7 Molecular emission spectroscopy

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Fluorescence spectrophotometry

KEYPOINTS

Principles

- Certain molecules, particularly those with a chromophore and a rigid structure, can be excited by UV/visible radiation, and will then emit the radiation absorbed at a longer wavelength. The radiation emitted can then be measured.

Applications

- Determination of fluorescent drugs in low-dose formulations in the presence non-fluorescent excipients.
- In carrying out limit tests where the impurity is fluorescent or can be simply rendered fluorescent.
- Useful for studying the binding of drugs to components in complex formulations.
- Widely used in bioanalysis for measuring small amounts of drug and for studying drug–protein binding.

Strengths

- A selective detection method and can be used to quantify a strongly fluorescent compound in the presence of a larger amount of non-fluorescent material.
- Can be used to monitor changes in complex molecules such as proteins which are being used increasingly as drugs.

Limitations

- The technique only applies to a limited number of molecules.
- Fluorescence is subject to interference by UV absorbing species, heavy ions in solution, and is affected by temperature.

Introduction

Figure 7.1 illustrates the behaviour of an excited electron in a fluorescent molecule. In a non-fluorescent molecule when an electron is excited to the electronic excited state, it returns back to the ground state by losing the energy it has acquired through conversion of the excess electronic energy into vibrational energy. If a molecule has a rigid structure the loss of electronic energy through its conversion into vibrational energy is relatively slow and there is a chance for the electronic energy to be emitted as ultraviolet or visible radiation. The energy emitted is of lower energy than the energy absorbed because, as indicated in Figure 7.1, the excited electron moves to the lowest energy vibrational state in the excited state before returning to the ground state. Thus fluorescence emission is typically shifted by 50–150 nm towards a longer wavelength in comparison with the wavelength of the radiation used to produce excitation. The fluorescence spectrum of a molecule is, ideally, a mirror image of the longest wavelength band in the absorption spectrum of the molecule but often the spectrum is distorted due to partial overlap between the absorption and the emission spectra. Vibrational fine structure of the fluorescence band may be observed if the molecule does not interact with the solvent strongly (cf. UV spectra) and can be observed in the fluorescence spectra of polycyclic aromatic hydrocarbons such as anthracene. The shape of the fluorescence spectrum is independent of the wavelength used for excitation since the transition producing the fluorescence spectrum is always from the first excited state to the ground state. In a molecule containing a number of UV absorption bands, the longest wavelength maximum is the one associated most strongly with the production of fluorescence. In addition, the wavelength usually used to produce excitation is close to the λ max of the longest wavelength absorption band in the spectrum of the analyte.

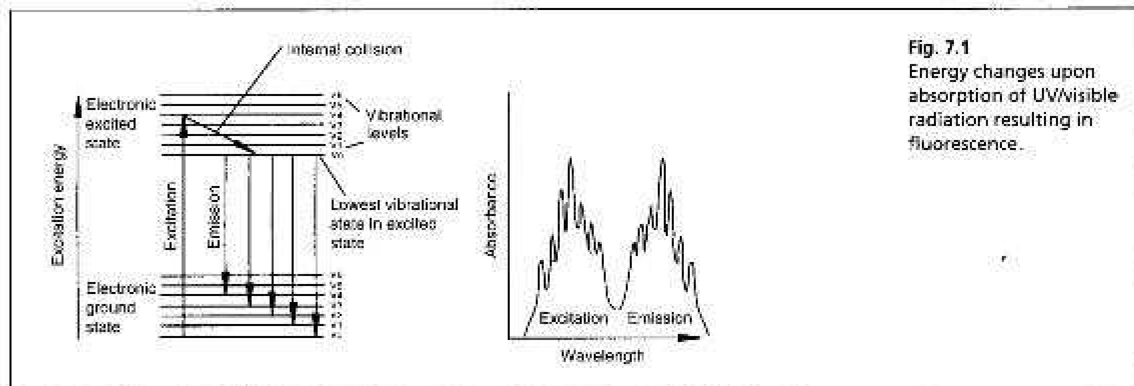

Fig. 7.1
Energy changes upon absorption of UV/visible radiation resulting in fluorescence.

Instrumentation

Figure 7.2 shows a schematic diagram of a fluorescence spectrophotometer. Since emission is being observed the light being emitted is observed at right angles to the light being used to excite the sample.

The instrument has two monochromators: one to select the wavelength to be used for excitation of the sample, the other to scan the wavelength range of the light emitted by the sample.

The lamp used, which is a quartz halogen lamp, produces radiation of high intensity to take advantage of the fact that the strength of the fluoresence is related to

the number of photons absorbed multiplied by the fluorescence quantum yield (ϕ). For strongly fluorescent compounds, ϕ is close to 1; for non-fluorescent compounds $\phi = 0$. The wavelength which gives maximum excitation is not necessarily exactly the same as the longest wavelength absorbance maximum in the compound since the intensity of light emitted by the quartz halogen lamp varies markedly with wavelength, unlike the deuterium and tungsten lamps used in UV/visible spectrophotometers. The lamp gives radiation of maximum intensity between 300 and 400 nm.

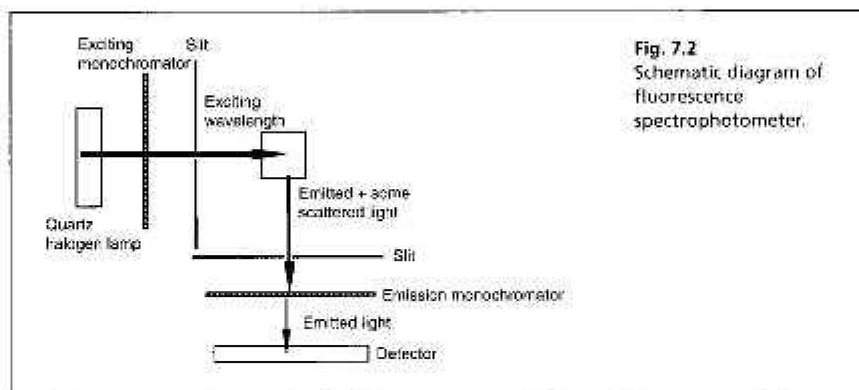

Fig. 7.2 Schematic diagram of fluorescence spectrophotometer.

Although the radiation emitted is observed at right angles to the exciting radiation, some of the exciting radiation can be detected by the emission detector because it is scattered by solvent molecules (Rayleigh scatter) or by colloidal particles in solution (Tyndall scatter). The presence of this scatter makes the use of the second monochromator necessary and also means that the fluorescence band has to be shifted by at least 20 nm beyond the excitation band for fluorescence measurements to be made without interference. Another, weaker, type of scatter which may be observed is Raman scatter. In Raman scatter, which is solvent dependent, the wavelength of the incident radiation is shifted to a longer wavelength by about 30 nm when methanol is used as a solvent and about 10 nm when chloroform is used as a solvent. Raman scatter is discussed in more detail later in this chapter.

Molecules which exhibit fluorescence

It is not entirely possible to predict how strongly fluorescent a molecule will be. For example adrenaline and noradrenaline differ in their structures by only a single methyl group but noradrenaline exhibits fluorescence nearly 20 times more intensely than adrenaline. Generally, fluorescence is associated with an extended chromophore/auxochrome system and a rigid structure. Quinine (Fig. 7.3) is an example of a strongly fluorescent molecule as might be expected from its extended chromophore and rigid structure. The chromophore in ethinyloestradiol is just an aromatic ring but the presence of a phenolic hydroxyl group in combination with rigid ring structure in the rest of the molecule renders it fluorescent (Fig. 7.3).

Figure 7.4 shows the fluorescence spectrum of ethinyloestradiol. When the fluorescence spectrum of the molecule is scanned with a wavelength of 285 nm being used for excitation, two maxima are seen. The maxima at 285 nm is due to scatter of the exciting radiation and the second more intense maximum at 310 nm is

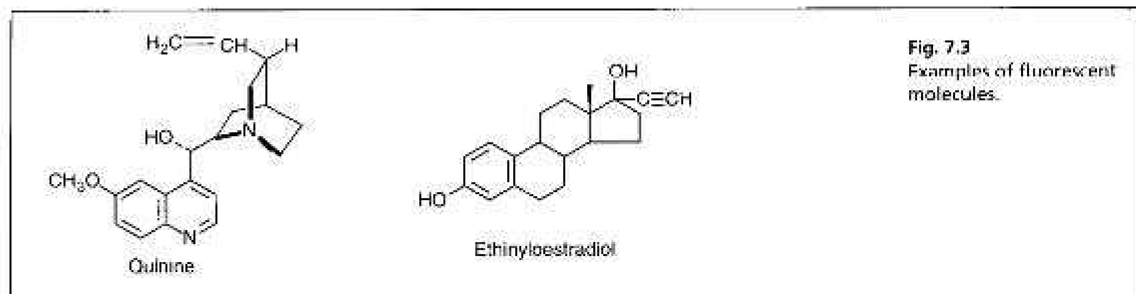

Fig. 7.3 Examples of fluorescent molecules.

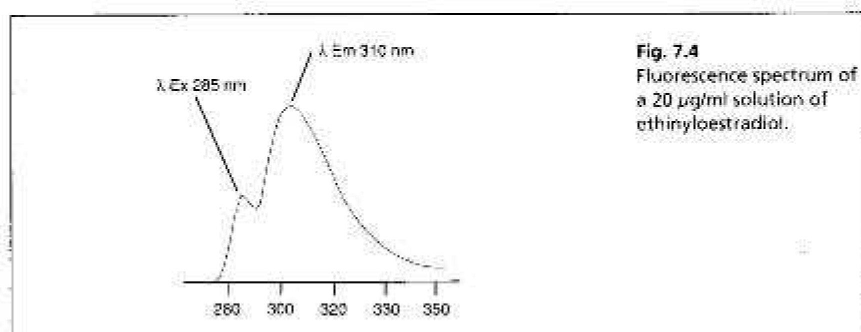

Fig. 7.4 Fluorescence spectrum of a 20 μg/ml solution of ethinyloestradiol.

due to fluorescence. The separation of the exciting radiation and emitted radiation is not great in this example, but this is partly because excitation is taking place at a relatively short wavelength where the displacement of wavelength with energy is lower. For example, the difference between 285 and 310 nm is 0.35 eV, whereas with an excitation wavelength at 385 nm, an energy displacement of 0.35 eV would give an emission wavelength at 443 nm.

Like ethinyloestradiol many other phenols exhibit fluorescence and as is the case for ethinyloestradiol, this fluorescence is pH dependent and does not occur under alkaline conditions when the phenolic group becomes ionised. Table 7.1 shows some examples of fluorescent drug and vitamin molecules.

Table 7.1 Examples of drugs which yield fluorescence spectra

Compound	Excitation	Emission	Limit of detection μg/ml
Pentobarbitone	265	440	0.1
Adrenaline	295	335	0.1
Chlorpromazine	350	480	0.1
Riboflavin	444	520	0.01
Procaine	275	245	0.01
Noradrenaline	285	325	0.006
Quinine	350	450	0.002

Factors interfering with fluorescence intensity

If the concentration of a solution prepared for fluorescence measurement is too high, some of the light emitted by the sample as fluorescence will be reabsorbed by other unexcited molecules in solution. For this reason, fluorescence measurements are best made on solutions with an absorbance of less than 0.02 at their maximum, i.e. solutions of a sample 10–100 weaker than those which would be used for measurement by UV spectrophotometry.

Heavy atoms in solution quench fluorescence by colliding with excited molecules so that their energy is dissipated, e.g. chloride or bromide ions in solution cause collisional quenching.

Formation of a chemical complex with other molecules in solution can change fluorescence behaviour, e.g. the presence of caffeine in solution reduces the fluorescence of riboflavin. This alteration of fluorescence upon binding is used to advantage when examining binding of fluorescent molecules to proteins or other constituents of cells.

Applications of fluorescence spectrophotometry in pharmaceutical analysis

Determination of ethinyloestradiol tablets

The BP utilises a fluorescence assay to determine ethinyloestradiol in tablets. The tablets contain low dosages of the drug so that interference by excipients is likely to cause problems in UV/visible spectrophotometric measurements. The sample is measured using an excitation wavelength of 280 nm and measuring the emission at 320 nm. As was seen when the fluorescence spectrum of ethinyloestradiol was discussed earlier, the optimum excitation wavelength for ethinyloestradiol is 285 nm and the emission maximum is 310 nm. Thus, this assay as described brings out two important points that may have been either consciously or empirically adjusted for in the design of the assay:

(i) The use of a slightly shorter excitation wavelength reduces possible interference from Raman scatter, which may overlap with the fluorescence spectrum and is dependent on the wavelength of the exciting radiation, whereas the fluorescence maximum is not.

(ii) The intensity of Rayleigh and Tyndall scatter at shorter wavelengths is greater and thus the emission is observed at the slightly longer wavelength of 320 nm to reduce interference from this source.

After the fluorescence of the sample extract in methanol has been determined, 1 M sodium hydroxide solution is added to the sample solution and the fluorescence is determined again. The addition of sodium hydroxide removes the fluorescence by ionising the phenol group of the ethinyloestradiol and thus any residual fluorescence which is due to excipients can be subtracted from the reading. In the BP assay the ethinyloestradiol content of the tablet extract is determined by comparison with the fluorescence of a solution containing a known amount of ethinyloestradiol standard analysed using the same conditions.

Calculation example 7.1

A methanolic extract from ethinyloestradiol tablets is measured using fluorescence spectrophotometry. A standard containing the pure drug is also measured under the same conditions. Calculate the content per tablet of the drug from the following data:

Weight of 20 tablets = 2.5673 g.

Weight of tablet powder taken for assay = 0.5257 g.

Volume of methanol extract of tablets = 50 ml.

Fluorescence reading of methanol extract of tablets = 64.1.

Fluorescence reading of sample after addition of 0.1 M NaOH = 3.5.

Concentration of standard solution of ethinyloestradiol = 4.85 μg/ml.

Fluorescence reading of standard solution = 62.3.

Fluorescence reading of standard solution after addition of 0.1 M NaOH = 4.1.

Corrected reading for tablet extract = 64.1 – 3.5 = 60.6.

Corrected reading for standard = 62.3 – 4.1 = 58.2.

Amount of ethinyloestradiol in tablet extract = 60.2/58.2 × 4.85 = 5.02 μg/ml.

Total amount in extract = 50 × 5.02 = 251 μg.

Number of tablets in tablet powder analysed = 2.5673/0.5257= 4.884.

Content of ethinyloestradiol per tablet = 251/4.885 = 51.4 μg.

Determination of dissolution rate of digoxin tablets

Some compounds which are not naturally fluorescent can be rendered fluorescent by simple chemical reactions. For instance digoxin can be converted to a fluorescent derivative by dehydration with HCl and followed by oxidation with H_2O_2. The drug has a narrow therapeutic index and it is important to ensure that the correct dose of drug is delivered by the dosage form. To ensure effective release of the drug from the tablet matrix, the BP indicates that dissolution testing should be carried out. The drug is given in low dosage (*ca* 100 μg per tablet) making measurement of the concentration released into the dissolution medium difficult. The BP assay for release indicates that 75% of the drug from six tablets should be released into 600 ml of dissolution medium after 2 h. The fluorescence measurements are made on the dissolution medium after derivative formation using an excitation wavelength of 360 nm and an emission wavelength of 490 nm. The drug in solution is quantified in comparison with a solution containing a known concentration of standard treated in the same way as the sample.

Determination of aluminium in water for injection as a fluorescent complex

Fluorescence measurements are useful in limit tests where the trace impurity is fluorescent or can be rendered fluorescent by chemical modification. An example is the determination of aluminium in water for use in haemodialysis solutions by formation of its salt with 8-hydroxyquinolone (Fig. 7.5) followed by quantification of the complex using fluorescence spectrophotometry. The excitation wavelength is set at 392 nm and the emission is measured at 518 nm. This type of fluorescent complex can be used to determine low levels of a number of metal ions.

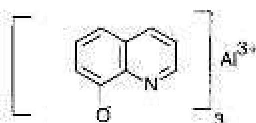

Fig. 7.5 Fluorescent complex of aluminium.

Determination of stability of peptide drugs in solution

The structural complexity of peptide drugs which are being produced increasingly by biotechnology means that additional quality control checks are necessary both for low level contaminants such as immunogenic proteins and for changes in the tertiary (three-dimensional) structure of the protein in solution which may affect its activity. During stability studies, peptide drugs are likely to form aggregates and this eventually results in precipitation. Such changes can alter the efficacy of the drug. In addition it is important to monitor for the inhibition of such changes where stabilisers and other formulation aids are added to the protein solution. Fluorimetry provides a method of following such changes in solution and was recently used in a study of the stability of recombinant fibroblast growth factor in solution.[1] Fluorescence in this peptide is largely due to the presence of tyrosine residues (excitation 277 nm and emission 305 nm) and a tryptophan residue (excitation 290 nm and emission 350 nm) in its structure. Protein denaturation is accompanied by a gradual fall in the emission peak of the tyrosine residues at 305 nm and a gradual rise in the emission peak of the tryptophan residue at 350 nm. This effect is shown in Figure 7.6 and illustrates the fact that the strength of fluorescence is dependent on the local environment of the chromophore.

Measurement of the effect was found to be capable of quantifying the amount of denatured protein in solution.

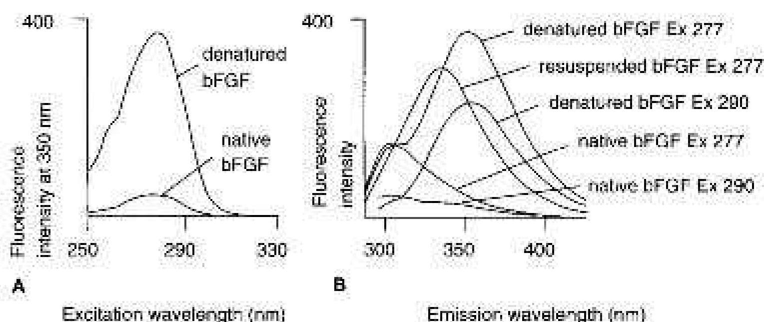

Fig. 7.6 The effect of denaturation on the fluorescence spectrum of fibroblast growth factor. An excitation spectra of native and denatured bFGF B Emission spectra of native and denatured bFGF. Reproduced with permission from J. Pharm. Biomed. Anal. (See Reference 1).

Fluorescent derivatives and flow injection analysis

Flow injection analysis is discussed in more detail in Chapter 3. Some simple chemical reactions which result in the formation of fluorescent derivatives are shown in Table 7.2. All of these reactions could be adapted to enable analysis by FIA.

Table 7.2 Examples of chemical conversion of drug molecules into fluorescent derivatives

Compound	Reagent	Excitation nm	Emission nm
Adrenaline	$K_3Fe(CN)_6$	410	530
Primary amines/amino acids	Fluorescamine	380	480
Chlorpheniramine	H_2O_2	350	436
Fluphenazine	H_2O_2	350	405

Raman spectroscopy

KEYPOINTS

Principles

- The Raman effect is analogous to fluorescence except that it is not wavelength dependent and does not require the molecule to have a chromophore. The energy shift in cm^{-1} due to inelastic scattering of laser radiation is measured rather than wavelength. The shifts measured correspond to the wavenumbers of the bands present in the middle-IR spectrum of the molecule.

Applications

- Has potential for indentifying complex samples, e.g. drugs in formulations and in pack
- Samples such as peptide pharmaceuticals can be analysed for changes in their three-dimensional structure
- Provides additional fingerprint identity information complementary to middle-IR spectroscopy.

Strengths

- Complementary to middle-IR spectroscopy but requires very little sample preparation since near-infrared (NIR) radiation with its good penetration properties can be used for the analysis
- Increasingly a readily available option on middle-IR FT–IR instruments.

Limitations

- Not yet fully established as a quantitative technique
- The solvent may interfere if samples are run in solution.

Introduction

All molecules can be polarised so that the electrons within them are displaced slightly in the direction of the applied field. This effect is not subject exactly to the laws of quantum mechanics, but the wavenumber of the displacement of radiation by a particular group is the same as the wavenumber of the radiation absorbed by that particular group in middle-IR spectroscopy. In fact the Raman effect is encountered when making fluorescence measurements in the UV visible region, although it is usually weak in comparison with Rayleigh and Tyndall scatter. It is analogous to fluorescence except that it is not wavelength dependent, does not require the molecule to have a chromophore and the energy shift in cm^{-1} is measured rather than in wavelength. Figure 7.7 illustrates the Raman effect; the radiation can be either shifted to slightly higher energy (anti-Stokes shift) or to slightly lower energy (Stokes shift). The Stokes shift is usually determined in Raman spectroscopy.

Comparison of the FT-Raman spectrum and FT–IR spectra of dichloroacetophenone (Fig. 7.8) illustrates the fact that the Raman shift for a particular group is similar in energy to the energy of IR absorption for the group in the middle-infrared region.[2] The two spectra provide complementary information.

The general rule is that those bands that absorb weakly in the middle-IR region will absorb strongly in the Raman region and vice versa. For example in dichloroacetophenone, it can be seen that the aromatic C–H groups which absorb IR radiation weakly give a strong Raman effect while the C=O group in the structure absorbs IR radiation strongly but gives a weak Raman effect.

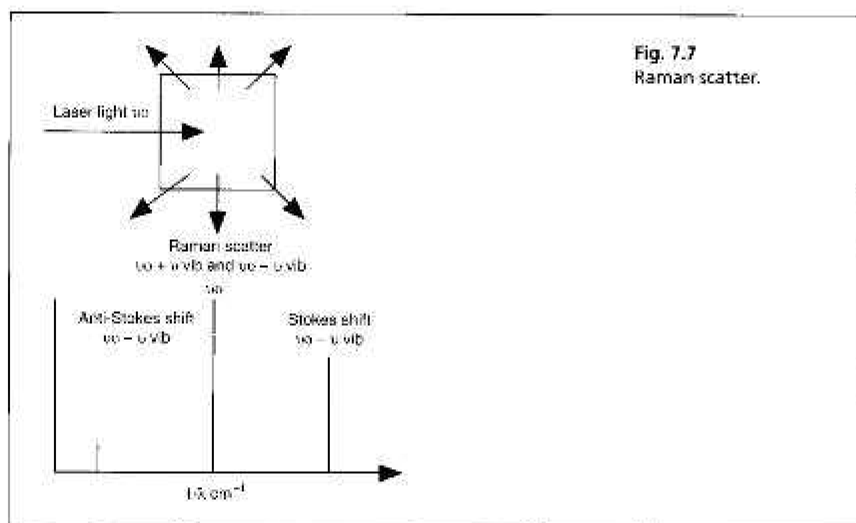

Fig. 7.7
Raman scatter.

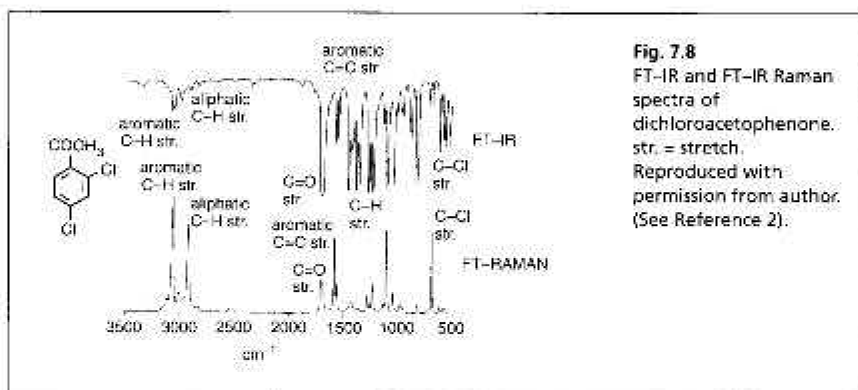

Fig. 7.8
FT–IR and FT–IR Raman spectra of dichloroacetophenone. str. = stretch. Reproduced with permission from author. (See Reference 2).

Instrumentation

The geometry of a Raman spectrometer (Fig. 7.9) is analogous to that for a fluorescence instrument. Since the Raman effect is weak but proportional to the intensity of energy applied, lasers are used to provide high-intensity radiation in the visible region, generally somewhere between 450 and 800 nm. Lasers provide several emission lines and in the case of a fluorescent molecule a line may be selected that gives Raman scatter where fluorescence does not interfere with the measurement. In recent years NIR lasers in conjunction with Fourier transform instruments have become available.[2] The use of NIR radiation has two advantages:

(i) Unlike UV/visible radiation, it does not excite fluorescence in molecules which can result in interference in measurements.
(ii) It has good penetration properties so that a sample in the solid phase can be examined without any sample preparation.

NIR Raman spectroscopy has good potential for the analysis of pharmaceutical formulations and biological materials.

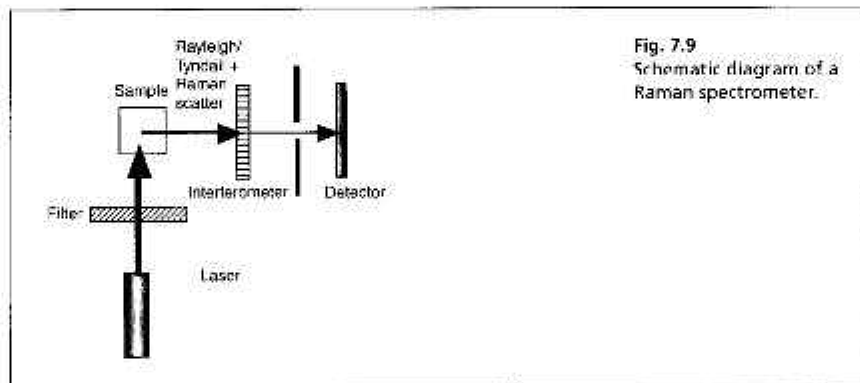

Fig. 7.9
Schematic diagram of a Raman spectrometer.

Applications

Rapid fingerprinting of drugs

The Raman spectra of heroin, morphine and codeine (Fig. 7.10) are highly characteristic because of the change in the bands due to the aromatic ring.[2] The FT–IR spectra of these compounds are quite similar. Near-infrared Raman spectroscopy can provide a rapid method for characterising drugs with minimal sample preparation and analysis time.

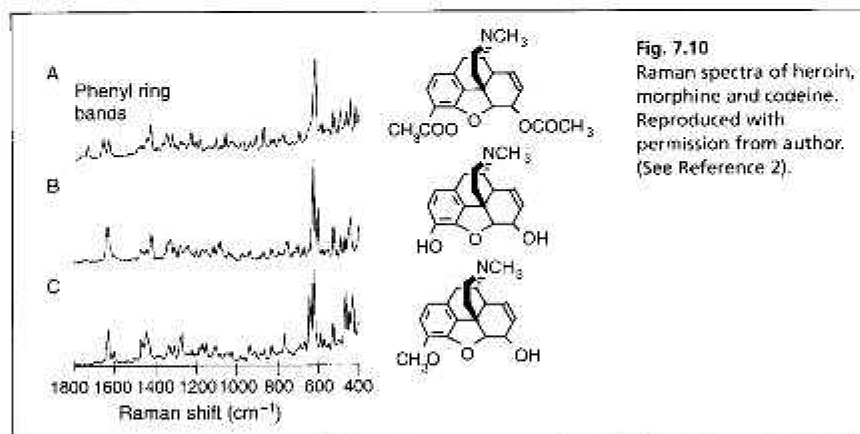

Fig. 7.10
Raman spectra of heroin, morphine and codeine. Reproduced with permission from author. (See Reference 2).

Analysis of drugs in their formulations

Drugs can be characterised directly in formulated materials. For example diclofenac formulated in sodium alginate was characterised by subtracting the spectrum of the alginate matrix from the spectrum of the formulation containing diclofenac (Fig. 7.11). It is also possible to analyse drugs which are packaged by subtracting the spectrum of the pack. This allows for instance a final quality control (QC) check on, for instance, blister-packed tablets.

A quantitative application

FT-Raman is potentially a quantitative technique but does not currently have the

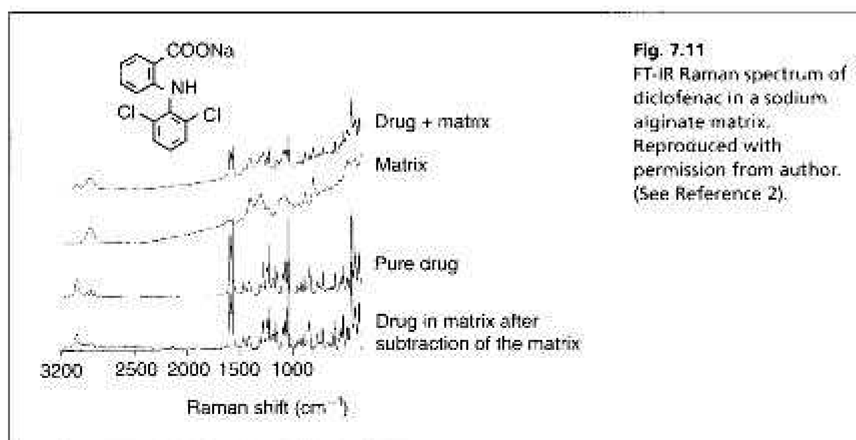

Fig. 7.11
FT-IR Raman spectrum of diclofenac in a sodium alginate matrix. Reproduced with permission from author. (See Reference 2).

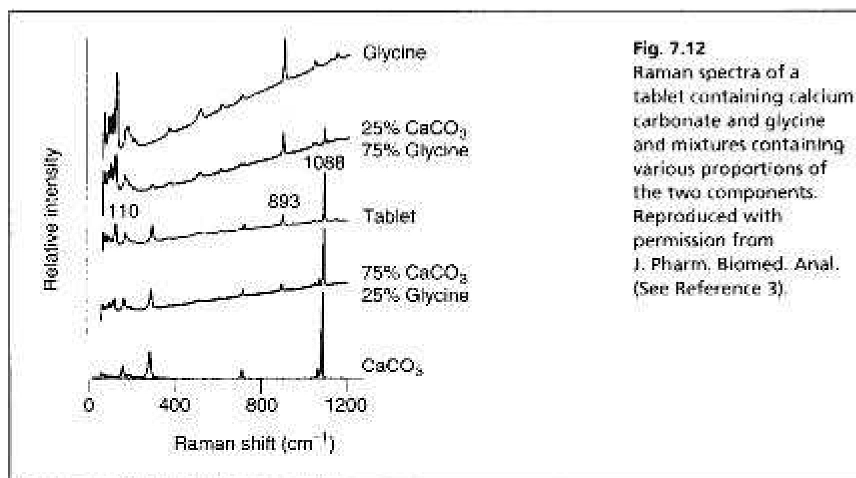

Fig. 7.12
Raman spectra of a tablet containing calcium carbonate and glycine and mixtures containing various proportions of the two components. Reproduced with permission from J. Pharm. Biomed. Anal. (See Reference 3).

sensitivity of NIR when it comes to determination of individual components in complex mixtures. Raman spectroscopy was used to determine glycine and calcium carbonate in an antacid tablet.[3] The intensity of the bands at 1088 cm^{-1} for calcium carbonate and 893 cm^{-1} for glycine were used as the basis for quantitation (Fig. 7.12). Precisions of $< \pm 3.5\%$ were achieved for the contents of the ingredients in the tablet.

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