1 Control of the quality of analytical methods

Box 1.1 Questions pharmaceutical analysis methods are used to answer

- Is the identity of the drug in the formulated product correct?
- What is the percentage of the stated content of a drug present in a formulation?
- Does this formulation contain solely the active ingredient or are additional impurities present?
- What is the stability of a drug in the formulation and hence the shelf-life of the product?
- At what rate is the drug released from its formulation so that it can be absorbed by the body?
- Do the identity and purity of a pure drug substance to be used in the preparation of a formulation meet specification?
- Do the identity and purity of excipients to be used in the preparation of a formulation meet specification?
- What are the concentrations of specified impurities in the pure drug substance?
- What is the concentration of the drug in a sample of tissue or biological fluid?
- What are the p*K*a value(s), partition coefficients, solubilities and stability of a drug substance under development?

Introduction

Pharmaceutical analysis procedures may be used to answer any of the questions outlined in Box 1.1 above. The quality of a product may deviate from the standard

required but in carrying out an analysis one also has to be certain that the quality of the analysis itself is of the standard required. Quality control is integral to all modern industrial processes and the pharmaceutical industry is no exception. Testing a pharmaceutical product involves chemical, physical and sometimes microbiological analyses. It has been estimated that £10 billion is spent each year on analyses in the UK alone and such analytical processes can be found in industries as diverse as those producing food, beverages, cosmetics, detergents, metals, paints, water, agrochemicals, biotechnological products and pharmaceuticals. With such large amounts of money being spent on analytical quality control, great importance must be placed on providing accurate and precise analyses. Thus it is appropriate to begin a book on the topic of pharmaceutical analysis by considering, at a basic level, the criteria which are used to judge the quality of an analysis. The terms used in defining analytical quality form a rather elegant vocabulary that can be used to describe quality in many fields, and in writing this book the author would hope to describe each topic under consideration with accuracy, precision and, most importantly, reproducibility, so that the information included in it can be readily assimilated and reproduced where required by the reader. The following sections provide an introduction to the control of analytical quality. More detailed treatment of the topic is given in the reference cited at the end of the chapter.[1]

Box 1.2 ICH guidelines

The requirements for control of the quality of methods of analysis (validation) have been addressed by the International Conference on Harmonisation of Technical Requirements For Registration of Pharmaceuticals for Human Use, or, more briefly, the ICH (www.ich.org). The ICH was initiated in Brussels in 1990 and brought together representatives of regulatory agencies and industry associations of Europe, Japan and the USA. The purpose of the organisation was to standardise the requirements for medicines regulation throughout the world. The standardisation of the validation of analytical procedures is one area that the ICH has addressed. The ICH indicated that the most important analytical procedures that require validation are:

- Identification tests
- Quantitative tests for impurities
- Limit tests for the control of impurities
- Quantitative tests of the active moiety in samples of drug substance or drug product or other selected component(s) in the drug product.

Control of errors in analysis

A quantitative analysis is not a great deal of use unless there is some estimation of how prone to error the analytical procedure is. Simply accepting the analytical result could lead to rejection or acceptance of a product on the basis of a faulty analysis. For this reason it is usual to make several repeat measurements of the same sample in order to determine the degree of agreement between them. There are three types of errors which may occur in the course of an analysis: gross, systematic and random. Gross errors are easily recognised since they involve a major breakdown in the analytical process such as samples being spilt, wrong dilutions being prepared or instruments breaking down or being used in the wrong way. If a gross error occurs the results are rejected and the analysis is repeated from the beginning. Random and systematic errors can be distinguished in the following example:

A batch of paracetamol tablets are stated to contain 500 mg of paracetamol per tablet; for the purpose of this example it is presumed that 100% of the stated content is the correct answer. Four students carry out a spectrophotometric analysis of an extract from the tablets and obtain the following percentages of stated content for the repeat analysis of paracetamol in the tablets:

Student 1: 99.5%, 99.9%, 100.2%, 99.4%, 100.5%
Student 2: 95.6%, 96.1%, 95.2%, 95.1%, 96.1%
Student 3: 93.5%, 98.3%, 92.5%, 102.5%, 97.6%
Student 4: 94.4%, 100.2%, 104.5%, 97.4%, 102.1%

The means of these results can be simply calculated according to the formula:

$$\bar{x} = \sum_i \frac{x_i}{n} \qquad \textbf{[Equation 1]}$$

Where $\bar{x}$ is the arithmetic mean, x_i is the individual value and n is the number of measurements.

These results can be seen diagrammatically in Figure 1.1.
Student 1 has obtained a set of results which are all clustered close to 100% of the stated content and with a mean for the five measurements very close to the correct answer. In this case the measurements made were both precise and accurate and obviously the steps in the assay have been controlled very carefully.
Student 2 has obtained a set of results which are closely clustered but give a mean which is less than the correct answer. Thus although this assay is precise it is not completely accurate. Such a set of results indicates that the analyst has not produced random errors, which would produce a large scatter in the results, but has produced an analysis containing a systematic error. Such errors might include repeated inaccuracy in the measurement of a volume or failure to zero the spectrophotometer correctly prior to taking the set of readings. The analysis has been mainly well controlled except for probably one step, which has caused the inaccuracy and thus the assay is precisely inaccurate.

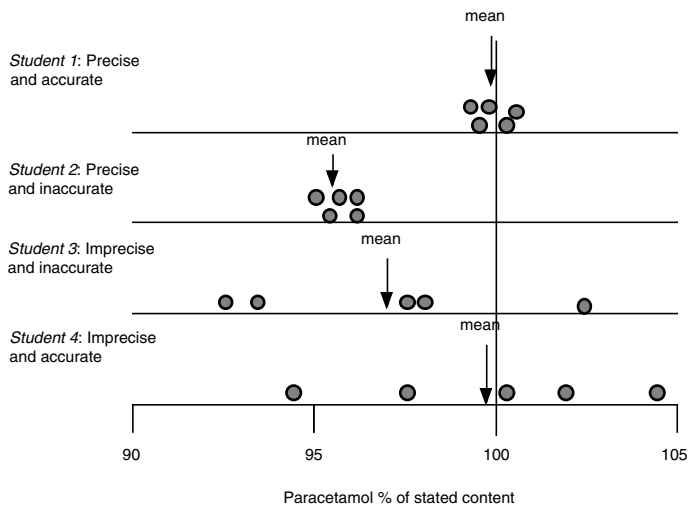

Fig. 1.1 Diagrammatic representation of accuracy and precision for analysis of paracetamol in tablet form.

Student 3 has obtained a set of results which are widely scattered and hence imprecise, and which give a mean which is less than the correct answer. Thus the analysis contains random errors or possibly, looking at the spread of the results, three defined errors which have been produced randomly. The analysis was thus poorly controlled and it would require more work than that required in the case of student 2 to eliminate the errors. In such a simple analysis the random results might simply be produced by, for instance, a poor pipetting technique, where volumes both higher and lower than that required were measured.
Student 4 has obtained a set of results which are widely scattered yet a mean which is close to the correct answer. It is probably only chance that separates the results of student 4 from those of student 3 and although the answer obtained is accurate, it would not be wise to trust it to always be so.

The best assay was carried out by student 1 and student 2 produced an assay which might be improved with a little work.

In practice it might be rather difficult to tell whether student 1 or student 2 had carried out the best analysis since it is rare, unless the sample is a pure analytical standard, that the exact content of a sample is known. In order to determine whether student 1 or 2 had carried out the best assay it might be necessary to get other analysts to obtain similar sets of precise results in order to be absolutely sure of the correct answer. The factors leading to imprecision and inaccuracy in assay results are outlined in Box 1.3.

Box 1.3 Some factors giving rise to imprecision and inaccuracy in an assay

- Incorrect weighing and transfer of analytes and standards
- Inefficient extraction of the analyte from a matrix, e.g. tablets
- Incorrect use of pipettes, burettes or volumetric flasks for volume measurement
- Measurement carried out using improperly calibrated instrumentation
- Failure to use an analytical blank
- Selection of assay conditions that cause degradation of the analyte
- Failure to allow for or to remove interference by excipients in the measurement of an analyte

Self-test 1.1

Suggest how the following might give rise to errors in an analytical procedure:

(i) Analysis of a sucrose-based elixir using a pipette to measure aliquots of the elixir for analysis.
(ii) Weighing out 2 mg of an analytical standard on a four-place analytical balance which weighs a minimum of 0.1 mg.
(iii) Use of an analytical standard that absorbs moisture from the atmosphere.
(iv) Incomplete powdering of coated tablets prior to extraction.
(v) Extraction of an ointment with a solvent in which it is poorly soluble.
(vi) Use of a burette that has not been rinsed free of traces of grease.

Answers: (i) Viscosity leads to incomplete drainage of the pipette; (ii) In any weighing there is an uncertainty of ± 0.05 mg, which in relation to 2 mg is ± 2.5%; (iii) The degree of moisture absorption is uncertain; (iv) Poor recovery of the analyte; (v) Poor recovery of the analyte; (vi) Distortion of meniscus making reading of the burette inaccurate.

Accuracy and precision

The most fundamental requirements of an analysis are that it should be accurate and precise. It is presumed, although it cannot be proven, that a series of measurements (y) of the same sample will be normally distributed about a mean (μ), i.e. they fall into a Gaussian pattern as shown in Figure 1.2.

The distance σ shown in Figure 1.2 appears to be nearly 0.5 of the width of distribution; however, because the function of the curve is exponential it tends to zero and does not actually meet the x axis until infinity, where there is an infinitesimal probability that there may be a value for x. For practical purposes approximately 68% of a series of measurements should fall within the distance σ either side of the mean and 95% of the measurements should lie with 2σ of the mean. The aim in an analysis is to make σ as small a percentage of the value of μ as possible. The value of σ can be estimated using the Equation 2:

$$s = \sqrt{\frac{\sum (x_i - \bar{x})^2}{(n-1)}} \qquad \textbf{[Equation 2]}$$

s = standard deviation
n = number of samples
x_i = values obtained for each measurement
$\bar{x}$ = mean of the measurements

Sometimes n rather than $n - 1$ is used in the equation but, particularly for small samples, it tends to produce an underestimate of σ. For a small number of values it is simple to work out s using a calculator and the above equation. Most calculators have a function which enables calculation of s directly and, on calculators, σ estimated using the above equation is usually labelled as σ_{n-1}. For instance, if the example of results obtained by student 1, where the mean is calculated to be 99.9%, are substituted into equation 2, the following calculation results:

$$s = \sqrt{\frac{(99.5-99.9)^2 + (99.9-99.9)^2 + (100.2-99.9)^2 + (99.4-99.9)^2 + (100.5-99.9)^2}{(5-1)}}$$

$$= \sqrt{\frac{(-0.4)^2 + (0)^2 + (0.3)^2 + (-0.5)^2 + (0.6)^2}{4}}$$

$$= \sqrt{\frac{0.16 + 0 + 0.09 + 0.25 + 0.36}{4}} = \sqrt{\frac{0.86}{4}} = \sqrt{0.215} = 0.46$$

s = 0.46% of stated content

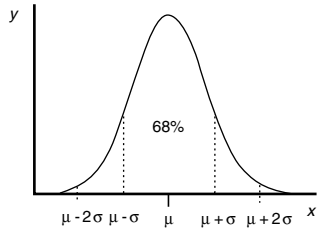

Fig. 1.2
The Gaussian distribution.

The calculated value for s provides a formal expression of the scatter in the results from the analysis rather than the visual judgement used in Figure 1.1. From the figure obtained for the standard deviation (SD), we can say that 68% of the results of the analysis will lie within the range 99.9 ± 0.46% (± σ) or within the range 99.44–100.36%. If we re-examine the figures obtained by student 1, it can be seen that 60% of the results fall within this range, with two outside the range, including one only very slightly below the range. The range based on ± σ defines the 68% confidence limits; for 95% confidence ± 2σ must be used, i.e. 95% of the results of student 1 lie within 99.9 ± 0.92% or 98.98–100.82%. It can be seen that this range includes all the results obtained by student 1.

The precision of an analysis is often expressed as the ± relative standard deviation (± RSD) (Equation 3).

$$\text{RSD} = \frac{s}{\bar{x}} \times 100\ \% \qquad \textbf{[Equation 3]}$$

The confidence limits in this case are often not quoted but, since it is the SD that is an estimate of σ which is being used, they are usually 68%. The advantage of expressing precision in this way is that it eliminates any units and expresses the precision as a percentage of the mean. The results obtained from the assay of paracetamol tablets are shown in Table 1.1.

Table 1.1 Results obtained for the analysis of paracetamol tablets by four analysts

Student	Mean (% of stated content)	S (% of stated content)	± RSD (68% confidence)
1	99.9	0.5	± 0.5%
2	95.6	0.5	± 0.5%
3	96.9	4.0	± 4.4%
4	99.7	4.0	± 4.0%

Self-test 1.2

Four analysts obtain the following data for a spectrophotometric analysis of an injection containing the local anaesthetic bupivacaine. The stated content of the injection is 0.25% weight in volume (w/v).
Analyst 1: 0.245% w/v, 0.234% w/v, 0.263% w/v, 0.261% w/v, 0.233% w/v.
Analyst 2: 0.236% w/v, 0.268% w/v, 0.247% w/v, 0.275% w/v, 0.285% w/v.
Analyst 3: 0.248% w/v, 0.247% w/v, 0.248% w/v, 0.249% w/v, 0.253% w/v.
Analyst 4: 0.230% w/v, 0.233% w/v, 0.227% w/v, 0.230% w/v, 0.229% w/v.
Calculate the mean percentage of stated content and RSD for each set of results at the 68% confidence level. Assuming the content really is as stated on the label, comment on the accuracy and precision of each set of results. Calculate the precision of each assay with regard to 95% confidence limits.

Answers: *Analyst 1*: 98.9% ± 5.8%: accurate but imprecise. At 95% confidence RSD = ± 11.6%; *Analyst 2*: 104.9 ± 7.7%: inaccurate and imprecise. At 95% confidence RSD = ±15.4%; *Analyst 3*: 99.6% ± 0.9%: accurate and precise. At 95% confidence RSD = ± 1.8%; *Analyst 4*: 91.9% ± 0.9%: inaccurate and precise. At 95% confidence RSD = ±1.8.

Validation of analytical procedures

The ICH has adopted the following terms as defining how the quality of an assay is controlled.

The analytical procedure

The analytical procedure provides an exact description of how the analysis is carried out. It should describe in detail the steps necessary to perform each analytical test. The full method should describe:

(i) the quality and source of the reference standard for the compound being analysed;
(ii) the procedures used for preparing solutions of the reference standard;
(iii) the quality of any reagents or solvents used in the assay and their method of preparation;
(iv) the procedures and settings used for the operation of any equipment required in the assay; and
(v) the methodology used for calibration of the assay and methodology used for the processing of the sample prior to analysis.

In fact it is difficult to be comprehensive in this short account, since the description of a fully validated method is a lengthy document.

Precision

The ICH guidelines define precision as follows:

> *"the precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions… The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements."*

This is broadly what was described in more detail above for the assay of paracetamol tablets. There is no absolute guideline for how good precision should be for the active ingredient in a formulation but, in general, a precision of $<\pm$ 1.0% is desirable. The precision achievable depends on the nature of the sample being analysed. The RSDs achievable in the analysis of trace impurities in a bulk drug or drugs in biological fluids may be considerably greater than ± 1.0% because of the increased likelihood of losses when very low concentrations of analyte are being extracted and analysed. The precision of the assay of a particular sample, in the first instance, is generally obtained by repeating the assay procedure a minimum of five times starting from five separate aliquots of sample (e.g. five weights of tablet powder or five volumes of elixir) giving a total of 25 measurements. Repetition of the sample extraction gives a measure of any variation in recovery during extraction from the formulation matrix.

One difficulty in defining the precision of an assay is in indicating which steps in the assay should be examined. Initially an assay will be characterised in detail but thereafter, in re-determining precision (e.g. in order to establish repeatability and intermediate precision), certain elements in the assay may be taken for granted. For example, the same standard calibration solution may be used for several days provided its stability to storage has been established or a limited number samples will be extracted for assay provided it has been established that the recovery of the sample upon extraction does not vary greatly. According to the ICH guidelines, precision may be considered at three levels: repeatability, intermediate precision and reproducibility.

Repeatability

Repeatability expresses the precision obtained under the same operating conditions over a short interval of time. Repeatability can also be termed intra-assay precision. It is likely that the assay would be repeated by the same person using a single instrument.

Intermediate precision

Intermediate precision expresses within-laboratory variation of precision when the analysis is carried out by different analysts, on different days and with different equipment. Obviously a laboratory will want to cut down the possibility for such variations being large and thus it will standardise on particular items of equipment, particular methods of data handling and make sure that all their analysts are trained to the same standard.

Reproducibility

Reproducibility expresses the precision between laboratories. Such a trial would be carried out when a method was being transferred from one part of a company to another. The data obtained during such method transfer does not usually form part of the marketing dossier submitted in order to obtain a product licence.

Accuracy

As described above, methods may be precise without being accurate.
The determination of accuracy in the assay of an unformulated drug substance is relatively straightforward. The simplest method is to compare the substance being analysed with a reference standard analysed by the same procedure. The reference standard is a highly characterised form of the drug which has been subjected to extensive analysis including a test for elemental composition. The methods for determining the accuracy of an assay of a formulated drug are less straightforward. The analytical procedure may be applied to: a drug formulation prepared on a small scale so that the amount of drug in the formulation is more precisely controlled than in a bulk process; a placebo formulation spiked with a known amount of drug or the formulated drug spiked with a known amount of drug. The accuracy of the method may also be assessed by comparison of the method with a previously established reference method such as a pharmacopoeial method.

Accuracy should be reported as percent recovery in relation to the known amount of analyte added to the sample or as the difference between the known amount and the amount determined by analysis. In general, at least five determinations, at 80, 100 and 120% of the label claim for drug in the formulated product, should be carried out in order to determine accuracy.

Standard operating procedure (SOP) for the assay of paracetamol tablets

The terms defined above are perhaps illustrated by using the example of the simple assay which we have mentioned before. The assay in Box 1.4 is laid out in the style of a SOP. This particular section of the operating procedure describes the assay itself but there would also be other sections in the procedure dealing with safety issues, the preparation and storage of the solutions used for extraction and dilution, the glassware required and a specification of the instrumentation to be used.

Box 1.4 Extract from a standard operating procedure for the analysis of paracetamol tablets

8. *Assay procedure*
8.1 Use a calibrated balance
8.2 Weigh 20 tablets
8.3 Powder the 20 paracetamol tablets and weigh accurately *by difference* a quantity of tablet powder equivalent to 125 ± 10 mg of paracetamol
8.4 Shake the tablet powder sample with *ca* 150 ml of acetic acid (0.05 M) for 10 min in a 500 ml volumetric flask and then adjust the volume to 500 ml with more acetic acid (0.05 M).
8.5 Filter *ca* 100 ml of the solution into a conical flask and then transfer five separate 5 ml aliquots of the filtrate to 100 ml volumetric flasks and adjust the volumes to 100 ml with acetic acid (0.05 M)
8.6 Take two readings of each dilution using a UV spectrophotometer and using the procedure specified in **Section 9**

The assay described in Box 1.4 assesses the precision of some of the operations within the assay. If a single analyst was to assess the *repeatability* of the assay, instructions might be issued to the effect that the assay as described was to be repeated five times in sequence, i.e. completing one assay before commencing another. If *between-day repeatability* were to be assessed, the process used for determining the repeatability would be repeated on two separate days. If the *within-laboratory reproducibility* were to be assessed two or more analysts would be assigned to carry out the *repeatability* procedure. In arriving at an SOP such as the one described in Box 1.4, there should be some justification in leaving out certain steps in the complete assay. For instance, weighing is often the most precise step in the process and thus repeat weighings of samples of tablet powder would not be necessary to guarantee precision; the precision of the extraction might be more open to question.

Each of the sections within an assay would have other SOPs associated with them, governing, for instance, the correct use and care of balances, as listed in Box 1.5.

Compound random errors

Systematic errors in analysis can usually be eliminated but true random errors are due to operations in an assay which are not completely controlled. A common type of random error arises from the acceptance of manufacturers' tolerances for glassware. Table 1.2 gives the RSD values specified for certain items of grades A and B glassware.

Table 1.2 Manufacturers' tolerances on some items of glassware

Item of glassware	Grade A	Grade B
1 ml bulb pipette	± 0.7%	± 1.5%
5 ml bulb pipette	± 0.3%	± 0.6%
100 ml volumetric flask	± 0.08%	± 0.15%
500 ml volumetric flask	± 0.05%	± 0.1%
full 25 ml burette	± 0.2%	± 0.4%

Box 1.5 Procedure for the use of a calibrated balance SOP/001A/01

This balance is a high-grade analytical balance. The balance is sited in a vibration-free area and disturbance by draughts should be avoided. It carries out internal calibration but as a double check it is checked with certified check weights. Any deviation of the check weight values from those expected indicates need for servicing of the balance. Check weight calibration should be carried out once a week according to the instructions in SOP/001C/01.

Caution: The logbook (form SOP/001 AR/01) must be filled in. Any spillages on the balance must be cleaned up immediately and recorded in the log. This balance is to be used only for analytical grade weighings.

Operation

1. When carrying out weighing of amounts < 50 mg use tweezers to handle the weighing vessel.
2. Make sure the door of the balance is shut. Switch on the balance and allow it to undergo its internal calibration procedure. When it is ready the digital read-out will be 0.0000. Wait 30 s to ensure that the reading has stabilised.
3. Introduce the weighing vessel onto the balance pan. Close the door. Wait 30 s to ensure that the reading has stabilised and then send the reading to the printer.
4. If the tare is used in the weighing procedure, press the tare button and wait until the balance reads 0.0000. Wait 30 s to ensure that the reading has stabilised. If it drifts, which under normal circumstances it should not, press the tare button again and wait for a stable reading.
5. Remove the weighing vessel from the balance, introduce the sample into the vessel and put it back onto the balance pan. Close the door and note the reading.
6. Remove the sample and adjust the sample size to bring it closer to the required amount. Re-introduce the sample onto the balance pan. Close the door and note the reading.
7. Repeat step 5 until the target weight is reached. When the required weight is reached wait 30 s to ensure that the reading has stabilised. Send the reading to the printer.

N.B. An unstable reading may indicate that moisture is being lost or gained and that the sample must be weighed in a capped vessel.

Date of issue: 6/10/95 **Signature:**

An estimate of compound random errors is obtained from the square root of the sum of the squares of the RSDs attributed to each component or operation in the analysis. If the analysis of paracetamol described in Box 1.4 is considered, then, assuming the items of glassware are used correctly, the errors involved in the dilution steps can be simply estimated from the tolerances given for the pipette and volumetric flasks. The British Standards Institution (BS) tolerances for the grade A glassware used in the assay are as follows:

500 ml volumetric flask 500 ml ± 0.05%
100 ml volumetric flask 100 ml ± 0.08%
5 ml one mark pipette 5 ml ± 0.3%

Standard deviation of error from glassware =

$$\sqrt{0.05^2 + 0.08^2 + 0.3^2} = \sqrt{0.0989} = 0.31\%$$

Thus it can be seen that the compound error from the glassware differs little from the largest error in the process. Of course the glassware errors can be eliminated by calibration of the glassware prior to use, but, in general, analysts will accept manufacturers' tolerances. The tolerated random error from glassware could be readily eliminated; other random errors such as variation in the extraction efficiency are more difficult to control.

Self-test 1.3

Estimate the compound random error in the following assay with respect to the dilution steps described and calculate the error as SD of the w/v percentage of the injection assuming it is exactly 2% w/v.

A 2% w/v injection was diluted twice using grade A 5 ml bulb pipettes and grade A 100 ml volumetric flasks as follows:

Dilution 1: 5 to 100 ml

Dilution 2: 5 to 100 ml

The uncertainty in the spectrophotometric reading was ± 0.2%.

Answer: ± 0.48% and ± 0.01% w/v

Reporting of results

In calculating an answer from the data obtained in an analysis it is important to not indicate a higher level of precision than was actually possible in the assay.

As mentioned in the previous section, when considering the accuracy of glassware used with the assumption that it complied with the BS grade A standard, it was obvious that there was some uncertainty in any figure < 1%. It might be possible to improve on this degree of precision by calibrating glassware; however, any improvement in precision in the real world would take time and hence have cost implications. Thus for the purposes of most analyses, and for the purposes of the calculations in this book, it would seem sensible to report four significant figures, i.e. to 0.1%. In the process of carrying out calculations, five figures can be retained and rounded up to four figures at the end of the calculation. Since in pharmaceutical analyses the percentage of the stated content of a drug in a formulation may be reported as being between 90 and 99.9%, if the first significant figure is 9, then at the end of the calculation a more realistic estimate of precision is given by rounding the answer up to three significant figures. The SD or RSD reported with the answer should reflect the number of significant figures given; since there is usually uncertainty in figures < 1% of the answer, the RSD should not be reported to below 0.1%. Taking this into consideration the correct and incorrect ways of reporting some answers are given in Table 1.3.

Table 1.3 Significant figures in the reporting of analytical results

Answer ± S Incorrect	RSD	Answer ± S Correct	RSD
% of stated content = 99.2 ± 0.22	0.22	% of stated content = 99.2 ± 0.2	0.2
% of stated content = 101.15 ± 0.35	0.35	% of stated content = 101.2 ± 0.4	0.4
0.2534 ± 0.00443% w/v	1.75	0.2534 ± 0.0044% w/v	1.7
1.0051 ± 0.0063% w/w	0.63	1.005 ± 0.006% w/w	0.6
1.784 ± 0.1242 μg/ml	6.962	1.784 ± 0.124 μg/ml	7.0

Other terms used in the control of analytical procedures

System suitability

System suitability should not be confused with method validation. System suitability tests are most often applied to analytical instrumentation. They are designed to evaluate the components of the analytical system in order to show that the performance of the system meets the standards required by the method. Method validation is performed once at the end of method development, whereas system suitability tests are performed on a system periodically to determine whether or not it is still working properly and is capable of carrying out the analysis. System suitability relates to the performance of the equipment. In selecting equipment, the four Qs rule can be applied.[2]

(i) Design qualification (fit for purpose). What is the equipment required to do?
(ii) Installation qualification. Does the equipment work in the way that the manufacturer claims?
(iii) Operational qualification. Does the equipment work for the analyst's particular application.
(iv) Performance qualification. Does the instrument continue to perform to the standard required?

In routine use it is point 4 that is checked, and, for a given procedure, an analyst will use several tests routinely, in order to monitor instrument performance, e.g. the resolution test during chromatography.

Analytical blank

This consists of all the reagents or solvents used in an analysis without any of the analyte being present. A true analytical blank should reflect all the operations to which the analyte in a real sample is subjected. It is used, for example, in checking that reagents or indicators do not contribute to the volume of titrant required for a titration, including zeroing spectrophotometers or in checking for chromatographic interference.

Calibration

The calibration of a method involves comparison of the value or values of a particular parameter measured by the system under strictly defined conditions with pre-set standard values. Examples include: calibration of the wavelength and absorbance scales of a UV/visible spectrophotometer (Ch. 4), calibration of the wavelength scale of an IR spectrometer (Ch. 5) and construction of chromatographic calibration curves (Ch. 12).

Limit of detection

This is the smallest amount of an analyte which can be detected by a particular method. It is formally defined as follows:

$$x - x_B = 3s_B$$

Where x is the signal from the sample, x_B is the signal from the analytical blank and s_B is the SD of the reading for the analytical blank. In other words, the criterion for a reading reflecting the presence of an analyte in a sample is that the difference

between the reading taken and the reading for the blank should be three times the SD of the blank reading. The SD of the signal from the sample can be disregarded since the sample and the blank should have been prepared in the same manner so that it and the sample produce a similar SD in their readings. A true limit of detection should reflect all the processes to which the analyte in a real assay is subjected and not be a simple dilution of a pure standard for the analyte until it can no longer be detected.

In the case of chromatographic separations there is usually a constant background reading called the baseline. In this case, a better definition of the limit of detection is that the analyte should give a signal > three times the standard deviation of the chromatographic baseline within a time range of 0.5 minutes before and after the peak.

Limit of quantification

The limit of quantification is defined as the smallest amount of analyte which can be quantified reliably, i.e. with an RSD for repeat measurement of <± 20%.
The limit of quantification is defined as: $x - x_B = 10s_B$. In this case the analyte should give a peak > ten times the standard deviation of the chromatographic baseline during chromatographic analysis.

Self-test 1.4

In which of the following cases has the limit of detection been reached?

Signal from sample	Sample SD	Signal from analytical blank	Analytical blank SD
1. Abs 0.0063	0.0003	0.0045	0.0003
2. Abs 0.0075	0.0017	0.0046	0.0018
3. 0.335 ng/ml	0.045 ng/ml	0.045 ng/ml	0.037 ng/ml

Answer: 2

Linearity

Most analytical methods are based on processes where the method produces a response that is linear and which increases or decreases linearly with analyte concentration. The equation of a straight line takes the form:

$$y = a + bx$$

where a is the intercept of the straight line with the y axis and b is the slope of the line. Taking a simple example, a three-point calibration curve is constructed through readings of absorbance against procaine concentration (Table 1.4).

Table 1.4 Data used for the construction of a calibration curve for the spectrophotometric determination of procaine

Procaine concentration mg/100 ml	Absorbance reading
0.8	0.604
1.0	0.763
1.2	0.931

The best fit of a straight line through these values can be determined by determining a and b from the following equations:

$$b = \frac{\sum_i (x_i - \bar{x})(y_i - \bar{y})}{\sum_i (x_i - \bar{x})^2}$$

$$a = \bar{y} - b\bar{x}$$

where x_i is the individual value for x, $\bar{x}$ is the mean value of x_i, y_i is the individual value for y and $\bar{y}$ is the mean of y_i.

From the data in Table 1.3:

$$\bar{x} = \frac{0.8 + 1.0 + 1.2}{3} = 1.0$$

$$\bar{y} = \frac{0.604 + 0.763 + 0.931}{3} = 0.766$$

$$b = \frac{(0.8 - 1.0)(0.604 - 0.766) + (1.0 - 1.0)(0.763 - 0.766) + (1.2 - 1.0)(0.931 - 0.766)}{(0.8 - 1.0)^2 + (1.0 - 1.0)^2 + (1.2 - 1.0)^2}$$

$$= \frac{0.0324 + 0 + 0.033}{0.04 + 0.04} = 0.818$$

$$a = 0.766 - 0.818 \times 1.0 = -0.052$$

Thus the equation for the best fit is:

$$y = 0.818x - 0.052$$

The statistical measure of the goodness of fit of the line through the data is the correlation coefficient r. A correlation coefficient of > 0.99 is regarded as indicating linearity. The correlation coefficient is determined from the following equation:

$$r = \frac{\sum_i \{(x_i - \bar{x})(y_i - \bar{y})\}}{\sqrt{\sum_i [(x_i - \bar{x})^2] \sum_i [(y_i - \bar{y})^2]}}$$

Substituting the values from Table 1.3:

$$r = \frac{(0.8 - 1.0)(0.604 - 0.766) + (1.0 - 1.0)(0.763 - 0.766) + (1.2 - 1.0)(0.931 - 0.766)}{\sqrt{[(0.8 - 1.0)^2 + (1.0 - 1.0)^2 + (1.2 - 1.0)^2][(0.604 - 0.766)^2 + (0.763 - 0.766)^2 + (0.931 - 0.766)^2]}}$$

$$r = \frac{0.0324 + 0 + 0.033}{\sqrt{0.08 \times 0.0534}} = 1.00$$

Thus, to three significant figures, the straight line fit through the values in Table 1.3 is perfect. For a fuller treatment of the mathematical determination and significance of a correlation coefficient see reference 1. The equation for the correlation coefficient is very useful in that it can be applied to correlations between curves of any shape and thus it can be used for spectral comparisons such as those carried out between diode array spectra obtained during high-pressure liquid chromatography (Ch. 12, p. 285).

Range

The term range can be applied to instrument performance (dynamic range) but, when applied to the performance of an assay, it refers to the interval between the upper and lower concentration of an analyte for which an acceptable level of precision and accuracy has been established. Typical ranges are: 80–120% of the

stated amount for a finished product; 70–130% of the expected concentration, e.g. for content of single tablets (the range may be even wider for some products, such as doses delivered by a metered dose inhaler) and 0–110% for dissolution tests where the drug is released from the dosage form over a time period.

Robustness

Robustness is evaluated in order to determine how resistant the precision and accuracy of an assay are to small variations in the method. The types of parameters which are assessed in order to determine the robustness of a method include: the stability of analytical solutions; the length of the extraction time; the effect of variations in the pH of a HPLC mobile phase; the effect of small variations in mobile phase composition; the effect of changing chromatographic columns; the effect of temperature and flow rate during chromatography.

Selectivity

The selectivity of a method is a measure of how capable it is of measuring the analyte alone in the presence of other compounds contained in the sample. The most selective analytical methods involve a chromatographic separation. Detection methods can be ranked according to their selectivity. A simple comparison is between fluorescence spectrophotometry and UV spectrophotometry. There are many more compounds which exhibit UV absorption than there are those which exhibit strong fluorescence; thus fluorescence spectrophotometry is a more selective method. Because selective methods are based on more complex principles than non-selective methods, they may be less robust, e.g. fluorescence spectrophotometry is more affected by changes in the analytical method than UV spectrophotometry.

Sensitivity

The sensitivity of method indicates how responsive it is to a small change in the concentration of an analyte. It can be viewed as the slope of a response curve and may be a function of the method itself or of the way in which the instrumentation has been calibrated. In Figure 1.3 the method having a *linear* response $y = 2.5x$ is five times more sensitive than the method exhibiting a linear response $y = 0.5x$.

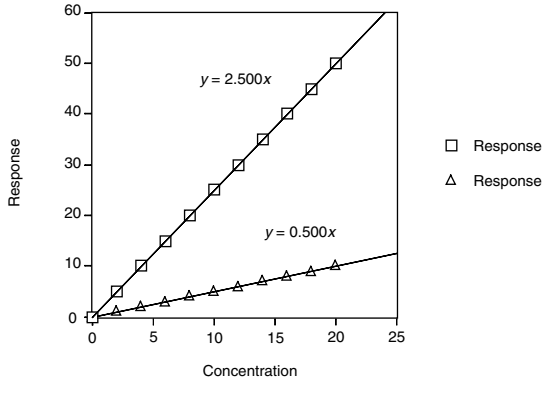

Fig. 1.3
Sensitivity as a function of the slope of a response curve.

Sensitivity and the *limit of detection* of a method are often confused. The limit of detection is due to a combination of *range* and *sensitivity*.

Weighing by difference

Weighing by difference is used to minimise weighing errors in an analytical procedure. The sample is weighed in a suitable vessel, e.g. a glass weighing boat with a spout, and then transferred immediately to the vessel in which it is going to be analysed or dissolved. The weighing vessel is then reweighed and the difference between the weights before and after transfer gives the weight of the sample. This method of weighing minimises errors due to, for example, the absorption of moisture onto the surface of the vessel. It also means that there is not a requirement for complete transfer of the sample that is to be analysed.

The points listed in Boxes 1.6 and 1.7 indicate how pharmaceutical preparations may come to be out of specification.

Box 1.6 Sources of impurities in pharmaceutical manufacture

- During the course of the manufacture of a pure drug substance, impurities may arise as follows:
  - (i) Present in the synthetic starting materials
  - (ii) Result from residual amounts of chemical intermediates used in the synthetic process and from unintended side reactions
  - (iii) Result from reagents, solvents and catalysts used in manufacture
- The process used to produce the formulated drug substance may introduce impurities as follows:
  - (i) Particulate matter from the atmosphere, machines and devices used in the manufacturing process and from containers
  - (ii) Impurities that are present in the excipients used in the formulation
  - (iii) Cross contamination may occur from other processes carried out using the same equipment, e.g. from mixers
  - (iv) Microbial contamination may occur
  - (v) The drug may react with the excipients used in the formulation
  - (vi) Impurities may be introduced from packaging, e.g. polymeric monomers.

Box 1.7 Processes leading to the deviation of the actual content from the stated content of a drug in a formulation

- Incomplete mixing of drug with formulation excipients prior to compression into tablets or filling into capsules
- Physical instability of the dosage form: tablets that disintegrate too readily; creams or suspensions that separate and over- or undercompression of tablets, leading to deviation from the required weight
- Chemical breakdown of the drug resulting from its reaction with air, water, light, excipients in a formulation or with packaging materials
- Partitioning of the drug into packaging materials.

Basic calculations in pharmaceutical analysis

The data obtained from an analysis can be carried out using computer-based methods. However, in order to have some idea of the correctness of an answer, it is

necessary to be able to carry out calculations in the traditional manner. There are various units used to express amounts and concentrations in pharmaceutical analysis and examples of these units will be considered below.

Percentage volume/volume (%v/v)

%v/v is most often encountered in relation to the composition of mobile phases used in high-pressure liquid chromatography. Thus, when 30 ml of methanol is mixed with 70 ml of water, a 30:70 v/v mixture is formed. Since some shrinking in volume occurs when two liquids are mixed, % v/v may only be approximate. Some chromatographers prefer to prepare mixtures of solvents by weighing them rather than by volume measurement and in this case the solvent mixture can be expressed as % weight in weight (%w/w).

Percentage weight in volume (%w/v)

%w/v is normally used to express the content of active ingredient in liquid formulations such as injections, infusions and eyedrops. The density of the solvent in this case is irrelevant; thus, a 1g/100 ml solution of a drug is 1% w/v whether it is dissolved in ethanol or water.

Self-test 1.5

Convert the following concentrations to %w/v.
(i) 0.1 g/100 ml
(ii) 1 mg/ml
(iii) 0.1 g/ml
(iv) 100 µg/ml

Answers: (i) 0.1% w/v (ii) 0.1% w/v (iii) 10% w/v (iv) 0.01% w/v

Dilutions

In order for an extract from a formulation or a solution of a pure drug substance to be measured it must be diluted so that it falls within the working range of the instrument used to make the measurement. Thus an understanding of dilution factors is fundamental to calculations based on analytical data.

Calculation example 1.1

An infusion stated to contain 0.95% w/v NaCl was diluted so that its Na content could be determined by flame photometry. The following dilutions were carried out:

(i) 10 ml of the sample was diluted to 250 ml with water.
(ii) 10 ml of the diluted sample was diluted to 200 ml with water.

The sample was found to contain 0.74 mg/100 ml of Na.
Atomic weights: Na = 23 Cl = 35.5

(Continued)

Calculation example 1.1 *(Continued)*

Calculate:

The % w/v of NaCl present in the infusion.
The % of stated content of NaCl.
Dilution factor = 10 to 250 (× 25), 10 to 200 (× 20). Total dilution = 25 × 20 = 500.
Therefore, in the original injection, conc. of Na = 0.74 × 500 mg/100 ml = 370 mg/100 ml.
Conc. of NaCl in the injection = 370 × (58.5/23) = 941 mg/100 ml = 0.941 g/100 ml = 0.941 % w/v.
% of stated content = (0.941/0.95) × 100 = 99.1%.
In the sample there are 941 mg/100 ml.

Calculation example 1.2

A 2 ml volume of eyedrops containing the local anaesthetic proxymetacaine. HCl is diluted to 100 ml and then 5 ml of the dilution is diluted to 200 ml. The diluted sample was measured by UV spectrophotometry and was found to contain 0.512 mg/100 ml of the drug. Calculate the % w/v of the drug in the eyedrops.

Dilution factors 2 to 100 (× 50), 5 to 200 (× 40). Total dilution 40 × 50 = 2000.
Original concentration = 2000 × 0.512 = 1024 mg/100 ml = 1.024g/100 ml = 1.024% w/v.

Self-test 1.6

A 5 ml sample of an injection of a steroid phosphate was diluted to 100 ml. Then 10 ml of the diluted injection was diluted to 100 ml and this dilution was further diluted 10 to100 ml. From measurement by UV the diluted sample was found to contain 0.249 mg/100 ml of the steroid. What was the original concentration of the injection in %w/v and in mg/ml?

Answers: 0.498 % w/v, 4.98 mg/ml

Self-test 1.7

A sample of an infusion was diluted 5 ml to 200 ml and then 10 ml to 200 ml. It was then analysed and was found to contain sodium at 0.789 mg/100 ml. Calculate the concentration of sodium in the original sample in %w/v. The sample was composed of a mixture of sodium lactate and sodium bicarbonate in equimolar amounts. Calculate the amount of sodium lactate and sodium bicarbonate in mg in 10 ml of the sample (Na = 23, lactate = 89, bicarbonate = 61).

Answers: 0.6312 % w/v, Na lactate = 153.7 mg/10 ml Na bicarbonate = 115.3 mg/10 ml

Preparation of standard stock solutions

In preparing a stock solution of a standard using a standard four-place balance, assuming there is no lack of availability of standard, it is best to weigh at least 100 mg of material, since an error of 0.1 mg in weight is only 0.1% of the weight taken.

Calculation example 1.3

Assuming that you wish to avoid pipetting less than a 5 ml volume, starting from a 102.1 mg/100 ml stock solution, how would you prepare the following concentrations for a calibration series?

0.2042 mg/100 ml, 0.4084 mg/100 ml, 0.6126 mg/100 ml, 0.8168 mg/100 ml, 1.021 mg/ml

When a large dilution (>10) is required it is best to carry it out in two stages.

In this case an initial dilution of 20 ml to 100 ml is carried out, producing a 10.21 mg/100 ml solution. Then 250 ml volumetric flasks can be used to carry out the following dilutions.

5 to 250 (× 50), 10 to 250 (× 25), 15 to 250 (× 16.67), 20 to 250 (× 12.5), and 25 to 250 (× 10), giving the dilution series above.

N.B. Once the volume required for the lowest concentration (5 ml) has been determined, then it can be multiplied × 2, × 3, × 4 and × 5 to give the series.

Self-test 1.8

A stock solution containing 125.6 mg of standard in 250 ml is prepared. Suggest how the following dilution series could be prepared (pipettes 5 ml or greater must be used).

0.1005 mg/100 ml, 0.2010 mg/100 ml, 0.3015 mg/100 ml, 0.4020 mg/100 ml, 0.5025 mg/100 ml

Answer: Number of possible answers, e.g. dilution 1, 10 ml to 250 ml. Then, from dilution 1, 5, 10, 15, 20 and 25 ml pipettes used to transfer into 100 ml volumetric flasks.

Percentage weight/weight (%w/w)

% w/w is a common measure used to express the concentration of active ingredient in a formulation such as a cream or to express the content of a minor impurity in a drug substance. Thus, a cream containing 10 mg (0.01g) of drug per gram is a: (0.01/1) × 100 = 1% w/w formulation. Equally, if a drug contains 0.5 mg (0.0005g) per gram of an impurity, the impurity is at a concentration of (0.0005/1) × 100 = 0.05% w/w. It is generally accepted that, for a drug, all impurities above 0.05% w/w should be characterised, i.e. their structures should be known and their toxicities should be assessed.

In determining impurities in a drug, 1 g of the drug might be dissolved in 100 ml of solvent. If an analysis was carried out and the drug solution was found to contain 3 mg/100 ml of an impurity, then the %w/w referring back to the original weight of drug substance would be:

(0.003/1) × 100 = 0.3% w/w

Self-test 1.9

(i) 0.1521 g of a corticosteroid drug was dissolved in 100 ml of methanol/water (1:1 v/v). The sample was analysed by HPLC and was found to contain a known impurity in a concentration of 0.354 mg/100 ml. What is the %w/w of the impurity in the drug?

(ii) 0.5321 g of a β-adrenergic blocking drug is dissolved in 50 ml of methanol/0.1% w/v acetic acid (20:80 v/v) and the sample is then diluted by taking 10 ml of the solution and diluting it to 50 ml. The diluted sample was analysed by HPLC and was found to contain 0.153 mg/100 ml of an impurity. What is the %w/w of the impurity in the drug?

Answers: (i) 0.233 % w/w
(ii) 0.0719 % w/w

Parts per million (ppm) calculations

Parts per million (ppm) on a w/w basis is 1mg/g (1µg/kg). It is a common measure used for impurities in drug substances, particularly heavy metals and solvents.
1 ppm is also 0.0001% w/w

Calculation example 1.4

The potassium content of an intravenous infusion containing sodium chloride was determined. The infusion was found to contain 0.9092% w/v NaCl. The undiluted infusion was measured for potassium content in comparison with a potassium chloride standard. The potassium content of the undiluted infusion was found to be 0.141 mg/100 ml. Calculate the potassium content in the sodium chloride in ppm.

0.9092 %w/v = 0.9092 g/100 ml
Potassium content = 0.141 mg/100 ml = 141 µg/100 ml.

Relative to the sodium chloride, there are 141 µg/0.9092 g = 141/0.9092 ppm = 155 ppm.

Self-test 1.10

125.1 mg of streptomycin sulphate are dissolved in 10 ml of water. A GC headspace analysis is carried out in order to determine the methanol content of the drug. A peak for methanol is produced which has 73.2% of the area of a peak for a methanol standard containing 0.532 mg/100 ml of methanol in water analysed under exactly the same conditions. What is the methanol content of the streptomycin sulphate in ppm and %w/w?

Answer: 311.3 ppm, 0.03113 %w/w.

Working between weights and molarity

Weights are much easier to appreciate than molar concentrations but sometimes, particularly in bioanalytical methods, molar concentrations are used.

Definitions

Molar : molecular weight in g/litre (mg/ml)
mMolar: molecular weight in mg/litre (µg/ml).
µMolar: molecular weight in µg/litre (ng/ml)
nMolar: molecular weight in ng/litre (pg/ml)

Calculation example 1.5

The metabolism of paracetamol (MW 151.2 amu) by liver microsomes was studied by preparing a 100 µM solution of paracetamol in 1 ml of incubation mixture. A 30.12 mg/100 ml solution of paracetamol in buffer was prepared. What volume of paracetamol solution had to be added to incubation mixture prior to making up the volume to 1 ml.

100 µM = 100 × 151.2 = 15120 µg/litre = 15.12 µg/ml.

30.12 mg/100 ml = 30120 µg/100 ml = 301.2 µg/ml = 0.3012 µg/µl.

Volume of paracetamol solution required = 15.12/0.3012 = 50.2 µl.

Self-test 1.11

(i) Calculate the concentrations in mg/ml and µg/µl of a 10µM solution of kanamycin (MW 484.5).

(ii) A solution containing diclofenac sodium (MW 318.1) at a concentration of 79.5 mg/100 ml in buffer was prepared. What volume of this solution was required in order to carry out a microsomal incubation containing a 25 µM concentration of the drug in 1 ml?

Answers: (i) 0.00485 mg/ml, 0.00485 µg/µl;
(ii) 10 µl

Additional problems

All answers to be given to four significant figures.
e.g. 1% w/v to four significant figures is 1.000 % w/v.

1. Convert the following concentrations to %w/v.
   (i) 10 mg/ml
   (ii) 100 mg/litre
   (iii) 0.025 g/ml
   (iv) 250 µg/ml
   (v) 20 µg/µl.

Answers: (i) 1.000% w/v
(ii) 0.01000% w/v
(iii) 2.500% w/v
(iv) 0.02500% w/v
(v) 2.000% w/v

2. An infusion which was stated to contain 0.5000% w/v KCl was diluted so that its K content could be determined by flame photometry. The following dilutions were carried out:
   (i) 10 ml of the sample was diluted to 200 ml with water.
   (ii) 10 ml of the diluted sample was diluted to 200 ml with water.

The sample was found to contain 0.6670 mg/100 ml of K.
(Atomic weights: K = 39.1 Cl = 35).

Calculate:
   (i) The % w/v of KCl present in the infusion
   (ii) The % of stated content of KCl.

Answers: (i) 0.5090 % w/v
(ii) 101.8%

(Continued)

Additional problems *(Continued)*

3. Oral rehydration salts are stated to contain the following components.

Sodium Chloride	3.5 g
Potassium Chloride	1.5 g
Sodium Citrate	2.9 g
Anhydrous Glucose	20.0 g

8.342 g of oral rehydration salts are dissolved in 500 ml of water. 5 ml of the solution is diluted to 100 ml and then 5 ml is taken from the diluted sample and is diluted to 100 ml. The sodium content of the sample is then determined by flame photometry. The sodium salts used to prepare the mixture were:
Trisodium citrate hydrate ($C_6H_5Na_3O_7,2H_2O$) MW 294.1 and sodium chloride (NaCl) MW 58.5.
Atomic weight of Na = 23.
The content of Na in the diluted sample was determined to be 0.3210 mg/100 ml.
Determine the % of stated content of Na in the sample.

Answer: 104.5%

4. A 5 ml volume of eyedrops containing the mydriatic drug cyclopentolate. HCl is diluted to 100 ml and then 20 ml of the dilution is diluted to 100 ml. The diluted sample was measured by UV spectrophotometry and was found to contain 20.20 mg/100 ml of the drug. Calculate the % w/v of the drug in the eyedrops.

Answer: 2.020% w/v

5. 0.5 % w/v of an injection is to be used as an anaesthetic for a 2-week-old baby weighing 3.4 Kg. The recommended dose for a bolus injection is 0.5 mg/Kg. The injection must be given in 1 ml. Calculate the amount of water for injection that must be drawn into the syringe with the appropriate volume of injection.

Answer: 0.6600 ml

6. 0.0641 g of a semi-synthetic alkaloid was dissolved in 25 ml of 1% w/v acetic acid and was analysed directly by HPLC. The solution was found to contain 0.142 mg/100 ml of an impurity. What is the level of impurity in %w/w and ppm.

Answers: 0.05538 % w/w, 553.8 ppm.

7. The level of ethyl acetate is determined in colchicine by headspace gas chromatography. A solution containing 4.361g/100 ml of colchicine in water was prepared. An aqueous standard containing 0.5912 mg/100 ml of ethyl acetate was also prepared. Headspace analysis of 2 ml volumes of the two solutions produced GC peaks for ethyl acetate with the following areas:

Colchicine solution: Peak area 13457
Ethyl acetate solution: Peak area 14689
Calculate the ethyl acetate content in the colchicine sample in ppm.

Answer: 124.2 ppm

References

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2. C. Burgess. Spectroscopy Europe 6, 10–13 (1994).