Analytical absorption spectroscopy in the ultraviolet (UV) and visible regions of the electromagnetic spectrum has been widely used in pharmaceutical and biomedical analysis for quantitative purposes and, with certain limitations, for the characterisation of drugs, impurities, metabolites and related substances. By contrast, luminescence methods, and fluorescence spectroscopy in particular, have been less widely exploited, despite the undoubted advantages of greater specificity and sensitivity commonly observed for fluorescent species. However, the wider availability of spectrofluorimeters able to present corrected excitation and emission spectra, coupled with the fact that reliable fluorogenic reactions permit non–fluorescent species to be examined fluorimetrically, has led to a renaissance of interest in fluorimetric methods in biomedical analysis.

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Theoretical background, UV and visible spectrophotometry
General considerations

Molecular absorption in the UV and visible region arises from energy transitions that involve the outer orbital or valency electrons. Spectra in liquid media are usually broad, relatively featureless bands, a result of the large number of closely spaced vibrational and rotational transitions. The fundamental band shape approximates Gaussian or log-normal Gaussian curves. Given the broad, overlapping profiles commonly encountered, the shape and precise location of individual bands are of limited usefulness in qualitative analysis. However, any fine structure detected in the spectra, coupled with solvent and pH effects, can be of diagnostic value. More informative spectra can be obtained for some volatile molecules of toxicological interest, such as benzene and polynuclear aromatic hydrocarbons; when examined in the vapour phase, vibrational and rotational fine structure can be readily seen superimposed on the broad spectral profiles. This is illustrated in Fig. 1 for 1,2,4,5-tetrazine. However, most drugs, metabolites and related compounds are relatively non-volatile; their spectra are observed necessarily in solution, or possibly in the solid phase by reflectance, or by compression to form a KBr disk, as used in infrared spectrophotometry.

UV and visible spectrophotometry find their primary application in quantitative analysis. The scope of absorption spectroscopy can be extended significantly by the use of colour reactions, often with a concomitant increase in sensitivity and/or selectivity. Such reactions are used to modify the spectrum of an absorbing molecule so that it can be detected in the visible region, well separated from other interfering components in the UV spectrum. Moreover, chemical modification can be used to transform an otherwise non-absorbing molecule into a stable derivative that possesses significant absorption.

Spectral selectivity can be enhanced further by a number of chemical or instrumental techniques, which include difference, higher-derivative and dual-wavelength spectrophotometry. Such methods, and certain graphic techniques such as the Morton-Stubbs method, can contribute in different ways to reducing the general problem of spectral interference in quantitative spectroscopy. Spectral interference can arise from so-called ‘irrelevant’ non-specific absorption, and also from absorption by other materials and impurities that may be present. When interference arises specifically from the spectral overlap of two or more well-defined components, a number of methods can be applied to measure the individual concentrations. These methods include the Vierordt multi-wavelength technique, least squares deconvolution and second- or higher-derivative spectrophotometry.
Spectral selectivity, and in some cases detection sensitivity, can be enhanced significantly by the various chemical and instrumental techniques outlined above. Such methods should, of course, be validated by applying the conventional analytical criteria of accuracy (against a reference method), linearity, precision and independence from interfering substances.

The scope of UV and visible spectrophotometry can be further extended when combined with a chromatographic separation step, such as high–performance liquid chromatography (HPLC). The development of rapid–scanning detectors based on the linear photodiode array permits spectra to be acquired during the elution of peaks. Computer–aided manipulation of these spectra has led to new strategies for the examination of chromatographic peak homogeneity, based on classic techniques in spectroscopy. The use of microcomputers enables the development of archive–retrieval methods for spectral characterisation (Fell et al. 1984).

Nomenclature

In the UV and visible spectrum, the energy of photons associated with electronic transitions lies in the range 147 to 630 kJ/mol. This energy ($\Delta E$) can be expressed in terms of the principal parameters that define electromagnetic radiation, namely frequency $\mu$ (Hz), wavelength $\lambda$ (nm) and wavenumber ($\text{cm}^{-1}$):

$$\Delta E = h\mu = \frac{hc}{\lambda} = hc\bar{\mu}$$  \hspace{1cm} (21.1)

where $h$ is Planck’s constant, and $c$ is the velocity of radiation in vacuo.

The positions of peaks are sometimes described in terms of wavenumber, which has the advantage of being a linear function of energy, but this term is much more frequently used in infrared spectrophotometry. The practical unit most often used in UV and visible spectrophotometry is wavelength, usually expressed in nanometres (nm). The older units of wavelength, millimicron (mμ) and Ångström (Å), are not recommended terms. The position of maximum absorbance of a peak is designated $\lambda_{\text{max}}$.

The wavelength span is conventionally divided into two ranges: the UV extends from 200 nm to about 400 nm; the visible range extends from about 400 nm to 800 nm. Outside these limits, the ‘far UV’ or ‘vacuum UV’ extends from 100 nm to 200 nm, and the ‘near infra–red’ from 1 μm to about 3 μm.
A molecular grouping specifically responsible for absorption is described as a chromophore, and is usually a conjugated system with extensive delocalisation of electron density. Any saturated group with little or no intrinsic absorption of its own, but that modifies the absorption spectrum when attached directly to a chromophore, is described as an auxochrome, examples being –OR, –NR₂, –SR. Auxochromes are considered to exert their effect through partial conjugation of their polarisable lone–pair electrons with those of the adjacent chromophore. If, however, the lone–pair of electrons is involved in bonding as, for example, in the case of a protonated quaternary ammonium group, the auxochromic effect vanishes. This property can be used for molecular characterisation, as discussed below.

**Laws of absorption spectrophotometry**

The extent of absorption of radiation by an absorbing system at a given monochromatic wavelength is described by the two classic laws of absorptiometry, which relate the intensity of radiation incident on the absorbing system \(I_0\) to the transmitted intensity \(I\), (Fig. 2). Lambert’s (or Bouguer’s) Law concerns instrumental factors, and states that at a given concentration \(c\) of a homogeneous absorbing system, the transmitted intensity \(I\) decreases exponentially with increase in path length \(b\). The complementary Beer’s Law deals with concentration and states that for a layer of defined path length \(b\), the transmitted intensity \(I\) decreases exponentially with the increase in concentration \(c\) of a homogeneous absorbing system. Combination of these observations gives the familiar Beer–Lambert Law:

\[
\log \frac{I_0}{I} = kcb
\]

\[\text{Eqn. (21.2)}\]

where \(k\) is the absorptivity of the system.

The logarithmic term is linearly related to concentration and path length, and is referred to as absorbance \(A\). The older terms, extinction \(E\) and optical density \(OD\) are not recommended, although they are often found in the literature. Transmittance \(T = I/I_0\) and percentage transmittance \([%T = 100(I/I_0)]\) are not linear functions of concentration and path length and can be related readily to absorbance:
The absorptivity ($k$) is a fundamental property of a molecule at a specified monochromatic wavelength. It has two connotations in European usage, and a third according to American convention. If the concentration is expressed in mol/L, $k$ is described as the molar absorptivity ($\varepsilon$, L/mol/cm), and defined as ‘the absorbance of a one molar solution in a cell of 1 cm path length’. It may also be quoted as the logarithm (base 10), the values typically being in the range 1 to 5.

When concentration is expressed in g/100 mL, $k$ is described as the specific absorbance and given the symbol $A^{1\%}_{1\text{cm}}$ or $A$ (1%, 1 cm), defined as ‘the absorbance of a 1% w/v solution in a cell of 1 cm path length’. It is usually written in the shortened form $A^1_1$ and is widely used in analytical chemistry. It was formerly known as the ‘specific extinction coefficient’, symbol $E^{1\%}_{1\text{cm}}$ or $E$ (1%, 1 cm).

American convention recognises the constant $k$ as ‘absorptivity’ ($a$, L/g/cm) defined as ‘the absorbance of a 1 g/L solution in a cell of 1 cm path length’.

These terms for absorptivity can be readily interconverted:

Since only the absorptivity ($k$) is a function of $\lambda$, the shape of a logarithmic absorption curve is independent of concentration and path length. Their only effect is to shift the log $A$ spectrum along the log $A$ axis. A disadvantage of the log $A$ plot is that fine structure near the top of the peak is compressed.

$$a = \frac{A^1_1}{10} = \frac{\varepsilon}{M_r} \quad (21.4)$$

where $M_r$ is the relative molecular mass. Thus, a compound with an $M_r$ of 100 and absorptivity $a$ of 20 at wavelength $\lambda$, in a particular solvent at a defined pH (if aqueous) and at a specified temperature, has a corresponding specific absorbance $A^1_1$ of 200 and a molar absorptivity $\varepsilon$ of 2000.

Absorbance and absorptivity are often expressed in logarithmic form in cases where spectra
are to be compared. The logarithmic form of the Beer–Lambert Law expresses the effects of absorptivity \((k)\), concentration \((c)\) and path length \((b)\) as additive terms.

\[
\log A = \log k + \log c + \log b \tag{21.5}
\]

Since only the absorptivity \((k)\) is a function of \(\lambda\), the shape of a logarithmic absorption curve is independent of concentration and path length. Their only effect is to shift the \(\log A\) spectrum along the \(\log A\) axis. A disadvantage of the \(\log A\) plot is that fine structure near the top of the peak is compressed.

**Validity of the Beer–Lambert law**

The validity of the Beer–Lambert Law is affected by a number of factors. If the radiation is non–monochromatic, that is its spectral bandwidth is greater than about 10% of the drug absorption bandwidth at half–height, the observed absorbance will be lower than the ‘true’ limiting value for monochromatic radiation. Thus, sharp bands are more susceptible than broad bands to absorbance error on this account. Moreover, if the absorbing species is non–homogeneous, or if it undergoes association, dissociation, photodegradation, solvation, complexation or adsorption, or if it emits fluorescence, then positive or negative deviations from the Beer–Lambert Law may be observed. Stray–light effects and the type of solvent used may also lead to non–compliance with the Beer–Lambert Law.

**Stray–light effects**

Stray light is radiation at wavelengths different from those desired. It may arise from light scattering or other defects within the instrument, or it may be caused by external radiation. If the stray light is not absorbed, the observed absorbance tends to a constant value as the concentration of drug is increased, thereby yielding a negative deviation from the Beer–Lambert Law.

Stray–light errors are more likely to be observed near the wavelength limits of an instrument, at which the radiation intensity of the source and the efficiency of the optical system are reduced, especially below 220 nm and at the crossover point between the UV and the visible lamps (about 320 to 400 nm). Errors may become serious if the solvent absorbs strongly or if a strongly–absorbing sample is measured by difference spectrophotometry.
Solvent effects

The solvent often exerts a profound influence on the quality and shape of the spectrum. For example, many aromatic chromophores display vibrational fine structure in non-polar solvents, whereas in more polar solvents this fine structure is absent because of solute–solvent interaction effects. A classic case is phenol and related compounds, which have different spectra in cyclohexane and in neutral aqueous solution. In aqueous solutions, the pH exerts a profound effect on ionisable chromophores because of the differing extent of conjugation in the ionised and the non-ionised chromophore.

The quality of spectral measurement is affected directly by the type and purity of the solvent used. Each solvent has a cut-off wavelength (which corresponds to about 10% transmittance) and this varies with solvent purity. A solvent should not be used below its cut-off wavelength, even though reference-cell compensation is employed, because of the greater risk of stray-light effects.

Some cautionary comments may be appropriate at this point. It is better to use single- or double-distilled water, and to avoid deionised water, which can be contaminated with absorptive fragments of ion-exchange resin or contain bacterial metabolites; these can contribute significantly to non-specific absorption at low wavelengths. Ethanol is normally used as the 96% v/v strength, since dehydrated alcohol is usually contaminated with traces of benzene added to form the azeotropic mixture for distillation. Acetonitrile can vary noticeably in quality, depending on the supplier; the grade supplied for use in HPLC is usually to be recommended. Acetone, sometimes used to clean cells, is highly absorptive and not always easily removed, despite its volatility and aqueous solubility. Chloroform and carbon tetrachloride absorb strongly at about 250 nm and should therefore only be used for measurements at wavelengths above about 280 nm. Given the safety considerations of chlorinated solvents, use of these is best avoided if possible. Ether, although transparent down to 220 nm, presents particular problems because of its volatility (unstable standard solutions) and inflammability. Although absorptivity is considered to be relatively insensitive to temperature changes, organic solvents in general suffer from high temperature-coefficients of expansion, so that for ultimate precision a cell provided with a thermostat may be required.

Fluorescence spectrophotometry
General considerations

Molecular fluorescence is an emission process in which molecules are excited by the absorption of electromagnetic radiation. The excited species then relax to the ground state, giving up their excess energy as photons.

There are several ways an excited molecule can give up its excess energy and relax to its ground state. Two of the most important of these mechanisms are non-radiative relaxation and fluorescent relaxation.

Non-radiative relaxation can occur through collisions between excited molecules and molecules of the solvent, by giving excess energy to solvent molecules. When relaxation takes place by fluorescence, bands of radiation are produced as the excited molecules relax to several energy states, which are very close in energy level and thus in wavelength. Fluorescence occurs only from the lowest vibrational level of an excited electronic state.

Note that molecular fluorescence bands are made up largely of lines that are longer in wavelength (lower in energy) than the band of absorbed radiation responsible for their excitation. This shift to longer wavelength is sometimes called the Stokes shift. For that reason, the absorption or excitation spectrum and the fluorescence spectrum for a compound often appear as approximate mirror images of one another. The most useful region for the fluorescence technique is 200 to 800 nm.

Fluorescence spectrophotometry is usually the method of choice for quantitative analytical purposes if applicable. It has assumed a major role in analysis, particularly the determination of trace contaminants in our environment, industries and bodies, because for applicable compounds fluorescence spectrometry gives high sensitivity and high specificity. The selectivity of fluorescence methods is greater than that of absorption methods, as fewer substances fluoresce than absorb radiation in the UV or visible region. Furthermore, fluorescence is more selective because both the emission and the absorption spectra can be obtained. Fluorescence is usually also more sensitive than absorption methods, as it is always easier to measure a small signal against a very small zero background than to measure a small difference between large signals. However, the phenomenon of fluorescence itself is subject to more rigorous constraints on molecular structure than is absorption.

Many drugs possess rather high quantum efficiencies for fluorescence, such as quinine and lysergic acid diethylamide (LSD).
Nomenclature

A term used in fluorescence is quantum efficiency, described by the quantum yield (i.e. the ratio of the number of molecules that fluoresce to the total number of excited molecules). Highly fluorescent molecules can have quantum efficiencies that approach unity.

All absorbing molecules have the potential to fluoresce. They do if fluorescent emission occurs at a greater rate than relaxation by non-radiative ways. The kind of relaxation process is highly dependent on the molecular structure. Compounds that contain aromatic rings give the most intense and most useful fluorescence emission. Substitution on an aromatic ring causes shifts in the excitation wavelength spectrum and in fluorescence efficiency. Substituents such as -NH₂, -OH, -OCH₃ and -NHCH₃ groups often enhance fluorescence, while -Cl, -Br, -I, -NO₂ or -COOH are electron-withdrawing groups that can lead to a complete reduction of fluorescence (e.g. aniline fluoresces while nitrobenzene does not). The molecular grouping responsible for fluorescence is sometimes described as a fluorophore.

Fluorescence is particularly favoured in rigid molecules, as molecular rigidity reduces deactivation by non-radiative processes (fewer internal vibrations). This is also the reason that certain organic chelating agents are more fluorescent when complexed with a metal ion.

Laws of fluorescence spectrophotometry

The power of fluorescent radiation $I_t$ is proportional to the radiant power of the excitation beam absorbed by the system:

$$I_t = K'(I_0 - I) \quad (21.6)$$

The constant $K'$ depends upon the quantum efficiency of the fluorescence. To relate $I_t$ to the molar concentration $c$ of the fluorescing molecule, Beer’s law can be used:

$$\frac{I}{I_0} = 10^{-ebc} \quad (21.7)$$

By substituting Equation (21.7) into (21.6) we obtain:
After expansion of the exponential term, and provided $\varepsilon_{bc} < 0.05$, we can write:

$$I_t = K' I_0 (1 - 10^{-\varepsilon_{bc}})$$  \hspace{1cm} (21.8)

or at constant $I_0$:

$$I_t = 2, 3 \ K' \varepsilon_{bc} I_0$$  \hspace{1cm} (21.9)

or at constant $I_0$:

$$I_t = Kc$$  \hspace{1cm} (21.10)

**Quenching and other special effects**

When the fluorescence of a species is attenuated as a result of its reaction with an analyte, the signal decreases. This effect is called quenching and can be used for quantification purposes, primarily for the determination of anions. Quenching can also be an unwanted effect in the case of dissolved oxygen (see later).

Also, if the analyte is too concentrated, self-quenching may occur when fluorescing molecules collide and lose their excitation energy by radiationless transfer. The fluorescence versus concentration curve may have a maximum and then actually show a decrease in fluorescent power with increasing concentration. It is imperative in quantitative determinations to be aware of this problem, since a given fluorescent power can correspond to two values of concentration.

Thus, a plot of the fluorescence power of a solution versus the concentration of the emitting species should be linear at low concentrations. Limiting factors for linearity are not only the concentration of the solute, but also factors such as the blank fluorescence, quenching and absorbance of exciting radiation by the solvent.

**Instrumentation**

The basic components of analytical instruments for absorption and fluorescence spectroscopy are alike in function and general performance requirements. Most spectroscopic instruments are made up of five components:
• stable source of radiant energy
• wavelength selector that permits the isolation of a restricted wavelength region
• sample container
• radiation detector, which converts radiant energy to a measurable signal (usually electrical)
• signal processor and readout.

Ultraviolet and visible spectrophotometry

Colorimeters

Colorimeters usually employ a single tungsten radiation source in combination with broad–band (approx. 30 nm) optical filters of nominal wavelength, or narrow bandwidth interference filters with a defined wavelength for use in the visible range. The range of linearity of the colorimeter may be constrained by the relatively broad spectral bandwidths employed, and therefore should be checked carefully for each type of assay.

Single-beam spectrophotometers

These differ from the colorimeter in using a prism or a high–quality diffraction grating monochromator, together with an additional intense source of UV radiation, usually a deuterium (or hydrogen) lamp. They are capable of high precision, particularly in the optimum absorbance range (0.3 to 0.6 absorbance units). The reference and sample cells must be moved manually in and out of the radiation beam at each wavelength, so it is not practicable to scan a spectrum using such a device.

Double-beam spectrophotometers

Double-beam spectrophotometers use similar high–quality optical components to those in the single–beam instrument. However, the radiation from the monochromator is split into two identical beams by a rotating mirror. One beam passes through the sample and the other through the reference cell, before being recombined to focus on the detector. Each signal is processed appropriately by the detector electronics to measure the absorbance 10 to 20 times per second, which gives full compensation for cell and solvent absorption. A scan motor drives the monochromator to give a constant wavelength change per second, which is synchronised with a recorder or digital plotter to present the spectrum. For broad
bands, scan speeds up to 2 nm/s can be employed. However, some computer-controlled spectrophotometers with fast data-processing capabilities can scan at rates approaching 20 nm/s, and still maintain spectral fidelity even for sharp peaks.

**Diode-array spectrophotometers**

These employ multichannel detectors. The most commonly encountered detector of this type is the linear photodiode array. The reversed-optics mode is employed, so that radiation is passed through the sample or reference cell, then dispersed by a diffraction grating polychromator and detected by a device that comprises several hundred diodes. Each photodiode registers the integrated intensity of radiation incident on it, which is determined by the spectral dispersion:photodiode ratio. If, for example, a 200 nm bandwidth of radiation is dispersed across 256 photodiodes, the nominal resolution per photodiode is 0.78 nm.

A spectrum in a specified range is acquired within 20 ms. The analogue signals from each photodiode are digitised and transferred to a computer, where they are corrected for dark current response and transformed to absorbance. A number of digital techniques are available to increase sensitivity, extending the use of rapid-scanning detectors to multicomponent analysis, reaction kinetics, tablet dissolution tests, process control and detection in HPLC (Fell et al. 1982).

**Fluorimetry**

**Single-beam fluorimeters**

A single-beam spectrofluorimeter consists of a radiation source (usually a mercury or xenon lamp), a primary filter (excitation), a sample cell, a secondary filter (emission) and a fluorescence detection system. In most such fluorimeters the detector is placed on an axis at 90° from that of the exciting beam. This right-angle geometry permits the exciting radiation to pass through the test specimen and not contaminate the output signal received by the fluorescence detector. However, the detector unavoidably receives some of the exciting radiation as a result of the inherent scattering properties of the solutions themselves, or if dust or other solids are present. Filters are used to eliminate this residual scatter. The primary filter selects the short-wavelength radiation able to excite the test specimen, while the secondary filter is normally a sharp cut-off filter that allows the longer wavelength fluorescence to be transmitted, but blocks the scattered excitation. Most fluorimeters use
photomultiplier tubes as detectors. The photocurrent is amplified and read out on a meter or recorder.

**Scanning spectrofluorimeters**

When at least one monochromator (grating or prism) is used instead of a filter, the instrument is called a spectrofluorimeter. The use of gratings instead of filters makes the instrument superior in wavelength selectivity, flexibility and convenience. More complex spectrofluorimeters employ two diffraction gratings (or prisms) to select the fixed excitation wavelength ($\lambda_{ex}$) and the fixed wavelength ($\lambda_{f}$), together with a high-intensity xenon source, scanning motors and electronic compensation for variations in source intensity as the wavelength is varied.

**Coupled techniques**

Where large numbers of samples must be analysed quickly, the use of automatic instruments becomes viable. One path is to use the automated sample handling in the flow injection analysis (FIA) technique.

FIA is an example of a continuous flow system: the sample becomes part of a flowing stream in which the unit operations of the analysis take place as the sample is carried from the injection point to a flow-through measuring device (such as a photometer) and finally to waste.

The intensity of the radiation that reaches the detector is recorded continuously; when an absorbing species is passing through, a sharp peak is generated with the height of the peak proportional to the analyte concentration. Sample sizes are mostly in the range 10 to 30 μL.

Instruments are available with the ability to carry out manipulations such as in-line heating, in-line distillation, in-line UV-digestion or in-line extraction, so that UV and/or visible spectroscopy can be used for a wide variety of compounds.

In multicomponent systems, the limited specificity for spectroscopic methods can be an important drawback and a restriction for analysis. With the technique of liquid chromatography, a wide variety of macromolecules and ionic species in complex mixtures can be separated. Detection systems depend upon the nature of the component of interest, but the most widely used detectors in liquid chromatography are based upon UV or visible...
radiation. Photometers often make use of the 254 nm and 280 nm lines from a mercury source, because many organic functional groups absorb in this region. However, to achieve an adequate specificity, often diode arrays are used, which are able to display an entire spectrum as an analyte exits the column [liquid chromatography with diode array detector (LC–DAD)]. Compared to single-wavelength detection, which provides no information about peak purity, the diode array’s full spectra comparison provides results with a far greater confidence level.

Generally, the LC–DAD technique is applied for toxicological screening. Substances are identified on the basis of both retention time and UV spectrum (Bogusz and Wu 1991; Lambert et al. 1995; Tracqui et al. 1995; Elliott and Hale 1998). The diode array can also be connected to a mass spectrometer (LC–DAD–MS), which increases the sensitivity and gives an extra confirmation of the component’s identity.

For components of interest that fluoresce, the chromatographic system can be equipped with a fluorimetric detector (LC–FL). In the case of weakly fluorescent or non-fluorescent drugs, a number of well-characterised derivatisation reactions are available. These include dansyl chloride (5-dimethylaminonaphthalene-1-sulfonyl chloride) for primary and secondary amines and phenolic hydroxyl groups, and fluorescamine {4-phenylspiro-[furan-2(3H), 1′-isobenzofuran]-3,3′-dione}, and o-phthalaldehyde for primary amines. Derivatisation reactions of this type have extended the scope of fluorescence detection in HPLC significantly.

The same type of detectors (DAD/fluorescence) can be coupled with capillary electrophoresis (CE–DAD or CE–FL) or ion chromatography to determine charged components in complex mixtures.

**Data processing and presentation of results**

**Single component systems**

Where only one component in the sample absorbs significantly, the wavelength is chosen to coincide with the centre of a broad maximum in the spectrum to minimise wavelength-setting errors. If the spectrum has no suitable maximum, a flat absorption minimum can be used, provided that the consequent loss of sensitivity is acceptable. Wavelengths near the extremities of the UV and visible ranges must be avoided, because of the effects of stray-light errors.
Accurate measurements of a drug in solution may be difficult because of non-specific absorption. In these circumstances, the geometric correction devised by Morton and Stubbs is sometimes applied. This assumes that the non-specific absorption varies linearly with wavelength over the range measured. Taking a solution of pure drug, two equi-absorptive points are selected, one at a lower wavelength ($\lambda_1$) and the other at a higher wavelength ($\lambda_3$) than that of the peak maximum ($\lambda_2$). Any irrelevant absorption in the sample increases the observed absorbance of one equi-absorptive point (usually $\lambda_1$) more than the other ($\lambda_3$). A simple geometrical calculation involving absorbances at $\lambda_1$ and $\lambda_3$ enables the absorbance at $\lambda_2$ to be corrected for the non-specific absorption (Donbrow 1967). The assumption of linearity of the irrelevant absorption can be tested by subtracting the theoretical curve for the calculated quantity of pure material and inspecting the residual difference spectrum.

The classic example of a pharmacopoeial assay based on the Morton–Stubbs correction is that for vitamin A alcohol and the ester (Stationery Office 2002, p. 1783). Other techniques proposed for the correction of non-specific absorption include difference spectrophotometry, second-derivative spectrophotometry, the use of orthogonal polynomials, and chemical or physical transformation of the drug to give absorption at a longer wavelength.

**Multicomponent systems**

The absorption spectra of two or more drugs of interest often overlap. Subject to certain conditions, the Vierordt method of simultaneous equations can be employed to obtain the individual concentrations (Glenn 1960). If each of n drugs obeys the Beer–Lambert Law over the concentration range of interest, and if the law of additivity of absorbances applies, then the total absorbance, $A^\lambda_T$, observed at any wavelength $\lambda$ is given by the sum:

$$A^\lambda_T = \sum_{i=1}^{n} A^\lambda_i = \sum_{i=1}^{n} k_i^\lambda c_i b$$  \hspace{1cm} (21.11)

where the subscript $i$ denotes each component in the system. The term $k_i^\lambda$ represents the absorptivity $a$ (L/g/cm), the specific absorbance ($A_{1\%}^{1\text{cm}}$), or the molar absorptivity $\varepsilon$ (L/mol/cm), as determined by the units selected for concentration $c_i$.

For a two-component system, two wavelengths $\lambda_1$ and $\lambda_2$ are selected (as discussed below) and two corresponding simultaneous equations set up:

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The selection of appropriate wavelengths and the use of accurate absorptivity values are clearly crucial. Generally, \( \lambda_1 \) is the \( \lambda_{\text{max}} \) for component 1, while \( \lambda_2 \) is the \( \lambda_{\text{max}} \) for component 2, provided that at these wavelengths the absorptivity of the overlapping component is small. If the spectra of both components are very similar, the errors of the method increase appreciably as the difference between the absorptivity ratios tends to zero.

Although this method should apply to the analysis of three or more components, in practice it is often difficult to select wavelengths that fulfil all the requisite conditions. However, computer–aided spectrophotometers exploit the ‘principle of overdetermination’, in which the number \( (m) \) of observation wavelengths exceeds the number \( (n) \) of components known to be present. This gives an \( n \times m \) matrix of data that can be solved readily by standard matrix algebra.

The limit test for amphotericin A (a tetraene, \( \lambda_{\text{max}} 300 \text{ nm} \)) in the antifungal antibiotic amphotericin (consisting primarily of amphotericin B, a heptaene, \( \lambda_{\text{max}} 380 \text{ nm} \)) is an example of such a two–component analysis (Stationery Office 2002, p. 129).

**Difference spectrophotometry**

Difference spectrophotometry is a method of compensating for the presence of extraneous materials in a sample that would otherwise interfere with the spectrum of the drug being determined. It involves the measurement of the absorbance difference, at a defined wavelength, between two samples in one of which a physical or chemical property of the drug has been changed. It is assumed that the spectrum of the drug can be changed without affecting the spectrum of the interfering material. Alternatively, the absorbance difference may be measured between the sample and an equivalent solution without the drug. Difference spectrophotometry is sometimes described as ‘differential spectrophotometry’, but this term is not recommended because of its possible confusion with derivative spectrophotometry.

Many suitable methods for physical and chemical modification of the drug absorbance have been reported. For example, the bathochromic effect (also discussed later) is used in the
difference spectrophotometric assay of barbiturates. The absorbance of the sample at about pH 10 ($A_{10}$), to which the mono–anionic species contributes ($A_B$), is used to compensate for the absorption of interfering endogenous materials ($A_M$) that have been carried through the extraction procedure. The sample absorbance at pH 13 ($A_{13}$), to which the di–anionic species ($A_D$) contributes, is measured at about 260 nm with reference to the sample absorbance at pH 10 ($A_{10}$), so that:

\[ A_{13} = A_D + A_M \]  
\[ A_{10} = A_B + A_M \]

Thus:

\[ \Delta A = A_{13} - A_{10} = A_D - A_B \]  

If:

\[ \Delta \varepsilon = (\varepsilon_D - \varepsilon_B) \]

Then:

\[ \Delta A = \Delta \varepsilon c \]

Thus, the difference absorbance can be related readily to concentration by prior calibration of the constant $\Delta \varepsilon$, or the concentration may be found by simple proportion:

\[ \frac{\Delta A_{\text{test}}}{\Delta A_{\text{standard}}} = \frac{c_{\text{test}}}{c_{\text{standard}}} \]

It should, however, be established that $\Delta A$ is a linear function of concentration ($c$) over the range required. It is convenient to select for the analytical wavelength a value that corresponds to a maximum in the difference spectrum, obtained by scanning the sample and reference solution over an appropriate wavelength range.

Difference spectrophotometry can be used for quality control in cases where the interfering material is well-defined, because an appropriate dilution of a suitable reference solution can be used in the reference cell. The difference absorbance is, however, susceptible to systematic error when there is uncertainty in the concentration of interfering materials in
the samples to be assayed. This error increases in proportion to the ratio of the molar absorptivity of the interference to that of the drug.

A further technique to correct for absorptive interferences by difference measurement is based on dual–wavelength spectrophotometry. In this method, two monochromatic beams at different wavelengths are passed through the same sample. One wavelength ($\lambda_1$) is generally characteristic of the drug, while the other ($\lambda_2$) is selected carefully so that the absorbance is equivalent to the level of absorptive interference ($A_{\lambda_m}^2$) anticipated at the analytical wavelength ($\lambda_1$). Thus, the second radiation beam is analogous to the reference cell employed in conventional difference spectrophotometry, and the difference in absorbance at the two wavelengths ($\Delta A$) represents the absorption of drug ($A_{\lambda_m}^2$) corrected for interference:

$$A_{\lambda_1}^2 = A_{n}^{\lambda_1} + A_{m}^{\lambda_2} \quad (21.20)$$

$$A_{\lambda_2}^2 = A_{m}^{\lambda_2} \quad (21.21)$$

$$\Delta A = A_{\lambda_1} - A_{\lambda_2} = A_{n}^{\lambda_1} \quad (21.22)$$

A classic application of this method is the correction of Rayleigh scatter in samples of biological origin.

**Derivative spectrophotometry**

In derivative spectrophotometry, the absorbance ($A$) of a sample is differentiated with respect to wavelength ($\lambda$) to generate the first, second or higher–order derivatives:

$$A = f(\lambda), \text{ zero order}$$

$$\frac{dA}{d\lambda} = f'(\lambda), \text{ first derivative}$$

$$\frac{d^2A}{d\lambda^2} = f''(\lambda), \text{ second derivative}$$
and so on.

Derivative spectra often yield a characteristic profile, in which subtle changes of gradient and curvature in the normal (zero order) spectrum are observed as distinctive bipolar features. The first derivative of an absorption spectrum represents the gradient at all points of the spectrum and can be used to locate ‘hidden’ peaks, since \( \frac{dA}{d\lambda} = 0 \) at peak maxima. However, second- and higher even-order derivatives are potentially more useful in analysis.

The even-order derivatives are bipolar functions of alternating sign at the centroid (i.e. negative for 2nd, positive for 4th, etc.), whose position coincides with that of the original peak maximum. To this extent, even-derivative spectra bear a similarity to the original spectrum, although the presence of satellite peaks that flank the centroid adds a degree of complexity to the derivative profile. A key feature is that the derivative centroid peak width of a Gaussian peak decreases to 53%, 41% and 34% of the original peak width, in the second, fourth and sixth orders, respectively. This feature can increase the resolution of overlapping peaks. However, the increasingly complex satellite patterns detract from resolution enhancement in higher derivative spectra.

An important property of the derivative process is that broad bands are suppressed relative to sharp bands. This effect increases with increasing order of the derivative, since the amplitude \( D_n \) of a Gaussian peak in the \( n \)th derivative is inversely related to the original peak width \( W \), raised to the \( n \)th degree:

\[
D_n \sim W^{-n}
\]  \hspace{1cm} (21.23)

Thus, for two coincident peaks of equal intensity, the \( n \)th derivative amplitude of the sharper peak \( (x) \) is greater than that of the broader peak \( (y) \) by a factor that increases with derivative order:

\[
\frac{D_{nX}}{D_{nY}} = \left[ \frac{W_Y}{W_X} \right]^n
\]  \hspace{1cm} (21.24)

This property leads to the selective rejection of broad, additive spectral interferences, such as Rayleigh scattering.

If the Beer–Lambert Law is obeyed, that is:
\[ A = \varepsilon b c \]  

(21.25)

\[ \frac{dA}{d\lambda} = \frac{d\varepsilon}{d\lambda} bc \]  

(21.26)

\[ \frac{d^2A}{d\lambda^2} = \frac{d^2\varepsilon}{d\lambda^2} bc \]  

(21.27)

And similarly for higher derivatives, where \( \varepsilon \) is the molar absorptivity (L/mol/cm), \( b \) the cell path length (cm) and \( c \) the concentration (mol/L).

For quantitative work, the amplitude of a derivative peak can be measured in various ways. Although the true derivative amplitude is that measured with respect to the derivative zero, the most common practice is to record the amplitude with respect to a satellite in the spectrum, which affords an extra degree of suppression of interference from extraneous substances. It is essential to run standards in bracketing sequence with the samples, and thus subject both to the same experimental conditions. It should be established that the graphic derivative adopted fulfils the analytical criteria of linear response with concentration, regression through or close to the origin, independence from interfering substances and optimum precision.

In general, methods for generating derivative spectra fall into two classes. These are optical methods, which operate on the radiation beam itself, and electronic or digital methods, which operate on the photometric detector output. The electronic analogue device generates the required derivative as a function of time as the spectrum is scanned at constant speed (d\( \lambda \)/dt), and therefore the derivative amplitude varies with the scan speed, slit width and gain. Moreover, the signal–to–noise ratio has been reported to degrade by approximately a factor of two in each successive derivative order.

Alternatively, a microcomputer, employing one of a number of digital algorithms, can be used to produce smoothed derivative spectra. This is carried out in real time or by post-run processing of the digitised spectrum. The digital approach is employed commonly in contemporary spectrophotometers, because of the widespread adoption of microprocessors for instrument control and data handling, coupled with the addition of powerful software for
further data processing.

Although transformation of a spectrum to its second- or higher-order derivative often yields a more highly characteristic profile than the zero-order spectrum, the intrinsic information content of the data is not increased; indeed, some data, such as constant ‘offset’ factors, are lost. However, the derivative method tends to emphasise subtle spectral features by presenting them in a new and visually more accessible way. The method is generally applicable in analytical chemistry and can be used equally for resolution enhancement of electrochemical, chromatographic or thermal analysis data.

Derivative spectrophotometry has found significant application in clinical, forensic and biomedical analysis (Gill et al. 1982). In forensic toxicology, the suppression of the absorbance from interfering substances by second-derivative spectrophotometry is well demonstrated in studies on amphetamine in a homogenised liver extract. Transformation of the zero-order spectrum (A) to its second derivative (B) using a rapid-scanning multichannel spectrophotometer permits the characteristically sharp benzenoid peaks of amphetamine to be detected and compared with an authentic standard (D), while the interfering background absorption is reduced substantially. The second- and fourth-derivative method for biological background correction can give a ten-fold increase in the detection limit of serum paraquat in cases of poisoning (Fell et al. 1981). The derivative method can be combined successfully with difference spectrophotometry, to give second-derivative difference spectra, in which enhanced discrimination against interfering substances and sharpened fine structural features are observed.

**Analytical fluorimetry**

The fluorescence properties of a compound are characterised by two spectra. An excitation spectrum is obtained by monitoring the fluorescence at a convenient fixed wavelength $\lambda_f$, while scanning the excitation monochromator at a fixed speed up to a wavelength no higher than $\lambda_f$. The excitation spectrum should, in principle, be comparable with the absorption spectrum. The fluorescence spectrum is obtained by illuminating the sample at a convenient fixed excitation wavelength $\lambda_{ex}$ and scanning the emission monochromator at a fixed speed over a wavelength range no lower than $\lambda_{ex}$. For interlaboratory comparisons, corrected fluorescence and excitation spectra should be obtained using one of the generally available digital or instrumental techniques.

Some spectrofluorimeters are able to scan the excitation and emission monochromators
synchronously to yield fluorescence spectra, which are generally simpler and considerably sharper than the conventional fluorescence spectrum. With computer-aided spectrofluorimetry, the acquisition and digital storage of fluorescence spectra are possible. These can then be manipulated in various ways to give the derivative spectrum in which fine structure is accentuated, the difference spectrum, or multiwavelength spectral deconvolution, to calculate the concentration of known overlapping components (Winfield et al. 1984).

In another digital technique, a series of fluorescence spectra are acquired while sequentially stepping the excitation wavelength. When these spectra are combined to give a matrix of \((I_f, \lambda_f, \lambda_{ex})\), a three-dimensional isometric projection is presented. This type of graphic presentation is described as an ‘emission-excitation matrix’ or ‘fluorogram’. The data can also be plotted as the equivalent two-dimensional plot of isointensity contours in the \((\lambda_f, \lambda_{ex})\) plane. Three-dimensional graphics are increasingly used for the qualitative comparison of fluorescent molecules, as in the example of promethazine and its principal degradation product, promethazine sulfoxide.

**Instrument performance checks**

While instruments with conventional optics may require frequent calibration because they have many moving parts, diode array spectrophotometers with no moving parts are extremely reproducible and stable, both in the short and the long term.

Following the recommendations of the European Pharmacopeia (Council of Europe 2002) for UV and visible spectrophotometry, the wavelength and the absorbance must be calibrated. The wavelength scale must be verified using the absorption maxima of holmium perchlorate solution, the line of a hydrogen or deuterium discharge lamp or the lines of a mercury vapour lamp. The permitted tolerance is ±1 nm for the UV range and ±3 nm for the visible range. The absorbance should be checked using suitable filters or a solution of potassium dichromate of 60 mg/L in 0.005 M sulfuric acid at the wavelengths indicated in the following Table:

<table>
<thead>
<tr>
<th>Wavelength (nm)</th>
<th>Specific absorbance ((A^1))</th>
<th>Maximum tolerance</th>
</tr>
</thead>
<tbody>
<tr>
<td>235</td>
<td>124.5</td>
<td>122.9–126.2</td>
</tr>
<tr>
<td>257</td>
<td>144.5</td>
<td>142.8–146.2</td>
</tr>
</tbody>
</table>
The level of stray light should be assessed, since it increases with instrument age. It may be detected at a given wavelength with suitable filters or a 1.2% w/v potassium chloride solution. The European Pharmacopoeia (Council of Europe 2002) requires that the absorbance be greater than two at a wavelength between 198 and 202 nm when compared with water as compensation liquid.

In some assays it is necessary to specify the minimum desirable resolution, since changes in the spectral bandwidth (or monochromator slit-width) can seriously affect the observed absorbance of sharp peaks. The European Pharmacopoeia (Council of Europe 2002) requires that the spectral bandwidth employed should be such that further reduction does not lead to an increase in measured absorbance. This is particularly important for drugs that have aromatic or strongly conjugated systems (e.g. diphenhydramine, phenoxyethylpenicillin, and amphotericin A and B). In such cases, a spectral bandwidth of more than 1 nm leads to a reduction in observed absorbance at the peak maximum (and conversely an increase in absorbance at a peak minimum), since the recorded absorbance is the mean of that over the whole bandwidth at that wavelength. Although increasing the slit width gives a better signal-to-noise ratio, a slit width of 2 nm is adequate for most bands, with 1 nm or 0.5 nm being used for very sharp peaks.

For qualitative analysis the resolution can be measured by recording the spectrum of a 0.02% solution of toluene in hexane. The minimum ratio of the absorbance at the maximum at 269 nm to that at the minimum at 266 nm is stated in the monographs of the European Pharmacopoeia (Council of Europe 2002).

According to the principles of good laboratory practice (GLP), the apparatus should be periodically inspected, cleaned, maintained and calibrated according to the laboratory’s Standard Operating Procedures. Records of procedures should be maintained. Calibration should, where appropriate, be traceable to national or international standards of measurements.

Examples of interesting options on the system are a logbook database in which lamp changes and defects are registered. Also, a key-lock function is now a common part of a quality system to avoid effects from unwanted keystrokes.
In fluorescence the calibration of excitation and emission monochromator wavelengths should be checked regularly by the use of sharp lines from the instrument’s own radiation source (e.g. xenon lines at 450.1, 462.4, 467.1 and 473.4 nm) or the use of sharp fluorescence peaks in solutions or glasses of trivalent lanthanide ions (Te, Eu). Other fluorescence standards in common use are ovulene and other polycyclic aromatic hydrocarbons with fine vibrational structures such as naphthalene and anthracene. In practice, it is necessary only to calibrate one monochromator, since the other can then be calibrated by the Rayleigh scattered radiation using a sample of colloidal silica in the sample position. The use of an auxiliary light source, such as a mercury light pen, is the least satisfactory method for wavelength calibration.

It is recommended that the performance of each computerised system be verified for the proper functioning of data acquisition, method-related calibration and reporting. Usually, test packages are commercially available from the supplier.

**Sample preparation and sample presentation**

The most frequent mode of sample presentation is as a dilute solution, although gases and solid surfaces can also be examined. Combinations of UV and visible spectrophotometry or spectrofluorimetry with HPLC are particularly advantageous for sensitive and selective detection of chromophores and/or fluorophores.

**Ultraviolet and visible spectrophotometry**

**Cells**

In the visible region, a matched pair of glass cells can be used, but they are inappropriate for the UV region because of the poor transmission properties of glass in this range. Fused silica or quartz cells have high transmittance from 190 to 1000 nm, and are therefore the cells of choice. The path length employed is usually 1.00 cm; longer path length cells are used for poorly absorptive drugs and/or where the concentration is low. Flow cells designed to minimise turbulent flow through the cell are used to monitor changes in absorbance during a reaction, for tablet dissolution studies, or for HPLC; care should be taken that the cell walls do not block the radiation beam, otherwise variable errors are introduced. Cells provided with a thermostat are used for studies on enzymatic and other processes in which temperature is a key parameter.
The meticulous handling and care of cells is a necessary condition for precise and accurate measurement. Cells should be cleaned carefully, filled with an appropriate solvent and matched for absorbance to less than 1%. Each pair of cells should be marked on the base in soft pencil to identify the set and its normal orientation. The tolerance on the path length of the cells used is ±0.005 cm (Council of Europe 2002).

When filled with the same solvent, the cells intended to contain the solution to be examined and the compensation liquid must have the same transmittance. If this is not the case, an appropriate correction must be applied. It is convenient to designate the more strongly absorbing cell as the ‘sample’ cell, the other cell being coded as ‘reference’. In this way the cell constant (i.e. the difference in absorbance at the measurement wavelength when filled with solvent) will be positive and can thus be subtracted from each absorbance reading. Moreover, the possibility of ‘oscillating error’ introduced by randomly changing the cell orientation during a series of measurements is eliminated. The ‘cell constant’ should be checked regularly at the measurement wavelength when filled with an appropriate solvent, or by scanning the baseline over the wavelength range.

Cells should be cleaned scrupulously after use. If they have contained aqueous solutions, they can be cleaned readily by repeated rinsing with distilled water or by soaking overnight in a very dilute solution of detergent; special detergents should be used to clean cells contaminated with biological material. Periodically, it is good practice to soak cells and

Transmittance ranges for various optical materials. (From Skoog et al. 1996, p. 529.)
stoppers thoroughly in a fresh solution of chromic acid, followed by copious rinsing with distilled water, to restore their matched performance. Cells that have been used with organic solvents require special care, with a sequence of solvents ending in spectroscopic ether being convenient to obtain dry, clean cells. In all cases, the manufacturer’s instructions should be followed, when available. Sharp glass or metal objects should not be introduced into a cell, lest the internal surface be scratched. The outside optical surfaces should be polished before use with a soft cloth or photographic lens tissue. Cells should be stored in pairs, dry and in a protective container.

**Solvents**

Solvents used in spectrophotometry must meet certain requirements to ensure successful and accurate results. The solvent chosen must dissolve the sample, yet be compatible with cuvette materials. The solvent must also be relatively transparent in the spectral region of interest. To avoid poor resolution and difficulties in spectrum interpretation, a solvent should not be used for measurements near or below its UV cut-off (i.e. the wavelength at which absorbance for the solvent alone approaches one absorbance unit).

Once a solvent is selected based on physical and spectral characteristic, its purity must be considered. The absorbance curve of a solvent, as supplied, should be smooth (i.e. have no extraneous impurity peaks in the spectral region of interest). Solvents especially purified and certified for spectrophotometric use are available from suppliers.

**Good Laboratory Practice**

According to GLP, chemicals, reagents and solutions should be labelled to indicate identity (with concentration if appropriate), expiry date and specific storage instructions. Information concerning source, preparation date and stability should be available. The expiry date may be extended on the basis of documented evaluation or analysis.

Records, including test-item and reference-item characterisation, date of receipt, expiry date, quantities received and used in studies should be maintained. Handling, sampling and storage procedures should be identified in order that the homogeneity and stability are assured to the degree possible, and that contamination and mix-up are precluded. Storage containers should carry information on identity, expiry date and specific storage instructions. The stability of test and reference items under storage and test conditions should be known for all studies.
Fluorimetry and spectrofluorimetry

Cells

Specimen cells used in fluorescence measurements may be round tubes or rectangular cells similar to those used in absorption spectrophotometry, except that they are polished on all four vertical sides. A convenient test specimen size is 2 to 3 mL, but some instruments can be fitted with small cells holding 100 to 300 μL or with a capillary holder that requires an even smaller amount of specimen.

Temperature regulation is often important in fluorescence spectrophotometry. For some substances, fluorescence efficiency may be reduced by as much as 1 to 2% per degree of temperature rise. In such cases, if maximum precision is desired, temperature-controlled sample cells are useful. For routine analysis, it may be sufficient to make measurements rapidly enough so that the specimen does not heat up appreciably from exposure to the intense light source (USP 2002).

Solvents

Change of solvent may markedly affect the intensity and spectral distribution of fluorescence. It is inadvisable, therefore, to alter the solvent specified in established methods without careful preliminary investigation. Many compounds are fluorescent in organic solvents, but virtually non-fluorescent in water; thus, a number of solvents should be tried before it is decided whether or not a compound is fluorescent. In many organic solvents, the intensity of fluorescence is increased by elimination of dissolved oxygen, which has a strong quenching effect. Oxygen may be removed by bubbling an inert gas, such as nitrogen or helium, through the test specimen.

Interpretation of spectra and qualitative analysis

Ultraviolet and visible spectrophotometry

Spectrophotometric measurements with UV or visible radiation are useful for detecting components that contain unsaturated groups or atoms such as sulfur or halogens. A drug, its impurity or a metabolite can also be transformed selectively so that the spectrum is shifted to the visible region and away from interference caused by another drug, formulation components, or biological substances, and thereby confer a further degree of
specificity.

However, specific identification of a compound can rarely be made on the basis of UV spectral evidence alone. Often, the spectrum serves as confirmatory evidence of identity, in support of other analytical data. The general approach usually followed in qualitative applications is first to establish by independent means (e.g. chromatography) that the material consists substantially of one absorbing component. Spectra are then recorded in aqueous acidic, basic and ethanolic or methanolic solution. The wavelengths of the principal peaks and the corresponding absorptivity values are noted for each solvent system. By comparison with data tabulated in ascending wavelength order, a number of compounds with absorbing properties similar to the test substance are selected (using a wavelength window of ±2 nm). One must be aware that, in general, the spectrum of metabolites of a component matches closely the spectrum of the component itself.

Further evidence can be deduced from the absorptivity ratios of peaks within a spectrum; moreover, the change in these ratios together with the shift in peak positions as the pH is changed can be diagnostic. If a drug molecule ionises reversibly (i.e. without degradation), the family of curves for a constant concentration in acidic and basic solvents displays one or more isosbestic points at characteristic wavelengths, at which the absorbance is constant at all values of pH.

Spectral shifts are among the most useful diagnostic features in drug molecules that possess ionisable groups. A marked bathochromic shift (or ‘red’ shift) to longer wavelengths in alkaline solution is observed not only for most of the phenolic drugs, such as the phenolic oestrogens, but also in the case of hydroxypyridines, ketones, benzodiazepines, pyridones and nitro-compounds. The bathochromic shift is often large (<10 nm) and accompanied by an increase in molar absorptivity (hyperchromic effect) and loss of any fine structure.

This effect has been exploited, both for qualitative and quantitative purposes, for the analysis of barbiturates. In acidic or neutral solution, barbiturates show little absorption above 230 nm, but in 0.05 M borax buffer (pH 9.2), ionisation yields an intense conjugated chromophore with a well-defined maximum near 240 nm ($A^1_{1} = 400$ to 450). In sodium hydroxide solution (pH 13), a second stage of ionisation occurs (except in N-substituted derivatives) to extend further the conjugation and give a peak maximum near 255 nm. However, solutions in alkali are unstable through ring-opening, so that measurements must be made rapidly.
Effect of pH on the UV spectrum of phenobarbital: A, non-ionised barbiturate in 0.1 mol/L hydrochloric acid; B, mono-anion in 0.05 mol/L borax buffer pH 9.2; C, di-anion in 0.5 mol/L sodium hydroxide pH 13.

The hypochromic or ‘blue’ shift to shorter wavelengths is shown by aromatic amines in acid solution and is highly characteristic for many drugs. On acidification, the protonated quaternary ammonium group no longer participates in the chromophore so that the spectrum is shifted to lower wavelengths, sometimes by as much as 30 nm, with a sharp fall in absorptivity (hypochromic effect).

Dissociation of C5-substituted barbituric acids: (A) undissociated free acid; (B) mono-anion; (C) di-anion.

In addition to their use in characterising a chromophore, pH-induced shifts can also be exploited to shift a spectrum along the wavelength scale to obtain an interference-free window to measure an ionisable species in a mixture.
**Solvent effects**

A solvent for UV and visible spectroscopy must be transparent throughout the region of interest and should dissolve a sufficient quantity of the sample to give well-defined peaks. Moreover, consideration must be given to possible interactions with the absorbing species. For example, polar solvents, such as water, alcohols, esters and ketones, tend to suppress vibrational fine structure and should thus be avoided when spectral detail is desired. Non-polar solvents, such as cyclohexane, often provide spectra that more closely approach that of a gas. In addition, the polarity of the solvent often influences the position of absorption maxima. Consequently, a common solvent must be employed when comparing spectra for the purpose of identification.

When using an HPLC-UV system, any effects on the spectral characteristics of a substance brought about by various mobile phases must be considered when comparing spectra generated in this manner with those recorded from acidic, neutral and alkaline solutions. Moreover, potential effects brought about during gradient elution HPLC, in which the composition of the mobile phases is constantly changing, should be borne in mind. With acetonitrile, methanol, ethanol, isopropanol and other water-miscible solvents, no essential changes in the spectra may occur and direct comparison with other databases is feasible. However, changes in pH can have a significant effect on the UV spectra of compounds involved in an acid-base equilibrium (e.g. carboxylic acids, phenols, thiophenols) and compounds with basic nitrogen atoms. Direct comparison of spectra with other compendia of data, including those listed in this volume, is only valid when the mobile phases have the same pH.

**Spectrofluorimetry**

For compounds with appropriate fluorescence properties, this technique gives high sensitivity and high specificity. High sensitivity results from the difference in wavelengths between the exciting and fluorescence radiation. This results in a signal contrasted with an essentially zero background; it is always easier to measure a small signal directly than a small difference between two large signals, as is done in absorption spectrophotometry. High specificity results from dependence on two spectra, the excitation and emission spectra, and the possibility of measuring the lifetimes of the fluorescent state. Although in biological samples the fluorescence intensity of interfering substances may be relatively high, the sensitivity and selectivity of the method are generally such that fluorescent drugs and their metabolites can be analysed more readily than by conventional spectrophotometry. Two compounds that are excited at the same wavelength, but emit at
different wavelengths, are readily differentiated without the use of chemical separation techniques. Likewise, two compounds may fluoresce at the same wavelength but require different excitation wavelengths. Also, a fluorescent compound in the presence of one or more non–fluorescent compounds is readily analysed fluorimetrically, even when the compounds have overlapping absorption spectra. Non–fluorescent or weakly fluorescent compounds can often be reacted with strong fluorophores, which enables them to be determined quantitatively.

Many fluorescent species contain ionisable groups with fluorescent properties sensitive to pH. In some cases only one of the ionised species may be fluorescent. Examples are the barbiturates, which only fluoresce at an elevated pH in the di–anionic form. Phenol fluoresces at pH 7, but at pH 12, when it is converted into its anion, there is no fluorescence. Therefore, the relationship of fluorescence intensity with pH should always be examined as part of the method development.

Fluorescence intensity and wavelength often vary with the solvent. In most molecules, fluorescence decreases with a decrease in solvent viscosity as the probability of intermolecular energy transfer tends to be enhanced. The same effect occurs with an increase in temperature.

Quantitative analysis - ultraviolet and visible spectrophotometry

Fundamental basis

The basic principle of most spectrophotometric measurements involves comparing, under well–defined conditions, the absorption of radiation by the substance in an unknown amount with the same absorption of radiation by a known amount of the material being determined. In general, to obtain the maximum sensitivity it is best to work with radiation of a wavelength that is approximately equal to that for which the solution exhibits a maximum selective absorption.

Assuming that the linear range for compliance with the Beer–Lambert Law has been established and that the drug concentration has been adjusted within the optimum range for the type of instrument concerned, two approaches to quantification may be employed. If an acceptable reference standard of the drug is available, and if the calibration graph passes through zero, measurement of replicates of the standard (at a comparable concentration)
and of the tests are performed in bracketing sequence (i.e. each group of samples is preceded and followed by the standard), under identical conditions of solvent and temperature and using the same pair of matched cells. Each result should be corrected for the cell constant; the concentration of the test sample is then found by reference to the results from the standards.

Alternatively, the specific absorbance is used to calculate the sample concentration, using the absorbance measured in the specified solvent. A check on the accuracy of the absorbance scale is clearly essential. Wavelength accuracy is not so important.

The practical usefulness of reference–specific absorbance values \( (A^1) \) clearly depends on a number of factors. These include the state of purity of the substance, the solvent conditions originally used to establish the reference data, the precise conditions employed in the reference instrument and the extent to which they correspond with those of a particular test laboratory. It is therefore wise to ascertain the status of any absorptivity data in the literature. In the monographs in Part 2, the reliability of all \( A^1 \) values has been assessed and indicated (see General Notices). However, if a sample of the drug concerned is available in pure form, it is good practice to establish periodically a ‘local’ value of the absorptivity and to use this in calculating sample concentrations.

**Linearity issues**

The validity of the Beer–Lambert Law should be established for each drug under the measurement conditions to be used over an appropriate concentration range. For single–beam instruments, the absorbance range for precise measurements is between about 0.3 and 0.6 absorbance units, the optimum being at 0.43 absorbance units. For double–beam spectrophotometers, the optimum range lies between 0.6 and 1.2 absorbance units. Five or more standard solutions, with absorbances that span the working range, should be measured in duplicate in a matched pair of cells against the solvent as reference; the residual absorbance difference between the cells when filled with solvent (the cell constant) should be subtracted from each individual measurement and checked regularly.

The linearity of an analytical method is determined by mathematical treatment of the absorbance data of the standard solutions across the claimed range of the method. The treatment is normally a calculation of a regression line \( y = ax + b \), with \( y \) being the absorbance and \( x \) the concentration, by the method of least squares. The linearity is usually expressed by means of a correlation coefficient \( r \), where:
When \( r = 1 \), there is a perfect correlation. Usually, \( r \) values better than 0.995 can be obtained.

The intercept should not differ significantly from zero. If a significant non-zero intercept is obtained, it should be demonstrated that there is no effect on the accuracy of the method.

Frequently, the linearity is evaluated graphically in addition or alternatively to a mathematical evaluation. The evaluation is made by visual inspection of a plot of absorbance \( A \) as a function of analyte concentration \( c \). If any systematic positive or negative deviation is found, additional points should be inserted and the linear working range established.

Deviations from linearity are sometimes difficult to detect, so two additional graphic procedures can be used. The first is to plot the deviations from the regression line against the concentration or the logarithm of the concentration, if the concentration range covers several decades. For linear ranges the deviation should be distributed equally between positive and negative values.

Another approach is to plot the function \( A/bc \) (the absorptivity) on the y-axis and the corresponding concentrations on the x-axis. The line obtained should be horizontal over the full linear range. Parallel horizontal lines are drawn on the graph that correspond to, for example, 95% and 105% of the horizontal line. The method is then linear up to the point where the plotted relative response line intersects one of these two lines.

**International Conference on Harmonisation validation criteria**

The main objective of validation of an analytical procedure is to demonstrate that the procedure is suitable for its intended purpose. The objective of an analytical procedure should be understood clearly, since this will govern the validation characteristics that need to be evaluated. Typical validation characteristics that should be considered according to
the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) are: accuracy, precision (repeatability and intermediate precision), specificity, detection limit, quantitation limit, linearity and range. All these characteristics are also listed and explained in the USP 25 (USP 2002). For the quantitative determination of impurities, all these criteria should be evaluated, while for limit tests the specificity and detection limit are most relevant. For assays, all characteristics except detection limit and quantification limit are normally evaluated.

For linearity, the ICH recommends a minimum of five concentrations to be measured. The range is allocated the following limits:

- For the assay of a drug substance or a finished (drug) product – normally from 80 to 120 % of the test concentration.
- For content uniformity – covering a minimum of 70 to 130% of the test concentration, unless a wider more appropriate range, based on the nature of the dosage form (e.g. metered dose inhalers), is justified.
- For dissolution testing –±20% over the specified range.
- For the determination of an impurity – from the reporting level of an impurity to 120% of the specification.
- If assay and purity are performed together as one test and only a 100% standard is used, linearity should cover the range from the reporting level of the impurities to 120% of the assay specification.

To determine the accuracy, a minimum of nine determinations over a minimum of three concentration levels that cover the specified range is recommended.

Repeatability should be assessed using a minimum of nine determinations that cover the specified range for the procedure (e.g. three concentrations with three replicates each) or a minimum of six determinations at 100% of the test concentration.

To determine the detection limit, the ICH recommendations correspond with the USP 25 (USP 2002) and can be determined, for example, based on signal-to-noise ratios, where 2:1 or 3:1 is generally accepted, or on the standard deviation of the blank.

To calculate the quantification limit, both ICH and USP 25 (USP 2002) recommend a signal-to-noise ratio of 10:1. Both USP 25 (USP 2002) and the ICH recommend explicitly validation of the quantification limit by the analysis of a suitable number of samples known to be near or prepared at the quantification limit.
Quantitative analysis - fluorimetry

Fundamental basis

Excitation spectra are usually used to confirm the identity of components and to select an optimum excitation wavelength for quantitative analysis. The emission spectrum is then used for analytical applications.

Spectrofluorimetry differs from absorption spectrophotometry in not yielding an absolute scale of values, as it depends on the number of excited molecules and their method of relaxation. For this reason it is essential to employ a reference standard for quantitative measurements.

The linear relationship between the power of fluorescence and the concentration of the fluorescing solute forms the basis for quantification, the linearity constant for which may be established by calibration with standards. A plot of fluorescence readings against the concentration of the reference solutions furnishes the calibration curve. Glass reference filters are also suitable as a calibration standard.

Linearity and baseline issues

In dilute solution, the fluorescence intensity \( I_f \) for defined values of \( \lambda_{ex} \) and \( \lambda_f \) is related linearly to molar concentration \( (c) \), according to the approximate relationship:

\[
I_f = Kc \text{ at } \lambda_{ex}, \lambda_f
\]

under constant instrumental conditions.

Sensitivity can be increased by working at high excitation powers to give larger signal-to-noise ratios. Since the source intensity can change from time to time, fluorescence signals are not measured as absolute parameters. They are expressed rather in terms of relative fluorescence. All measurements are made relative to reference standards of known concentration. All readings must be corrected for background fluorescence.

A necessary condition is that the total absorbance \( (=\varepsilon bc) \) of the system should not exceed 0.05 absorbance units; otherwise progressively greater negative deviations from linearity are observed. At high drug concentrations, fluorescence intensity reaches a plateau. Beyond
this, fluorescence intensity actually decreases with increasing concentration, because of inner-filter effects, in which ground-state molecules absorb the fluorescence emitted by excited molecules.

It is essential to establish the range of linearity of the calibration curve of $I_f$ versus $c$, using at least five standard solutions, for which the condition that absorbance at the wavelength of maximum excitation is $<0.05$ absorbance units holds. Samples are usually analysed by single-point bracketing, taking a standard conveniently close to the anticipated sample value and calculating the result by simple proportion.

The sequence of standard measurements before and after measuring the sample permits any baseline drift to be compensated. For additional assay security, two-point bracketing can be employed, in which two standard solutions, one higher and the other lower than the concentration observed for the sample, are used in bracketing sequence.

Instrumental limitations caused by instability of the radiative source can be overcome by the ratio mode operation. A small fraction of the exciting radiation is directed to a reference photodetector, which is chosen primarily for wide wavelength response. The output signal is used as a monitor and, as the excitation radiation increases or decreases in power because of fluctuations in the source, there is a corresponding increase or decrease in relative fluorescence. [1] M. Bogusz and M. Wu, Standardized HPLC/DAD system, based on retention indices ...

Références