock solution is required, how can this be done? This is a small weight and it would be wasteful to weigh out mg quantities, and it may be preferable to calculate the volume of water that should be added to provide a 10 mM solution. First, calculate how many moles are there in the bottle.

The form in which tubocurarine is supplied is a tubocurarine hydrochloride pentahydrate with a MW of 771.72, so 10 mg = 10/771.72 mmol = 0.013 mmol or 13 μmol.

13 μmol dissolved in 13 mL gives a 1 mM solution, so 13 μmol in 1.3 mL is a 10 mM solution.

1.5.3 Dilutions

Drug dilutions are made serially, usually with a constant dilution factor. If this factor is 10, then this is a logarithmic dilution (a dilution factor of 1:10 or dilution ratio of 1:9; 1 vol of stock solution + 9 vol of diluent, for a total of 10 parts). If the dilution factor is 2 (a dilution factor of 1:2 or dilution ratio of 1:1 means 1vol. stock solution + 1vol. diluent, for a total of 2 parts). The concentration that is most important, and is cited in reports of experiments, is the final concentration present in an organ bath or enzyme reaction mixture. This is simply the volume of drug added to the organ bath or assay (V\text{added}) as a fraction of the total volume (V\text{total}) multiplied by the original concentration of the drug C\text{stock}.

\[
\text{Final concentration} = \left( \frac{V\text{added}}{V\text{total}} \right) \times C\text{stock}
\]

Particular care must be to ensure that all the units are the same. For example, if 10 μL of a 1 mg/mL solution is added to 20 mL organ bath, the same units must be used throughout. 10 μL is expressed as 0.01 mL. The final concentration in the organ bath is:

\[
\left( \frac{0.01}{20} \right) \times 1 = 5 \times 10^{-4} \text{ mg/mL or 0.5 μg/mL}
\]
Frequently, it is advantageous to work in molar units throughout, starting with the stock solutions. This prevents calculation errors and it is easy to arrive at the final molar concentrations.

A few examples are given to illustrate some typical calculations.

1. 30 μL of 1 mM d-tubocurarine (d-TC) was added to a 25 mL organ bath. What was the organ bath concentration?
   
   **Answer:** Final bath concentration \(= \frac{0.03}{25} \times 1 = 1.2 \text{ μM}.\)

2. What weight of sodium chloride should be weighed out to make 20 L of 0.9% saline? What is the molarity of this solution (MW = 58.44)?
   
   **Answer:** 0.9% saline is 0.9 g NaCl per 100 mL of water = 180 g per 20 L. 0.9% means 9 g/L, so molarity of 0.9% NaCl = \(\frac{9}{58.44} = 0.154\text{ M}.\)

3. How would you make up 10 mL of a 50 μM solution. Assume that the smallest quantity that can be weighed on the balance is 1 mg, and make any additional solutions in a volume greater than 1 mL.
   
   **Answer:** A 50 μM solution contains 50 nmol/mL, so 10 mL contains 500 nmol = 0.5 μmol. MW of ACh is 181.7, so 0.5 μmol is \(181.7 \times 0.5 = 90.85\text{ μg}.\) This quantity is too small to be weighed on the balance, so make 10 mL of 5 mM by weighing 9 mg of ACh and dissolving this in 10 mL. Dilute 0.1 mL of 5 mM ACh in 9.9 mL water to give 10 mL of 50 μM.

4. An anti-hypertensive drug (MW 368) is recommended to be administered i.v. at 5 mg/kg. What volume of a 10 mM solution should be administered to an animal which weighs 132 g?
   
   **Answer:** Weight to be given = \(5 \times 0.132 = 0.66\text{ mg}.\) To convert 10 mM to mg/mL, 10 mM of drug = \(10 \times 368 = 3680\text{ mg/L} = 3.68\text{ mg/mL}.\) So \(0.66/3.68 = 0.18\text{ mL} \) should be administered to the animal.

1.5.4 Logarithms

Logarithmic relationships between variables are common in pharmacology. It is therefore important to have a clear understanding how to manipulate logarithms in calculations. When expressing numbers as logarithms, a base number must be specified. There are two commonly used base numbers, 10 (\(\log_{10}\)) and \(e = 2.7183\) (\(\log_e\), ln or natural logs). \(\log_{10}\)}
are most widely used in pharmacology. The logarithm of a number \(x\) is the base number \(b\) to the power of that number (i.e. \(b^x\)). For example,

\[ \log_b x = b^y \]

and

\[ \log_{10} 100 = 10^2, \text{ so } 2 \text{ is the } \log_{10} \text{ of } 100 \]

The term antilog is more accurately described as the exponent, or the power to which the base must be raised to equal the original number.

\[ \text{anti } \log_{10} y = 10^x \]
\[ \text{anti } \log_{10} 2 = 10^2 = 100, \text{ so } 100 \text{ is the antilog of } 2 \]

Using a calculator, the exponent or antilog is found by using the inverse log function. The order in which numbers and functions are entered varies between different models of calculator, and should be checked for each model.

Logarithms are frequently first encountered in chemistry in the pH scale which is used to express the hydrogen ion concentration. For practical purposes, pH is defined as the negative logarithm to the base 10 of the hydrogen ion concentration \([H^+]\). Thus in a solution of pH 7, the \([H^+]\), or more accurately the hydronium ion \([H_3O^+]\) is \(\text{antilog } 7 = 10^{-7} \text{ M}\). Note that an increase in one unit of pH scale means that the \([H^+]\) decreases 10-fold, because

\[
\text{Change in}[H^+] = [10^{-7}] - [10^{-8}] = 10^{-1} = 0.1
\]

In pharmacology, a logarithmic scale is used to express the potency of a drug, be it agonist or antagonist (see Section 2.1). If an agonist has a potency \(EC_{50} 10^{-6} \text{ M}\), then it is said to have a pD\(_2\) of \(-\log_{10} 10^{-6} = 6\). In order to compare the potency of this drug with another drug with a pD\(_2\) of 8, then it may be thought that this would simply be 6/8 = 0.75, so drug A is 0.75 times less potent than drug B. \textit{This is incorrect!} When the log of a number is divided by the log of another, they are in fact subtracted, and the answer is the log of the difference:

\[
\frac{\text{pD}_2 A}{\text{pD}_2 B} = (-\log 6) - (-\log 8) = \log 2 = 100
\]

So drug A is in fact 100\(\times\) less potent than drug B.
1.6 ESSENTIAL STATISTICS

This section is not intended to give a comprehensive account of statistics as applied to biomedical experiments, since this has been done in books dedicated to the topic. For a more detailed explanation of statistical procedures, recommended books are those by Ennos (2012) and Motulsky (2003). The latter refers to the explanatory manuals (available as books and online) for GraphPad Prism, and is particularly useful since the software was written by bioscientists for bioscientists, and there are regular updates and analytical tips available on their website.

From the outset, it should be clear that there are three very different types of data:

- continuous or quantitative data
- ranked data
- non-continuous or categorical data

1.6.1 Continuous Data – \( t \)-test, ANOVA, Non-parametric Tests and Regression

Continuous (or quantitative) data consist of measurements of variables such as contraction, blood pressure temperature, time or concentration. Since the basic aim of statistics is to be able to extrapolate from sample measurements of a population to the entire population, and then assess the probability that one population (treated or untreated) differs from another. To do this it is necessary to estimate the variability of sample measurements (precision), and the distribution of individual measurements. The precision is described by the variability by descriptive statistics, the mean and standard deviation. It is vital to know whether or not the sample measurements are distributed “normally”, where a bell-shaped Gaussian curve is obtained. It is simple to check the distribution of the sample measurements by simply plotting a frequency curve. It will be apparent how important it is to have a sufficient number of sample measurements. A bare minimum of 10 will start to form a pattern, but really 10 times this is needed to obtain a reliable frequency distribution curve. A table of descriptive statistics will give information about the variation of the samples in the population. This can be carried out by any statistical software package. It will consist of information about the mean and variability and confidence limits. It will convey information as to whether the distribution is symmetrical or skewed on either side.
of the mean. The standard deviation expresses the variation of samples around the mean, and depends how “flat” the bell-shaped curve is. If there is small variation about the mean it will reflect a sharp bell-shaped curve. The standard deviation (SD, S or $\sigma$) is defined as

$$SD = \sqrt{\frac{\sum (x - x_{\text{mean}})^2}{N - 1}}$$

where $x =$ each value, $x_{\text{mean}} =$ mean of values and $N =$ number of values.

If the results are evenly distributed in a Gaussian manner, then 68% of the results fall within SD of the mean, and 95% of the values fall within 2SD of the mean.

The standard error of the mean (SEM)

$$SEM = \frac{SD}{\sqrt{N}}$$

This indicates the precision with which the true population mean has been estimated.

The appropriate statistical test to analyse quantitative data depends on the aim and design of a study. The study may have one of two basic aims.

To calculate the probability that two populations are different from each other, that is to say that they have different means (e.g. treated compared with control). The appropriate test will depend on (a) whether the measurements are “normally” distributed, and (b) the numbers of populations (e.g. treatments). Parametric tests are used to test normally distributed data, otherwise non-parametric tests must be used.

Comparing Two Populations

The method of testing for a difference between treatments is to propose a “null hypothesis”, which states that the two populations have the same mean and distribution (i.e. that they are both from a single population). Statistical tests will calculate probability that this is true or false. If it is true, the null hypothesis is accepted, and it can be said that there is a high probability that there is no difference between the populations. It is only when the null hypothesis is rejected that it can be stated that there is probability that there is a difference. The probability level commonly taken is 95%, which can be expressed as $P = 0.05$ or 1 in 20. This
is accepted as being the minimum level of probability that is regarded as statistically significant. For normally distributed, parametric populations, a Student’s $t$-test can be used. To test the null hypothesis, the value of a $t$-statistic is calculated. This is a measure of how different the means are relative to the variability. In general, the $t$-statistic is calculated as the difference between the two means divided by the standard error of the difference between the means:

$$t = \frac{M_A - M_B}{SEM}$$

The $t$-statistic is tested against a critical value (obtained from tables or embedded in a computer program). The larger the $t$-statistic the greater the distance between the means, and the more likely that the null hypothesis will be rejected. The smaller the value of the $t$-statistic, the more likely the null hypothesis will be accepted.

For parametrically distributed populations, the $t$-statistic is calculated in a slightly different manner depending on the design of the experiment.

1. Comparing the average of a set of samples of a population with an expected value, such as that of a larger population.
2. Comparing two sets of paired values, such as before and after treatment measurements. Samples are said to be paired if they are the same animals, subjects or cells on which the control and treatment measurements are taken.
3. Comparing two sets of unpaired groups. The sets are unpaired if the control and treated sets are different animals, subjects or cells.

If the populations are not distributed in a Gaussian manner, a parametric test should be used. These tests compare the ranks of each sample, irrespective of the treatment group to which they belong. Examples are the Mann–Whitney test and the Wilcoxon signed-rank test.

**Example of a Student’s $t$-test**

According to a report, the angiotensin receptor antagonist, losartan, inhibited platelet aggregation. This was curious because angiotensin receptors had not been reported on platelets, but if this was true it would document an additional beneficial effect of losartan, which is
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Table 1.1  Inhibition of collagen-stimulated platelet aggregation (measured as decrease in optical density) by losartan.

<table>
<thead>
<tr>
<th>Control</th>
<th>Losartan</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.155</td>
<td>0.120</td>
</tr>
<tr>
<td>0.100</td>
<td>0.130</td>
</tr>
<tr>
<td>0.144</td>
<td>0.129</td>
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<tr>
<td>0.103</td>
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<tr>
<td>0.136</td>
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<tr>
<td>0.124</td>
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<td>0.141</td>
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<tr>
<td>0.125</td>
<td>0.137</td>
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</table>

widely prescribed for hypertension and heart failure. A single concentration of 10 mM was used in the study. To investigate any inhibitory effect on a platelet agonist, a sub-maximal collagen concentration of 7.81 μg/mL was selected. A platelet aggregation experiment was carried out in which there were three control measurements, followed by three measurements in the presence of 10 mm losartan. A t-test was performed to statistically assess any differences between the treatments (to calculate the chances that the null hypothesis was true or false). The results obtained are shown in Table 1.1, and plotted as a bar graph in Figure 1.1.

An unpaired, two-tailed t-test was carried out using GraphPad Prism v.4. The options selected were unpaired since each sample was a separate sample of the platelet suspension. If each sample was tested before and after losartan, a paired test would have been selected. A two-tailed test was appropriate since there was no reason to think that the variance would extend in one direction only. The following results were obtained (see Table 1.2).

Software packages designed to perform statistical test produce a large array of data that must be interpreted. Prism is more user-friendly than many programs, such as Microsoft Excel, since it has many aids to interpretation. There are three sections to the results table – results of the unpaired t-test, a list of the differences and an F-test to test whether the variances are equal for both groups. The results of the t-test indicate that \( t = 1.936 \), and the degrees of freedom (d.f.) = 20 – 2 = 18. From this, a
Figure 1.1 Inhibition of collagen-stimulated platelet aggregation by losartan. The large bars show the means of the two samples and the error bars indicate the SEM, \( n = 10 \). A \( t \)-test is carried out to test whether there is a statistical difference between the two samples.

Table 1.2 Results of the \( t \)-test of the data Table 1.1 as produced by GraphPad Prism v.4.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column A vs. Column B</td>
<td>Control vs. losartan</td>
</tr>
<tr>
<td>Unpaired ( t )-test</td>
<td></td>
</tr>
<tr>
<td>( P ) value</td>
<td>0.0688</td>
</tr>
<tr>
<td>( P ) value summary</td>
<td>Not significant</td>
</tr>
<tr>
<td>Are means significantly different? (( P &lt; 0.05 ))</td>
<td>No</td>
</tr>
<tr>
<td>One- or two-tailed ( P ) value?</td>
<td>Two-tailed</td>
</tr>
<tr>
<td>( t ), d.f.</td>
<td>( t = 1.936 ), d.f. = 18</td>
</tr>
<tr>
<td>How big is the difference?</td>
<td></td>
</tr>
<tr>
<td>Mean ± SEM of column A</td>
<td>0.1236 ± 0.005916, ( N = 10 )</td>
</tr>
<tr>
<td>Mean ± SEM of column B</td>
<td>0.1365 ± 0.003067, ( N = 10 )</td>
</tr>
<tr>
<td>Difference between means</td>
<td>−0.0129 ± 0.006664</td>
</tr>
<tr>
<td>95% confidence interval</td>
<td>−0.02690–0.001101</td>
</tr>
<tr>
<td>R squared</td>
<td>0.1723</td>
</tr>
<tr>
<td>( F )-test to compare variances</td>
<td></td>
</tr>
<tr>
<td>( F ), DFn, Dfd</td>
<td>3.722, 9, 9</td>
</tr>
<tr>
<td>( P ) value</td>
<td>0.0634</td>
</tr>
<tr>
<td>( P ) value summary</td>
<td>Not significant</td>
</tr>
<tr>
<td>Are variances significantly different?</td>
<td>No</td>
</tr>
</tbody>
</table>
$P$ value of 0.0688, which is greater than 0.05 so there is more than a 95% (1 in 20) chance that the groups are the same, thus the null hypothesis is accepted. The relatively large $F$ value suggests that the variances in the two groups are not different.

**Analysis of Variance**

An analysis of variance (ANOVA) compares measurements or multiple-dependent continuous variables (such as weight, enzyme activity, dose or time). For comparing two dependent variables with an independent variable, a one-way ANOVA is used. For comparing more than three variables, a two-way ANOVA is used. To illustrate the application of these tests, it may be desired to expand on the investigation as to whether a losartan inhibited platelet aggregation. In the simple experiment where a single concentration of losartan was compared with a control group without losartan, the results of a Student’s $t$-test showed that there was no difference between the two groups. This was thought to inclusive since only one concentration of both agonist and losartan were used. If it was wished to expand the experiment by including two concentrations of losartan, three variables would have to be compared, control and the two losartan concentrations. The results of this experiment would best be analysed using a one-way ANOVA. It may be thought that a more complex test could be avoided by applying multiple $t$-tests, but it should be appreciated that this is not the same thing as using a one-way ANOVA. However, it was thought that since the losartan concentration selected was maximal, it may be more informative to examine the effect of one losartan concentration over the range of agonist concentrations that cover its concentration–response curve. Here the control and losartan groups are tested at seven concentrations of agonist (collagen). This now requires the use of a two-way ANOVA, followed by a Bonferroni post-test. Table 1.3 shows the results of such an experiment.

**Regression**

Regression analysis is used to evaluate how closely two variables are related to each other. Linear regression is used to test whether two variables are linearly correlated. Here the probability that one variable is directly proportional to the variation of another variable. Often two variables are related by a more complex relationship than a straight line. In this case, the relationship between two variables can be tested
Table 1.3 Results from an experiment where the aggregation of platelets was tested at seven different concentrations of the agonist, collagen, both in the presence and absence of losartan. Each measurement was performed in triplicate.

<table>
<thead>
<tr>
<th>Collagen (µg/mL)</th>
<th>Aggregation (ΔE/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>0.00</td>
<td>0.077</td>
</tr>
<tr>
<td>1.95</td>
<td>0.610</td>
</tr>
<tr>
<td>3.90</td>
<td>0.092</td>
</tr>
<tr>
<td>7.81</td>
<td>0.155</td>
</tr>
<tr>
<td>15.60</td>
<td>0.366</td>
</tr>
<tr>
<td>31.25</td>
<td>0.458</td>
</tr>
<tr>
<td>62.50</td>
<td>0.480</td>
</tr>
<tr>
<td>125.0</td>
<td>0.464</td>
</tr>
</tbody>
</table>

how well the data fit a particular model. This is non-linear correlation. The most frequently encountered example of this in pharmacology is the log concentration (or dose)–response relationship which produces a sigmoid curve. A frequent misinterpretation of a correlation between two variables is that this proves causation of one event by another. In pharmacology, an increase in concentration of a drug may cause an increase in response, but an increase in response does not cause an increase in drug concentration. In this case, the response would be termed a dependent variable, and dose an independent variable; the response depends on the drug concentration, but not vice versa. Graphically, the independent variable is plotted on the horizontal axis (x-axis) and the dependent variable plotted on the vertical (y-axis). For example dose (independent) against response (dependent).

In a linear regression a straight line can be fitted to the graph by minimizing the variability of each point from a line. A straight line is described by the equation

\[ y = mx + c \]

where \( m \) = the slope (\( \Delta y/\Delta x \)) and \( c \) is the intercept of line with \( y \) when \( x = 0 \).

When deciding how to plot data to which you wish to perform a linear regression, it is important to define which are the independent (\( x \)) and dependent (\( y \)) variables. A regression of \( x \) on \( y \) will not give the same result as \( y \) on \( x \).

The more closely the two variables are related to each other is described by the Pearson coefficient (\( R \)). A coefficient of 1 indicates that
the variances of the individual observations from the line are extremely small, and as this value decreases, the larger are the variances from the line. This is sometimes described as the “goodness of fit”. A major use of a linear correlation graph is in predicting the magnitude of one parameter from another. A very common application is the standard curve for a spectrophotometric assay. This allows the prediction of the concentration of a substance from its absorbance by using the formula

$$y = mx + c$$

where $y = \text{absorbance of unknown sample}$ and $x = \text{concentration of unknown sample}$. It is important to realize that this prediction is only valid within the absorbance range of the standards that provides a linear correlation between absorbance and concentration (see Figure 1.2).

**Non-linear Correlation** For many data, the dependent variable is not linearly related to the dependent variable. Here a suitable model must be selected, and test how well the data fits this model. This may be, for example, a hyperbola (as ideally found in many binding experiments) or

![Graph](image.png)

**Figure 1.2** Linear regression analysis calculates the probability that the dependent variable ($y$) is linearly related to the independent variable ($x$). The “goodness of fit” is expressed by how near the Pearson coefficient ($R$) is to the value of one.
an exponential growth curve. GraphPad prism actually gives the option of selecting one of a large number of models. Details of the equations for the different non-linear models, and further explanation, are given by Motulsky and Christopoulos (2003). In pharmacology, a typical model is the log concentration (or dose)–response relationship, and binding data as used in ELISA assays This is a sigmoid curve when the log of the independent variable (concentration) is plotted against the response (see Figure 1.3).

It is important to evaluate how well the curve fits the data. Help with doing this is given by 95% confidence interval (CI) and an R-squared value. For a good fit, narrow CI values and a high (close to 1) R² value is expected. Some common sense is also required in assessing the fit of the curve. Frequently, there is insufficient data to fit the curve to reasonable parameters. If the minimum value is negative or the maximum value is astronomical, this is clearly absurd, and any EC₅₀ value deduced from

![Graph of sigmoid curve](image)

**Figure 1.3** Non-linear correlation is useful in pharmacology for calculating the probability that the log concentration and response are related by a sigmoid curve. This provides the “best-fit” line from which the EC₅₀, maximum and minimum response values and Hill slope can be obtained. The points were fitted to the equation for a sigmoid dose–response curve, variable slope, using GraphPad Prism v.4. (GraphPad Software, Inc, San Diego, CA, USA.)
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Table 1.4  Results of a Bonferroni post-test, following a two-way ANOVA.

<table>
<thead>
<tr>
<th>Collagen (mg/mL)</th>
<th>Difference</th>
<th>t</th>
<th>P value</th>
<th>Summary</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0000</td>
<td>−0.0190</td>
<td>0.6906</td>
<td>P &gt; 0.05</td>
<td>Not significant</td>
</tr>
<tr>
<td>1.950</td>
<td>0.0130</td>
<td>0.4725</td>
<td>P &gt; 0.05</td>
<td>Not significant</td>
</tr>
<tr>
<td>3.900</td>
<td>−0.05033</td>
<td>1.829</td>
<td>P &gt; 0.05</td>
<td>Not significant</td>
</tr>
<tr>
<td>7.810</td>
<td>0.0006667</td>
<td>0.02423</td>
<td>P &gt; 0.05</td>
<td>Not significant</td>
</tr>
<tr>
<td>15.60</td>
<td>−0.1350</td>
<td>4.907</td>
<td>P &lt; 0.001</td>
<td>***</td>
</tr>
<tr>
<td>31.25</td>
<td>−0.1273</td>
<td>4.628</td>
<td>P &lt; 0.001</td>
<td>***</td>
</tr>
<tr>
<td>62.50</td>
<td>−0.1487</td>
<td>5.404</td>
<td>P &lt; 0.001</td>
<td>***</td>
</tr>
<tr>
<td>125.0</td>
<td>−0.1933</td>
<td>7.027</td>
<td>P &lt; 0.001</td>
<td>***</td>
</tr>
</tbody>
</table>

the curve is invalid. Using GraphPad Prism, it is possible to constrain the values for maximum and minimum to fixed values, but usually it means that more data must be obtained.

Comparing concentration–response curves is problematic, as discussed by Motulsky and Christopoulos (2003). An easy approach to this is to perform a two-way ANOVA, but this can only be done with caution. A problem is that in performing ANOVA, concentration is considered as any other non-continuous treatment, like a series of drugs. When dealing with a continuous variable, like concentration or time, consideration should be given as to whether there is a trend. This can be applied to the experiment already discussed above to illustrate the use of a two-way ANOVA (Figure 1.3). Here two concentration–response curves can be drawn, one for the control curve for collagen alone, and the other for the curve in the presence of losartan. It is seen that the curve for losartan-treated platelets clearly has a lower maximum value. Applying a two-way ANOVA (Table 1.4) confirms that the points near the maximum value have a low P value (P < 0.001), so it seems reasonable to accept this result. This is illustrated in Figure 1.4.

1.6.2 Discontinuous Data – $\chi^2$ and Fisher’s Exact Test

These are non-continuous measurements such as the numbers of cells, tissues or animals that possess an attribute like alive or dead. In terms of digital data terminology, it is the effect of a treatment on producing one of the two states. Here contingency tables are constructed in order to carry out a Chi-squared ($\chi^2$) test or Fisher’s exact test.
Figure 1.4 Comparison of the log concentration–response curves for control and losartan treatments, and results of a two-way ANOVA. $P = 0.001$ is indicated by $\ast\ast\ast$, each point shows the mean of triplicate observations, and the error bars indicate the SEM.

These tests are used to test whether a treatment significantly affected the outcome of an event in two similar groups but which were exposed to two different treatments, for example, whether treatment with a drug influenced the occurrence of a disease. They are used for analysing the difference between frequency distributions. These tests are used to analyse the so-called non-continuous data. The analysis may be by a $\chi^2$ test or Fisher’s exact comparison test. The $\chi^2$ test is inaccurate for small numbers of observations, but is easier to calculate by hand. The Fisher’s exact test is more accurate but involves more complex calculations.

Experiments to be analysed by $\chi^2$ test or Fisher’s exact test yield an outcome that is a categorical variable such as alive/dead or disease/no disease. To perform these tests, they must be arranged in a contingency table (see Table 1.5). This may be applied to a wide range of experimental designs.

Table 1.5 Example of a contingency table.

<table>
<thead>
<tr>
<th></th>
<th>Response</th>
<th>No response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
There are five types of experimental designs that can be analysed using a contingency table.

- **Prospective study.** In this design, two closely matching groups are selected. One group is exposed to a treatment at a high dose, and the other to a lower dose. At the end of the study, the numbers in each group showing a response, such as developing cancer are noted. The results are then displayed in a contingency table before applying the appropriate statistical test.

- **Cross-sectional study.** Here a single, random group of subjects is selected. They are then sorted into two groups according to whether they have been subjected to a treatment. The numbers in each group showing a response is assessed, and the results displayed in a contingency table.

- **Retrospective case control.** This design is similar to a cross-sectional study, but may be regarded as superior since the numbers in the two groups are under more control. Two groups are selected according to whether they have been subject to a treatment, and it is then assessed whether they show a response.

- **Experiment manipulating variables.** This design resembles the first design, the prospective study. Two groups of closely matched subjects are selected, but in this case, one group receives a treatment and the other acts as a control. At the end of the study, the numbers in each group showing a response are assessed.

- **Accuracy of a diagnostic test.** Here two groups are selected according to whether they have been diagnosed with a characteristic or disease, such as AIDS for example. All subjects then undergo a diagnostic test for the disease and classified as to whether they register positive or negative in the test. The results can then be displayed in a contingency table, showing the numbers with and without the disease against whether they registered positive or negative in the test.

*The $\chi^2$ Test*  Here it is determined whether the observed frequencies of an event differ significantly from the expected frequencies. It should be remembered that this test is inaccurate for small numbers in each group (say <10).

$$\chi^2 = \sum \frac{(\text{observed} - \text{expected})^2}{\text{expected}}$$
Table 1.6 Contingency table for vitamin C data.

<table>
<thead>
<tr>
<th></th>
<th>Survived</th>
<th>Died</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15</td>
<td>35</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>31</td>
<td>17</td>
</tr>
</tbody>
</table>

The $\chi^2$ statistic is compared with a table of $\chi^2$ values for degrees of freedom (which is $n - 1$) against $P$ values. Usually the value for $P = 0.05$ is taken. If the calculated $\chi^2$ value is greater than the tabulated value, then the null hypothesis is disproved and treatment does have a protective effect at a certain probability level – if $P < 0.05$ for the $\chi^2$ statistic at the relevant degrees of freedom, then there is a 95% probability (or chance) that the treatment produces a response.

This is best explained by an example. In a classic experiment, it was tested whether vitamin C protected guinea pigs from the effects of rabies virus infection (Banic, 1975). A random sample of guinea pigs was taken and all were infected with the same dose of rabies virus. Some were given vitamin C and others were not. The numbers of animals surviving and dying were recorded in the two groups. The results were displayed in a 2 x 2 contingency table (see Table 1.6).

Table 1.7 shows the calculation of the $\chi^2$ value. The assumption is made that vitamin C has no effect (null hypothesis). The expected odds of dying for the two groups is calculated. Of a total of 98 animals, a total of 52 died. Therefore, the chances of dying is $(52/98) = 0.5306$ or
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53%. For the random sample of 48 animals in the vitamin C group, we would expect $48 \times 0.5301 = 25.469$.

Fisher’s Exact Test This is another test that can be used to statistically test the data in a contingency table, and must be used if there are small numbers in each group (<10 per cell in the table) to provide an accurate estimate of the $P$ value. A disadvantage may be that it requires the use of a computer program since the calculation of the $P$ value is more complex than in $\chi^2$ test.

REFERENCES
