A mass spectrometer works by generating charged molecules or molecular fragments either in a high vacuum or immediately before the sample enters the high–vacuum region. Instruments typically maintain vacuums of about 10^{-6} mmHg, since ionised molecules have to be generated in the gas phase to be able to manipulate them using magnetic or electrostatic fields. In classic mass spectrometry (MS) only one method could produce the charged gaseous molecules, but now quite a number of alternatives are available. Once the molecules are charged and in the gas phase, they can be manipulated by the application of either electric or magnetic fields to enable the determination of their relative molecular mass and the relative molecular mass of any fragments produced by the molecules breaking up. A number of useful introductory texts that describe mass spectrometers and mass spectral interpretation are available (Williams and Fleming 1980; McLafferty and Turecek 1993; Davis and Frearson 1994; Chapman 1995; Watson 1997; Lee 1998; Smith and Busch 1999).

Sommaire

Theory

A magnetic sector mass spectrometer is illustrated in Fig 1. For MS that follows chromatographic separation, other methods of ion separation are generally preferred on the basis of the cost of separation in a magnetic field.

One method of introducing a sample into a mass spectrometer is to insert it on the end of a heated probe so that it is volatilised with assistance from the high vacuum in the instrument. Once the analyte molecules are in the vapour phase, the source provides energy to cause them to ionise. The original method for producing ionisation, which is still used for routine analyses, is to bombard the analyte with electrons produced by a rhenium or tungsten filament. The electrons produced by the filament are accelerated towards a positive target with an energy of, most commonly, 70 eV. The analyte vapour is introduced into the instrument between the filament and the target. Since the electrons used to promote ionisation are of much higher energy than the strength of the

bonds within the analyte (which are of the order 4–7 eV), extensive fragmentation of the analyte usually occurs. In magnetic sector mass spectrometers the ions are separated by application of an electrostatic field followed by a magnetic one, or the reverse in reverse–geometry instruments.

In a straight geometry magnetic sector mass spectrometer the ions generated in the source are pushed out of the source by a repeller potential, which has the same charge as the ions. These ions are then accelerated and focused into a beam using a series of electrostatic lenses, which can be tuned to give optimum sensitivity. As the ions leave the ion source they have a range of kinetic energies because they were formed at different points in the source. The kinetic energies of the ions are focused into a narrow range using an electrostatic field applied at right angles to their direction of travel. As a result they take a circular path through the electrostatic analyser. Only ions with a narrow range of velocities then pass through the slit into the magnetic analyser. The width of the slits in the instrument can be varied to control its resolving power. Having focused the ion beam, the ions are then separated in the magnetic analyser according to the equation below. By varying the magnetic field B, ions with a range of mass/charge (m/z) values can be collected and detected using either an electron- or photomultiplier. A typical sweep time for the magnetic field across a mass range of 50–800 atomic mass units (a.m.u.) would be 1 s but faster speeds are required if high–resolution chromatography is being used.

where B is the magnetic field strength, r is the radius of path through the magnet and V is the accelerating velocity. It is also possible to vary the potential of the electrostatic field and keep B constant. In this case, the mass range is limited by the range of kinetic energies of the ions generated in the source and scans may be only be over a mass range of 5–10% of the mass of the ion being measured. However, the electrostatic region is not subject to hysteresis in the same way as the magnetic field and better high–resolution measurements can result from varying the electrostatic field. Although magnetic sector instruments are still widely used to determine the elemental compositions of ions, they may be superseded eventually by time–of–flight (TOF) instruments.

Relative molecular masses and elemental composition

The nitrogen rule

Most relative molecular masses of analytes are even numbers, unless the molecule contains a nitrogen atom. Compounds that contain a single nitrogen atom have odd–number relative molecular masses, two nitrogen atoms in a structure produce an even relative molecular mass, three an odd relative molecular mass and so on. Other elements (e.g. boron) can produce odd–number relative molecular masses, but nitrogen is the most common

within the structures of drug molecules.

Isotopes

Table 1 summarises the isotope abundances of some elements commonly found in drug molecules. Chlorine and bromine have abundant isotopes; the presence of ^{35}Cl and ^{37}Cl or ^{79}Br and ^{81}Br produces characteristic 3:1 or 1:1 molecular ion patterns, respectively. These isotope patterns are very distinctive and are particularly so if a molecule contains more than one chlorine or bromine atom. The ^{13}C isotope has quite a high abundance, 1.1% of the abundance of the ^{12}C isotope. For instance, androstenedione has a molecular ion at *m*/*z* 286 (Fig 2) and, since the molecule contains 19 carbon atoms, it has an isotope peak at m/*z* 287, which is about 21% (19 × 1.1%) of the ion at *m*/*z* 286, which is the probability of the occurrence of one ^{13}C atom in its structure. By the same logic, there is an ion at *m*/*z* 288, which is about 4% of the 286 ion and results from two ^{13}C atoms in androstenedione. Androstenedione also contains 26 hydrogen atoms, so its mass will be higher by about 0.2 a.m.u. from its nominal mass, based on hydrogen having an exact mass of 1.0078 (Table 2). These two effects have to be taken into account when dealing with molecules of very high relative molecular mass, such as proteins. The deviation of the mass of hydrogen from unity makes a significant difference to the exact mass of a protein, and the abundance of ^{13}C in protein molecules makes the isotope patterns of protein spectra complex. The ^{15}N isotope and the ^{34}S isotope also add to the complexity of the isotope pattern of protein spectra. Often, the average mass of a molecule is quoted; this is not an accurate mass, but rather an average of the contribution of all the isotopes towards its mass. Thus, HCl has an average mass of 36.5, since it contains 75% of ^{35}Cl and 25% of ^{37}Cl.

The relatively new technique of gas chromatography–combustion–isotope ratio MS to evaluate the content of ^{13}C or ^{15}N has potential for forensic toxicology, such as in the differentiation of endogenous and exogenous testosterone in sports drug testing.

Accurate mass measurement

Magnetic sector instruments can be used to determine the mass of a molecule to several decimal places. Based on the convention that carbon (^{12}C) has an exact mass of 12, the atoms of other elements do not have exact masses as seen in Table 1. Thus, a high–resolution mass spectrometer would be able to distinguish between CO, $CH_2{=}CH_2$ and N_2, which all have a rounded mass of 28 a.m.u. The difference in mass between $CH_2{=}CH_2$ and N_2 is

0.0252 a.m.u., which relative to a mass of 28 is 900 parts per million (ppm; 1 ppm relative to 28 = 0.000 028). Most double–focusing magnetic sector instruments should be able to measure masses to 1 ppm accuracy. To carry out accurate mass measurements, the instrument usually has to be calibrated at the same time as the sample is being measured.

Fig 3 shows the mass spectrum of chloroquine phosphate obtained using fast atom bombardment (FAB) ionisation. FAB is a soft ionisation technique, so that the spectrum is quite simple and the molecular ion for chloroquine (+H) is seen at *m*/*z* 320 for the chlorine 35 isotope and *m*/*z* 322 for the chlorine 37 isotope. A feature of FAB is that matrix peaks are usually also present in the spectrum. Two clear peaks can be seen at *m*/*z* 277 and *m*/*z* 369; these result from the formation of cluster ions from the glycerol matrix in which the sample is dissolved. These two peaks, and other glycerol cluster ions, have a known elemental composition/exact mass ratio and can be used to calibrate the mass axis of the instrument with high precision, so that an elemental composition for the two molecular ions of chloroquine can be obtained. Other commonly used calibrants are volatile ones used in heated probe work [perfluorokerosene (PFK) and perfluorotributylamine (PFTBA)] and polyethylene glycol and Ultramark (used in calibrating the masses of high molecular weight compounds).

Figure 3. Fast–atom bombardment mass spectrum of chloroquine in a glycerol matrix.

Resolution

Resolution in MS is rather analogous to the separation of peaks in chromatography and is of similar importance when quantitative measurements are being made, since overlap can produce quantitative inaccuracies. Unit resolution implies that two masses are completely separated (0% peak/valley ratio). For example, if 100 is separated completely from 101, this is termed 100 resolution. Magnetic sector instruments can effect resolution of up to 20 000 at 50% peak/valley, that is they can resolve masses of 20 000 and 20 001, so there is a valley of 50% of the peak height between them. However, greater resolution is achieved by narrowing the slits in the instrument, which means lower sensitivity because fewer ions pass through. Quadrupole instruments only provide resolution of about 500. Time of flight (TOF) instruments are now beginning to approach the level of the resolving power of magnetic sector instruments.

Instrumentation

Magnetic sector instruments

The magnetic sector instrument is discussed above in the section that gives the theoretical background to ion generation.

Quadrupole instruments

Fig 4 illustrates a quadrupole mass spectrometer. For many years, quadrupoles were the only alternative to magnetic sector instruments. The principal features of quadrupole instruments are:

- Two varying electrostatic fields, one direct current (DC) and one at varying radiofrequency, are applied at right angles to each other via the four rods of the quadrupole. This creates a resonance frequency for each *m*/*z* value in a mass spectrum. The full mass range is scanned by varying the resonant frequency of the quadrupole.
- A simple quadrupole mass spectrometer only gives about 500 resolution and thus masses of ions separated by a quadrupole are only measured to one decimal place.
- Quadrupole instruments are of higher sensitivity than magnetic sector instruments, as they do not require all the ions that enter the quadrupole to have the same kinetic energy, and thus ion transmission is not reduced by slits.
- Since quadrupoles do not suffer from hysteresis, as magnetic sector instruments do, they are ideal for the rapid switching between ions required in selected ion monitoring mode. In this mode a few significant ions in the mass spectrum of an analyte are monitored to achieve high sensitivity by ignoring background ions not derived from the analyte of interest.
- In a quadrupole instrument, larger diameter rods produce increased sensitivity. Narrower or longer rods increase the resolving power.

Ion–trap instruments

Ion-traps (Ghosh 1995) have been developed in the past 15 years only and their performance has improved greatly in the past 5 years. The analyte is ionised using one of the available methods and the ions are transferred into the trap in which they are then confined by application of a radiofrequency voltage to the circular electrode (Fig 5). The energy of the ions in the trap is quenched by helium, which is introduced into the trap to give a pressure of about 1 mmHg, so that they focus near the centre of the trap (i.e. their centrifugal energy is reduced). The ions can

then be mass–selectively ejected by increasing a radiofrequency potential applied to the endcap electrodes (which also have a DC potential applied to them) to produce a mass spectrum as the ions are detected by the electron or photomultiplier tube. The power of the technique resides in the amount of control that can be exerted on the ions in the trap. The ions can be confined within the trap and excited by changing the radiofrequency potential applied to the ring electrode so that their kinetic energy increases and they fragment more extensively through collision with the helium atoms in the trap. Selectivity can be introduced by ejecting all ions apart from, for instance, the molecular ion of the analyte of interest. The analyte is thus freed from any interfering peaks in the background and can be subjected to additional fragmentation by changing the radiofrequency potential of the trap. This technique provides a low–cost alternative to tandem MS (see below).

Ion cyclotron resonance mass spectrometer

This instrument operates in a similar way to an ion trap. The ions are formed and then introduced into a trap and orbit within a circular magnetic field. As they circulate within the trap they pass close to two opposing electrode plates and thus induce a small alternating electric current within the circuit attached to the two plates. The amplitude of this current is proportional to the number of ions in the trap and its frequency is the same as the cyclotron frequency of the ions. The ions are thus detected without colliding with the detector via the current (the image current) they induce. The ions in the trap can be excited by application of a radiofrequency pulse of radiation, which boosts the image current, which in turn falls off as the ions return to their unexcited state. The fall off in the current can yield frequency information by using Fourier transformation, which leads to the construction of a mass spectrum. This process is analogous to Fourier–transform nuclear magnetic resonance spectroscopy, and can yield extremely high–resolution mass measurement, about ten times greater than the best performance of a magnetic sector instrument. The resolving power of this type of instrument is good enough to allow the accurate mass measurement of proteins.

Time–of–flight instruments

TOF (Cotter 1997) was an early technique that produced ion separations. It is a very good technique for the analysis of high relative molecular-mass compounds such as proteins. The principle of TOF is quite simply that the larger an ion, the longer it will take for it to reach the detector of a mass spectrometer after acceleration through an electric field. For the technique to be effective, the time period during which the ions leave the ion source has to be well defined. One ionisation process, matrix assisted laser desorption ionisation (MALDI), uses short pulses of laser energy focused on the sample dissolved in a matrix that absorbs ultraviolet (UV) light. Alternatively, electrospray ionisation (ESI; see below) may be used in conjunction with a gating mechanism that allows ions to enter the separation field for only a very short period of time. The ions formed in the source have varying kinetic energies and, to avoid broad mass peaks, a device called a reflectron may be used to focus the kinetic energies of

the ions that enter the TOF analyser. The greater the kinetic energy of an ion the further it penetrates into the reflectron, and thus the faster ions are retarded by the reflectron, which allows the slower ions to catch up. Improvements in reflectron focusing and gating mechanisms have enabled TOF to become capable of accurate mass measurement.

Tandem mass spectrometry

The most common form of tandem MS started with three quadrupole mass spectrometers linked together, but it can now be carried out at a lower cost using ion–trap instruments. It is the most powerful mass spectrometric technique because it enables analytes to be separated rapidly by using a mass spectrometer rather than chromatography. In a triple quadrupole instrument, the three quadrupoles are linked in series. MS 1 (Fig 6) separates the ions in a mass spectrum in the usual way. Then a selected ion (or ions) may be transferred to the second quadrupole, which simply acts as an ion guide rather than producing separations, in which they are allowed to collide with atoms of a heavy gas, such as argon, to produce additional fragmentation. The fragmentation pattern produced is analysed using MS 2.

The most common method for promoting fragmentation of an analyte is collision with an inert gas. High–energy collisions are promoted by acceleration of the ions prior to entering the collision chamber. In this case, the gas used to promote fragmentation is usually helium and the fragmentation is fairly reproducible because the kinetic energy of the ions is controlled. Low–energy collisions rely on the inherent kinetic energy of the analyte ions and are less reproducible because the kinetic energy of the ions is variable. In this case, either argon or xenon is used as collision gas and the fragmentation varies according to gas type, temperature and pressure.

Photodissociation may also be used to promote ion fragmentation, the photoenergy being provided by a laser. This technique gives very reproducible fragmentation and is particularly applicable to ion–trap analyses in which the ions can be trapped and irradiated.

Coupled techniques

Introduction

The most powerful mass spectrometric techniques involve the coupling of separation techniques with mass spectrometers. The development of ionisation techniques is linked intimately to the development of coupled techniques. Thus, in the majority of applications the ionisation technique has been developed and refined in

conjunction with a separation technique. This is particularly true of the ionisation techniques used in conjunction with liquid chromatography (LC).

Gas chromatography–mass spectrometry

Gas chromatography–mass spectrometry (GC–MS; Message 1984; Kitson et al. 1996; Neissen 2001) was the earliest technique in which chromatography and MS were coupled. The original type of gas chromatograph had a packed GC column with a gas flow rate passing through it of about 20 mL/min, so the major problem was how to interface the GC without losing the mass spectrometer vacuum. This was solved by use of molecular separators. In one of these, known as the jet separator, the column effluent was passed across a very narrow gap between two jets and the highly diffusible gas was largely removed, whereas the heavier analyte molecules crossed the gap without being vented. This problem of how to dispose of the GC carrier gas no longer exists, since GC capillary columns provide a flow rate of 0.5 to 2 mL/min of gas which can be introduced directly into the mass spectrometer without a loss of vacuum. The GC–MS interface is very simple and no special design is required. The GC effluent is simply introduced in the same way as a direct insertion probe would be introduced into the instrument. If the same carrier gas pressure and column are used when a GC method is transferred to a GC–MS one, the retention times produced by the GC–MS method are shorter because of the effect of the vacuum at the mass spectrometer end of the column. Most GC software incorporates a flow calculator that enables an estimate to be made of the effect of a mass spectrometer vacuum on the linear velocity of the GC carrier gas.

Although many analyses are now carried out by LC–MS, GC–MS is still important for many drug analyses. In the negative–ion mode, it is probably the most sensitive analytical technique available for analytes that are strongly electron capturing. The main areas of application for GC–MS are in the characterisation of volatile compounds, such as flavourings, fatty acids and unidentified residual solvents. In the forensic field many narcotics can be analysed by GC–MS with a high degree of selectivity, as can other abused drugs such as anabolic steroids. An advantage of GC–MS is that electron impact (EI) spectra can be obtained, which are correlated readily to library spectra that have been built up over many years. The disadvantage of the technique is that more sample preparation is required in comparison with LC–MS techniques.

Liquid chromatographic interfaces with mass spectrometers

The interfacing of a liquid chromatograph to a mass spectrometer proved much more difficult than for a gas chromatograph, since each mole of solvent produces 22.4 litres of solvent vapour even at atmospheric pressure. The technique has made huge advances in the past ten years and many types of interface are available, the most successful of which are the electrospray and atmospheric pressure ionisation sources (Neissen 1998). The advances in technique have meant that splitting of the eluent flow from a high–pressure liquid chromatography

(HPLC) column is now unnecessary and instruments can cope with flow rates of up to 1 mL/min, although mobile phases with a high water content may need to be introduced at flows of less than 1 mL/min.

Fast atom bombardment mass spectrometry

FABMS is a popular technique for direct insertion–probe MS, but it is also available as a LC–MS interface. Before the popularisation of ESI, FAB was the major technique for the ionisation of large molecules. The liquid chromatographic interfaces, such as the frit–FAB interface in which the eluate flows from the end of the chromatographic column onto the centre of a porous frit (which is the FAB target), are only able to tolerate flows of about 10 µL/min, which is a major disadvantage. FAB ionisation was also used as the basis for the only thin layer chromatography–MS interface developed, but this interface was not really popularised. Now the application of FAB is confined largely to promoting the ionisation of molecules to determine their elemental composition, as illustrated for chloroquine in Fig 3.

Electrospray ionisation mass spectrometry and atmospheric pressure chemical ionisation mass spectrometry

ESI (Cole 1997; Snyder 1996) and atmospheric pressure chemical ionisation (APCI) are the most widely used interfaces between liquid chromatography and mass spectrometers. They are carried out using the same source. In the ESI mode the sample is sprayed in solution into the source via a needle held at a high potential of 3 to 5 kV. The formation of an aerosol from the sample is assisted by a flow of heated nitrogen, which enters the instrument along a direction co–axial with the needle. The sample enters the instrument through a narrow orifice in a metal cone, which leads to a chamber at an intermediate level of vacuum; it then passes through a second orifice into a high–vacuum region. Fig 7 illustrates the production of the electrospray. At the tip of the capillary, positive ions are separated from their negative counter ions, which are pulled towards the capillary. The isolated positive ions generated are repelled by the capillary. This force breaks up the surface tension of the liquid in which the sample is dissolved, to generate a cone (Taylor cone) that breaks up into charged droplets. This process is charge dependent and, since the analyte is usually of low concentration, electrolytes must also be in the liquid at a minimum concentration of 10^{-5} M to assist in promoting ESI. Once the sample has formed into charged droplets, the excess of positive charges in the droplets produces repulsion, which causes the droplets to break up further. This is assisted by evaporation of solvent from the droplets, which increases their charge and thus further promotes their break up. At the final stage, the analyte is believed to abstract one or more protons from the solvent (or donate a proton if a negative ion is formed) to give a positively charged gas–phase ion. If analytes can be protonated at multiple sites, they will carry several charges. Thus, a protein of molecular weight 10 000 a.m.u. that carries 10 charges appears to the mass analyser to have a mass of 1000 a.m.u. Fig. 26.8 shows the

electrospray mass spectrum of porcine insulin. The charge on a given ion may be obtained from two adjacent ions in the mass spectrum according to the formula:

where n is the charge on M_B, and M_A and M_B are adjacent ions with M_A the higher in mass. Thus, for the spectrum of porcine insulin shown below.

Thus the charge on the ion at m/z 964 is 6+ giving a relative molecular mass of 5784 for hexaprotonated porcine insulin. Insulin has six basic centres and, in this case, the predominant ion is that with all six centres protonated. Protonation of all the basic centres of a protein does not always occur.

To carry out APCI the instrument used for ESI simply has to be reconfigured to introduce a corona discharge pin at the point where the stream of solvent that contains the analyte enters the instrument. Although APCI is carried out on the same instrument as ESI, it is quite a different process. It does not depend on the production of ions by evaporation, but it rather uses chemical species to promote the ionisation process in a manner analogous to the production of ions under positive ion chemical ionisation (PICI; see below) conditions. In this case the reagent ions that promote the ionisation include N^+_2 and H_3O^+. APCI tends to be less sensitive than ESI, but it is useful for molecules that will not ionise readily (e.g. neutral drugs such as steroids).

Capillary electrophoresis–mass spectrometry

The preferred mode of ionisation for interfacing a capillary electrophoresis (CE) instrument with a mass spectrometer is ESI. The technique has been improved by the development of a method to introduce a sheath flow of mobile phase, which augments the nanolitre per min flows through the CE column to form a stable electrospray. During CE, a potential of 25 to 30 kV is applied across the capillary between the inlet and the outlet, the outlet being at ground potential. In ESI, the needle is normally held at a potential of 3 to 5 kV so that the potential applied across the CE has to be increased to compensate for the increase in potential at the outlet end. The CE interface is one of the most recent developments in MS and it is expected to have a wide range of applications in obtaining impurity profiles of both conventional synthetic drugs and biotechnological drugs.

Data processing

Considerations with regard to processing data obtained from mass spectrometers overlap those involved in the processing of chromatographic data obtained using other types of detector. Thus, from the chromatographic standpoint, the correct settings of integration parameters, such as peak width and threshold, are important to ensure that processing parameters do not confound the accuracy of the data obtained.

Figure 9. GC–MS trace of a mixture of estradiol, estrone and testosterone showing extracted ion traces for their molecular ions and the total ion current.

The difficulties of fused peaks in total ion current (TIC) chromatograms can often be circumvented by the use of selected ions that are characteristic of the compound of interest. For example, in the chromatogram shown in Fig 9, the peaks for testosterone, estradiol and estradione partially overlap. However, it is possible to extract the molecular ions for each individual compound and Fig 9 shows the extracted ion traces for the molecular ions of the three analytes with very little interference between them. However, interference is not necessarily completely eliminated, as the testosterone peak produces some response in the selected ion trace for estrone since m/*z* 270 occurs in the mass spectrum of testosterone through the loss of water from its molecular ion at *m*/*z* 288.

The use of extracted ion traces is common in LC–MS data processing used for the screening of drug metabolites. The background produced by ions derived from the LC solvent in LC–MS mode means that when metabolites are only present in low amounts the molecular ions of potential metabolites can be predicted and used to generate extracted ion traces to check for their presence. However, chromatograms such as these do not show the same sensitivity as single–ion monitoring does.

Data processing in the generation of accurate mass data is a little more complex since the mass spectrum of the compound of interest has to be calibrated against a standard, which is introduced into the mass spectrometer at the same time as the sample. For example, three of the calibrant ions are picked manually by the operator and the computer can construct a calibration curve based on the known masses of all the major calibrant ions (see Fig 3).

System stability tests

Chromatographic tests

Chromatographic systems interfaced with mass spectrometers are subjected to the same system–suitability tests used in conjunction with other types of chromatographic detectors.

Calibration of the mass axis

A test of fundamental importance in MS is to calibrate the mass axis of the mass spectrometer with a suitable tuning compound or mixture. In GC–MS systems the most popular tuning compound is PFTBA. The use of a fluorinated tuning compound has two advantages. On the carbon–12 scale of relative atomic mass, fluorine is very close to its nominal mass of 19, whereas the mass of hydrogen is considerably greater than its nominal mass of unity. Secondly, since PFTBA is electron capturing, it can be used to tune in the negative–ion chemical ionisation mode. In the electron–impact mode, PFTBA produces a number of abundant ions below a mass of *m/z* 219 and weaker ions above this value. Tuning should be carried out on a minimum of three ions that cover the mass range of interest. Typically, the ions at *m/z* 69, 219 and 502 generated by PFTBA are used to calibrate the mass axis in EI mode. These ions are used also to determine resolution between masses. There is always a trade–off between resolution and sensitivity, and if the mass window is narrowed to reduce peak width, sensitivity is lost. The PFTBA ions at *m/z* 69, 219 and 414, and 452, 595 and 633 are suitable for instrument tuning in PICI and negative–ion chemical ionisation, respectively. Instruments can either be automatically or manually tuned. With quadrupole mass spectrometers and other low–resolution instruments, such as ion traps, the ions used in tuning are assigned masses to the nearest whole number. When high–resolution calibration is being carried out, masses of four or five decimal places are assigned to calibration ions; thus, for instance, the CF^{+}_{3} ion at *m/z* 69 would be assigned its exact mass of *m/z* 68.9952. All the major ions in the spectrum of PFTBA would be used to carry out a high–resolution calibration, and thus enable accurate mass assignment.

In LC–MS the tuning mixtures used are less well defined than those used in GC–MS, and generally laboratories and manufacturers develop their own. Typical examples include valine, tri-tyrosine and hexa-tyrosine, which provide ions at *m/z* 118, 508 and 997, respectively, in the positive ESI mode. To determine proteins in positive ESI mode, the instrument may be tuned to the known average masses of the multiply charged ions derived from horse myoglobin, which gives ions at *m/z* 848.5, 1060.4, 1211.8, 1413.5 and 1696. In the negative ESI mode, polyethylene glycols may be used for tuning or a mixture of flavonoids, quercetin, quercitrin and rutin in the range *m/z* 300 to 610. Polyethylene glycols are used for calibration in ESI mode and they are also used to calibrate TOF instruments.

Sensitivity checks

There is no specific test for checking the sensitivity of a mass spectrometer. The counts provided by the tuning compound provide an indication of sensitivity, but beyond that the usual checks are of the sensitivity of the system when interfaced with chromatography. Manufacturers usually have their own favourite compounds for assessing sensitivity, such as methyl stearate to assess EI sensitivity in GC–MS mode or octafluoronaphthalene to assess negative–ion chemical ionisation (NICI) sensitivity in GC–MS mode. However, these particular analytes are not typical of the average drug molecule for which chromatographic factors contribute to the overall sensitivity. Sensitivity checks are usually carried out by diluting the compound of interest until it is no longer detectable by the particular system being used.

Sample preparation and presentation

The preparation of samples for GC–MS and LC–MS analysis does not differ from the types of preparation that would be carried out prior to any chromatographic method, that is extraction to remove interferences by the sample matrix as far as possible. Some extra considerations apply, for instance in GC–MS mode, as very often derivative formation may be necessary. For example, trimethylsilyl derivatives provide fairly abundant $[M-15]^+$ ions, which may be useful. Also, fluoroacylated derivatives may be prepared so that the sample is suitable for analysis in the negative ion mode. If a chromatographic step is not used in conjunction with MS, it is important that the sample be relatively free of non-volatile materials, such as salts that would prevent it from evaporating from the direct insertion probe. The presence of non-volatile salts in samples may also interfere in LC–MS in the ESI or APCI mode, since the non-volatile salt will elute from the column and deposit on the cone, and so reduce instrument sensitivity quite rapidly. The cone can be continually washed to remove buffer salts, but, despite this, sensitivity tends to fall quite rapidly with time.

Data interpretation

Introduction

The interpretation of a mass spectrum (Williams and Fleming 1980; McLafferty and Turecek 1993; Davis and Frearson 1994; Chapman 1995; Watson 1997; Lee 1998) is intimately bound up with the type of ionisation used to generate the mass spectrum. With EIMS, the fragmentation patterns for molecules are generally quite complex. Under other types of ionisation, fragmentation is less extensive and more predictable, as described below.

Electron–impact mass spectrometry

EI ionisation uses high–energy electrons at 70 eV, which produce extensive fragmentation of the bonds within the analyte. It is still very commonly used in standard chemical composition analyses, but is not as readily applicable if the molecule being analysed is very non-volatile or unstable. In these cases, a range of other ionisation techniques can be applied.

Fig 10 shows a generalised scheme for decomposition of a molecule under EI conditions:

- $M^{\bullet+}$ represents the molecular ion that bears one positive charge, since it has lost one electron, and the unpaired electron that results from the loss of one electron (represented by a dot).
- $M^{\bullet+}$ may lose a radical that, in a straightforward fragmentation not involving rearrangement, can be produced by the breaking of any single bond in the molecule. The radical removes the unpaired electron from the molecule to leave behind a cation A^+.
- This cation (A^+) can lose any number of neutral fragments, such as H_2O or CO_2, but no further radicals.
- The same process can occur in a different order with a neutral fragment (H_2O, CO_2, etc.) being lost to produce the radical cation $B\bullet^+$, and since this ion still has an unpaired electron it can lose a radical to produce C^+, which can thereafter only lose neutral fragments.

An EI spectrum is shown for heroin in Fig 11 and the fragmentation pathways that give rise to the spectrum are shown in Fig 12. The molecular ion can be seen at m/*z* 369 (and therefore must contain a nitrogen atom) and the fragments at *m/z* 327 and 310 correspond to ions B and A in Fig 10. The ion at m/*z* 268 corresponds to ion C in Fig 10, and can also be derived via loss of acetate from ion B. The ion at m/*z* 284 results from the loss of two neutral fragments. Like many molecules the simply explained losses only occur within about 100 a.m.u. of the molecular ion, and below *m/z* 268 the fragment ions are derived from complex rearrangements of the structure of the molecule. An advantage of EI is that the complex fragmentation pattern produced can be used as a fingerprint to identify the molecule, for instance to confirm the identity of traces of heroin in a forensic sample or the identity of an anabolic steroid in a urine sample. Thus, with EI spectra there is plenty of scope to identify an unknown via interpretation. Table 3 shows some of the common losses from molecular ions that may be used in the interpretation of an EI mass spectrum.

The fragmentation patterns of large molecules may be difficult to interpret. For instance, steroid molecules give particularly complex patterns. Most of the ions in the EI mass spectrum of hydrocortisone (Fig 13) arise from

rearrangements of the structure that involve migrations of hydrogen atoms. These types of spectra give a characteristic fingerprint and the base peak ion (most abundant ion) at m/*z* 123 occurs in many steroids. However, it may vary in mass from a hydrogen atom higher to one lower than 123. The base peak in the mass spectrum of testosterone occurs at *m*/*z* 124, even though it has exactly the same A and B rings as hydrocortisone. However, the mass spectrum of prednisolone, which has an additional unit of unsaturation in the A ring, is consistent with hydrocortisone and yields a base peak ion at *m*/*z* 121.

In many drug molecules, the use of EI ionisation results in a low abundance of the molecular ion. Thus chloroquine under EI conditions gives less than 1% abundance of molecular ion (Fig 14) and its mass spectrum is dominated by an alpha cleavage fragment, which arises as shown in Fig 15.

The spectra of heroin, hydrocortisone and chloroquine illustrate the three most common types of spectra observed in drug molecules. The spectra of heroin and hydrocortisone are most useful, since they are complex, provide a fingerprint and are more open to interpretation than simple spectra such as the spectrum of chloroquine. Other dominant fragmentation modes exist, such as the formation of a tropylium ion, which occurs in benzyl compounds, but these are not as common as those mentioned above and are discussed elsewhere (Williams and Fleming 1980; McLafferty and Turecek 1993; Davis and Frearson 1994; Chapman 1995; Watson 1997; Lee 1998).

If more information is required about the relative molecular mass of a drug that fragments easily, such as chloroquine, softer ionisation techniques are used. FAB ionisation is one option (the FAB spectrum of chloroquine is shown in Fig 3). However, FAB combines less satisfactorily with chromatographic interfaces than other ionisation methods; quite a number of methods promote ionisation, some of which are discussed below.

Positive ion chemical ionisation

PICI can be carried out with a number of reagent gases (methane, isobutene and ammonia are most commonly used), and it is most applicable in GC–MS. The gas is introduced into the source of the mass spectrometer continuously during the analysis and the source configuration is changed so that the gas is contained within a small chamber within the source. Gas is introduced to give a source pressure of about 1 mmHg and bombarded with electrons produced by the same filament as used for EI ionisation at energies of about 200 eV. The electrons cause the reagent gas to ionise and further ions are produced through the gas reacting with itself. In the case of methane, three major reagent ions are produced, namely CH^{+}_{5} (*m*/*z* 17), $C_2H_5^+$ (*m*/*z* 29) and C_3H_5 (*m*/*z* 41). These reagent ions can either transfer a proton to the analyte, thus ionising it, or combine with it to produce adduct ions.

When chloroquine is ionised under PICI conditions the spectrum shown in Fig 16 is produced, which can be compared with the EI spectrum in Fig 14. In the PICI spectrum the $[M+H]^+$ ion is the base peak and ions seen at

m/*z* 348 and *m*/*z* 360 are from the addition of *m*/*z* 29 and *m*/*z* 41 reagent ions to chloroquine. There is also an ion at *m*/*z* 284, which is caused by ionisation via abstraction of the chlorine atom from chloroquine by the reagent ions. PICI is not commonly used in chromatographic analyses, since it is usually of low efficiency, but it can sometimes be a useful method to help identify impurities in drugs; it is also a useful way to generate an abundant molecular ion for elemental composition determination when using high–resolution MS.

Negative ion chemical ionisation

NICI spectra are generated in the same way as PICI spectra, except that the instrument is set to focus and detect negative ions. Most negative–ion spectra are not true chemical ionisation spectra, but are rather electron capture spectra. Molecules that contain electronegative and/or electron–rich groups, such as halogens, oxygens or conjugated double bond systems, have a high affinity for electrons and thus capture the low–energy electrons produced by collision with methane. Thus, NICI is only suitable for molecules that contain electron–capturing groups, but for this type of molecule it is an extremely efficient and selective mode of ionisation. The NICI spectrum of chloroquine is shown in Fig 17. As in PICI, the spectrum is dominated by the molecular ion, in this case at *m*/*z* 319.

Mass spectrometry in qualitative analysis

Analysis of rosemary oil

Fig 18(A) shows the chromatogram obtained from the analysis of a sample of rosemary oil by GC–MS. The mass spectrum shown (B) is for the peak at 14.37 min. This spectrum was computer matched against the NIST library spectrum (details from http://www.hdscience.com) and gave two match figures of 932 and 941. Spectra are matched with the library by comparing the intensities of ions across the mass range scanned and a correlation coefficient is obtained. Any match of between 900 and 1000 is regarded as excellent. The higher second match figure in this case was obtained by matching the library spectrum to the unknown, omitting any peaks in the unknown that did not occur in the library spectrum, which is why the correlation number is slightly higher.

Library search matches are very useful for identifying unknown volatile materials; another area in which GC–MS is useful is in the identification of unknown residual solvents. Even though solvents are relatively simple molecules, there is quite a wide range of possibilities when an unknown is picked up, so library matching is very useful in such cases. In addition to solvents, many manufacturing intermediates have a relatively low molecular weight and are volatile and thus suitable for GC–MS analysis.

Impurity profiling

The Food and Drug Administration now require that impurities of greater than 0.1% be identified in pharmaceuticals, in which MS with chromatography can assist. Such impurities can arise either from the manufacturing process or from degradation of the drug. Fig 19 shows a GC–MS trace for a commercial sample of the β-blocker oxprenolol. The compound contains three major impurities, one of which is resolved poorly from the very large peak obtained for the drug. The manufacture of β-blockers is by fairly standard routes, which helps the identification of manufacturing impurities. The mass spectrum of oxprenolol is shown in Fig 19. Under EI conditions it gives a relatively weak molecular ion at *m*/*z* 265 and the spectrum is dominated by the fragment that arises from alpha homolytic cleavage next to the amine in the side chain, which is typical of many amines (as in chloroquine).

Let us take, as an example, the impurity peak 3. The EI spectrum of this impurity (Fig 20) shows only two major ions at m/*z* 158 and *m*/*z* 72. Its GC retention time is longer than that of oxprenolol and, on the non–polar GC column used, this suggests that the impurity has a higher molecular weight than oxprenolol. Under PICI conditions, the base peak of the mass spectrum of the impurity is an ion at *m*/*z* 308; the ion at 158 is still present, although much weaker. Taking into account the final step in the synthetic route to oxprenolol, the product possibly arises from the presence of a small amount of diisopropylamine in the isopropylamine used in the final step of the synthesis. Impurities 1 and 2 are isomers of oxprenolol; impurity 1 could arise via opening of the epoxide ring in the final step of the synthesis to form a primary alcohol, but both might also arise from isomers of the dihydroxybenzene present in the starting material for the synthesis.

Application of LC–MS in qualitative analysis

Determination of a degradation product from salmon calcitonin by ESI with triple quadrupole tandem MS

With the advent of biotechnologically produced pharmaceutical products, LC–MS (Neissen 1998) has come into its own as a quality control method. The complexity of proteins means that quality control by any other method is very difficult.

Salmon calcitonin (SC) is a single–chain 32 amino acid polypeptide that is active in humans and is used to treat osteoporosis. The potency and duration of action of the drug depend on eight residues at the N-terminus, the formation of a S–S bridge between cysteine residues at positions 1 and 7 and the presence of a proline residue at the C-terminus. SC is formulated in aqueous solution for injection and thus is susceptible to degradation with time. Examination of a 2–year–old ampoule by LC–MS revealed an impurity peak in the LC–MS chromatogram of a few

per cent running before the SC (Silvestro and Savu 1996). Fig 21 shows the mass spectrum of the impurity and the mass spectrum of SC, which gives a doubly charged molecular ion at 1716 a.m.u. with which is associated a small ion at 1727 a.m.u. from the addition of sodium (23/2) to the molecular ion. The impurity gives a doubly charged molecular ion at *m/z* 1725, which differs from SC by 9 a.m.u. and suggests the addition of water to the molecule. To find out the position of the modification, the SC impurity was isolated and treated with trypsin, which selectively cleaves polypeptides next to arginine and lysine residues. SC is expected to produce seven fragments from trypsin treatment. The impurity produced a similar pattern of degradants to SC. One of the fragments produced by the impurity, which corresponded to the 1–18 fragment in SC, gave a single–charge molecular ion, which indicated a difference of 18 a.m.u. from the corresponding SC fragment. It was possible to induce fragmentation of this ion by changing the cone voltage, and the series of ions produced when compared with those derived from SC suggested that hydrolysis of the S–S bridge between residues 1 and 7 had occurred. Storage of the SC in ampoules filled with an inert gas minimised the formation of this degradant.

Identification of drug metabolites

Metabolism is an important component in the drug discovery and development process, and LC–MS has an important role in identifying drug metabolites (Oliveira and Watson 2000). The pathways of phase 1 and phase 2 drug metabolisms are well known, and it is possible to derive useful information even from a single quadrupole instrument by using extracted ion chromatograms to search for predicted metabolites. For example, formation of a monoglucuronide of a drug results in a shift of 176 a.m.u. from the molecular ion of the parent. However, the process of searching for metabolites is easier if tandem MS is available. The preferred mode of metabolite profiling in the preliminary analysis of metabolites is to use product–ion scanning. The predicted ion for a metabolite is selected by the first quadrupole, subjected to fragmentation in the collision cell and the fragments are analysed by the third quadrupole. This enables acquisition of clean metabolite spectra that are free from any interfering solvent background. If a quantitative analysis is required, selected reaction monitoring may be carried out, in which a critical transition is monitored. For example, the transition produced by loss of a glucuronide moiety from a glucuronide metabolite might be monitored if it gives a very specific response for that particular metabolite. Constant neutral–loss scanning is especially useful for searching for a particular class of metabolite, since it can readily detect metabolites resulting from both phase 1 and phase 2 metabolisms. For example, glucuronide metabolites for which the loss of the glucuronide moiety (–177 a.m.u.) is a major fragmentation pathway might be monitored. If the masses of the metabolites fall in a range (e.g. between 400 a.m.u. and 700 a.m.u.), the first quadrupole is set to scan between 400 and 700 a.m.u. and the second quadrupole to scan in the range 400−177 a.m.u. to 700−177 a.m.u. In this way, any metabolites that are, for example, methylated or undergo additional hydroxylation followed by glucuronidation are picked out, as well as simple glucuronides.

Ion–trap instruments can also be used to good effect in drug–metabolism studies and have approximately ten times the sensitivity of triple–sector quadrupole instruments when used to examine full–scan spectra. This is

advantageous in the first phase of metabolite identification when the metabolites are unknown. Another advantage of trap instruments is that fragmentation of selected ions can be carried out several times with all the ions, apart from the molecular ion of the metabolite of interest, being ejected from the trap before the next fragmentation. This process produces clean spectra for the metabolite.

Some applications of mass spectrometry in quantitative analysis

Mass spectrometric detectors are able to carry out precise and accurate quantification of analytes. However, it is generally necessary to use an internal standard in analyses, since the instrumentation is more subject to sensitivity fluctuations than simpler detectors, such as the UV–visible detectors used in HPLC analyses. The selection of an internal standard has to be made carefully so that its mass spectrometric behaviour is reproducible and closely similar to that of the analyte. The internal standards labelled with stable isotope (described below) are ideal, since they mimic the analyte very closely, but often a close structural analogue of the analyte will suffice.

The most common application of MS to quantitative analysis of biomedical samples is in the quantitative determination of drugs and their metabolites in biological fluids and tissues. The advantage of MS in this area is that its selectivity means it is less subject to interference by other compounds extracted from the biological matrix along with the compound of interest. The greatest accuracy in such analyses is afforded by using as internal standards analogues of the compound being measured that are labelled with stable isotopes. An isotopomeric internal standard of a drug co–elutes with it from a chromatographic column (sometimes deuterated compounds elute very slightly earlier than the unlabelled compound) and should have an almost identical response factor. Fig 22 shows the NICI mass spectra of the trimethylsilyl oxime derivative of prednisolone and its tetradeuterated analogue. The deuterated analogue of prednisolone can be used as an internal standard in the determination of prednisolone in a biological matrix. On the basis of the mass spectra shown the ions at *m*/*z* 457 and 472 are monitored for prednisolone and those at *m*/*z* 461 and 476 for the tetradeuterated internal standard.

Figure 22. NICI spectra of trimethylsilyl prednisolone oxime and its tetradeuterated isotopomer.

Since isotopomeric internal standards co–elute with the analyte, they aid in the recovery of the analyte from the chromatographic system (carrier effect). Fig 23 shows a selected ion chromatogram of prednisolone methyl oxime/trimthylsilyl (MO/TMS) derivative (Knapp 1990) (monitored as the sum of the ions m/*z* 457 and 472, see Fig 22), which was extracted from aqueous humour after addition of 10 ng of tetradeuterated prednisolone (the MO/TMS derivative was monitored as the sum of the ions m/*z* 461 and 476, see Fig 22); the analysis was carried

out using GC–MS. Similar types of approaches can be taken in LC–MS analysis.

Figure 23. Prednisolone extracted from aqueous humour in comparison with D4 prednisolone (10 ng) added as an internal standard. (Both as their trimethylsilyl oximes.)

Collections of data

Electron–impact libraries

The most comprehensive collections of mass spectral data are based on EI mass spectra that have been acquired over many years and are used most commonly in conjunction with GC–MS analysis. The most popular libraries are the NIST library (details from http://www.hdscience.com), which contains the mass spectra of 130 000 compounds, and the Wiley Registry of Mass Spectral Data (http://www.wileyregistry.com), which contains 390 000 reference spectra. The mass spectrum of a molecule in the library may be called up by using either its CAS number, its name or its relative molecular mass. These libraries use peak–based matching to compare the mass spectrum of an unknown against the library spectrum. Spectra are matched in a manner analogous to the way two UV spectra are matched, except in this case the *m*/*z* values and the intensity of the ions of library spectrum and the unknown are matched. To simplify matching, a threshold can be set to eliminate, for example, ions of less than 1% intensity from the mass spectra being compared. A perfect match to a library spectrum has a value of 1000 and a value above 900 is regarded as a good match; a poor match has a value below 600. Other sources of EI mass spectra, such as the Eight Peak Index (Royal Society of Chemistry 1991), and a collection of mass spectra of drugs and poisons (Pfleger et al. 2000) are available in book form, but the computer–based libraries offer a greater degree of convenience in most cases.

Libraries associated with LC–MS ionisation methods

Mass spectra obtained under the ionisation conditions used in LC–MS, such as electrospray, show little fragmentation, and the established libraries of EI spectra are of little use in searching such spectra. The use of tandem techniques increases the degree of fragmentation of molecules, but these do not reproduce exactly the EI spectrum of a molecule. Currently no comprehensive libraries have been for methods, such as ESI and APCI, used to ionise compounds after LC separation; generally, these types of library are user-generated for a specific purpose.

There is one area in which comprehensive databases based on MALDITOF or electrospray–ion trap spectra have been built up, and this is in the field of proteomics (James 2000; Kinter and Sherman 2000). The databases built up from MALDITOF data have been in operation for a longer time than those based on ion–trap data. The standard approach used in conjunction with MALDITOF is to separate proteins on gel electrophoresis, and thus obtain an approximate p*I* value (the pH of the isoelectric point of the protein), which can also be used in identification. The protein is cut from the gel, a proteolytic digest (most often using trypsin) is carried out and the peptide fragments generated are analysed using MALDITOF. The pattern of peptides obtained can be matched against one of a number of databases by using a linking program, such as ProteinProspector (http://prospector.ucsf.edu/). The linking program searches one of the large protein–sequence databases, such as SwissProt, for the proteins that contain amino acid sequences most closely matching those of the unknown. Additional information can be obtained by varying the laser power during the ionisation step or by post–source decay using the reflector to produce additional fragmentation of the peptides in the digest, and this information can be used to further refine the database search. A similar process is used for the ion–trap instruments using ESI. In this case the peptide digest can be separated by HPLC prior to its introduction into the mass spectrometer. A program such as TurboSEQUEST can be used to search protein sequence databases and MS–MS spectra can be obtained from the peptides in the digest to refine the search.