CHAPTER 8

Mass Spectrometry in Medical Research

8.1 CHARACTERISATION AND QUANTITATION OF DRUGS AND METABOLITES

8.1.1 Introduction

In recent years, there has been an appreciable shift in focus in drug discovery away from the design, synthesis, characterisation and evaluation of organic drug molecules. Today's pharmaceutical and academic research laboratories seek to gain a global understanding of the genetic basis of disease states (through functional genomics), potential causative proteins or markers (functional proteomics), and the evaluation of drug candidates and therapies. Pharmacological studies of drug absorption, excretion and metabolism are also performed in the context of a complete description of human biology (metabolomics). The identification and characterisation of these biomarkers or targets can be performed by mass spectrometry using approaches described in Chapter 7.

The traditional characterisation and evaluation of organic drug targets, nonetheless, continues to be an important area of medical research. In these studies, molecules are designed to "target" a biological molecule, tissue or system to stimulate, confer or prevent some function or activity. Such molecules are usually constructed by synthetic routes either as a single lead compound or as part of a chemical library of related compounds. Potential drug targets are assessed based upon their bioavailability (or the extent to which the administered dose reaches its target), half-life, and therapeutic index. The half-life of the compound represents the time in which it takes for 50% of the concentration of the compound to be excreted or metabolised *in vivo*. The therapeutic index represents a measure of the desired function or activity versus any undesired side effects. The most effective drugs are those with a high therapeutic index, a high bioavailability, and often a low half-life.

8.1.2 Sample Preparation Techniques in Drug Discovery

When dealing with biological and *in vitro* samples, the preparation of the sample for mass spectrometric analysis is of paramount importance in order to achieve analytical success. A wide variety of approaches including precipitation and centrifugation methods, ultrafiltration, solid-phase extraction, blotting and immobilisation are all employed subject to the sample at hand and its biological source. The precipitation of biological components from an extract can be achieved by adding denaturing solvents such as methanol, acetonitrile or acetone. The recovery of these components follows the rapid mixing of the solution and their density centrifugation to the base of the sample vial.

Ultrafiltration provides another means with which to separate the components of biological mixtures by passing the solution through molecular weight cut-off filters. Under centrifugation the larger molecular weight species remain trapped on the top of the filter while smaller compounds pass through with the solvent. This process can be performed in a series of stages so that a sample mixture is effectively partitioned into sets of compounds spanning several molecular weight ranges.

Solid-phase extraction (SPE) takes advantage of the separation characteristics exploited in liquid chromatography. Samples are loaded onto small cartridges, pre-packed with chromatographic supports suitable for reverse-phase, ion exchange or ion exclusion chromatography. The sample solution is passed through the cartridge under gravity, by vacuum or using centrifugal force. Solvents of differing compositions are then added in succession to effect the partitioning of solutes between the solid and solution phases.

Blotting and immobilisation procedures are also used widely in drug discovery applications to isolate particular components from biological sources. Drug targets can also be absorbed or immobilised on films and screened against an array of drug compounds ahead of their analysis. The screening of drugs using mass spectrometry is the subject of Section 8.4.

8.1.3 Qualitative Analysis of Organic Drugs and their Metabolites

The identification or characterisation of a drug compound by mass spectrometry is dependent upon the structural features of that compound. For most small to moderately sized (~1000 Da.) compounds this is achieved by either GC-MS or LC-ESI-MS. The former approach predated the development of the ESI technique and has to some extent been superseded by it. However, certain volatile or derivatised molecules are particularly suited to GC-MS in which the resulting EI (or CI) mass spectrum provides both molecular weight and structural information.

The latter is usually achieved through the use of tandem mass spectrometry in LC-ESI-MS experiments since the "soft" nature of the ESI technique results in most compounds resisting fragmentation. Such approaches can be used for the characterisation of trial drugs, those approved for pharmaceutical use, or illegal drugs or narcotics.

As an illustration, cocaine and 6-acetylmorphine can be detected in a single human hair by GC-MS. A cutting from a single hair was washed and heated in methanol. The volatile components were passed into the ion source of a GC-MS and both cocaine and 6-acetylmorphine were detected by means of selected ion monitoring (SIM) (Chapter 5, Section 5.4.2) of the fragment ions at *m/z* 182 (Figure 8.1) and 268 respectively.

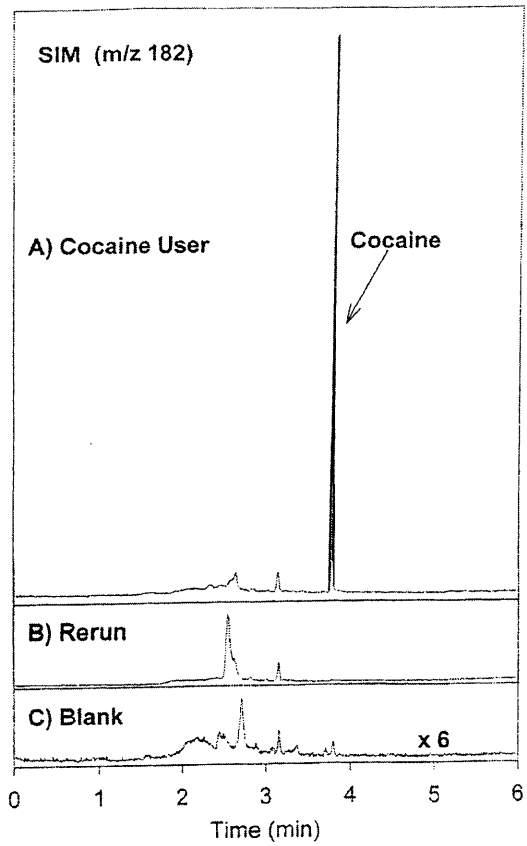

Figure 8.1 *SIM of the m/z 182 fragment of cocaine from: (A) a 1-cm piece of a cocaine user's hair (subject 2), (B) The rerun of the same hair sample used in (A) demonstrating the near 100% recovery, and (C) a 1-cm piece of hair obtained from a drug free individual*
(Source: S.B. Wainhaus, N. Tzanani, S. Dagan, M.L. Miller and A. Amirav, Fast Analysis of Drugs in a Single Hair, *J. Am. Soc. Mass Spectrom.*, 1998, **9**, 1311, Figure 4)

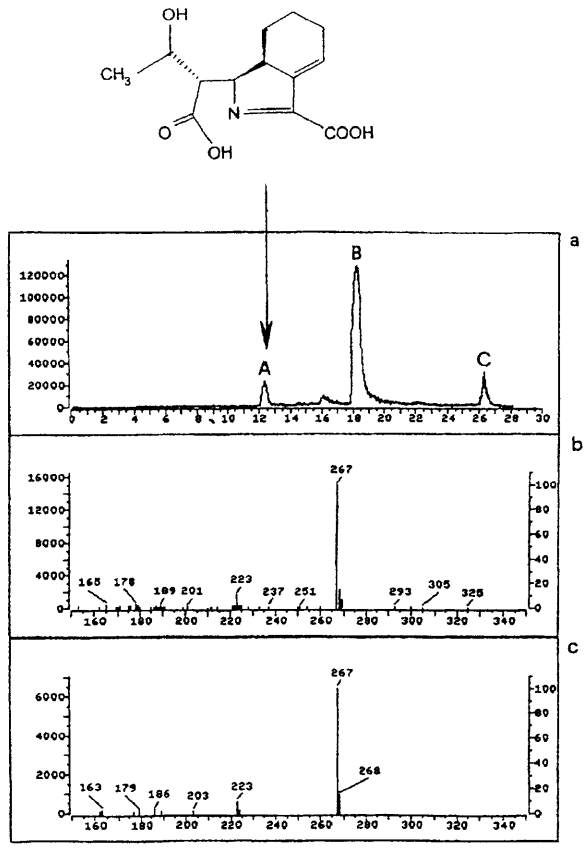

Figure 8.2 *Particle beam/chemical ionisation data for: (a) total ion current (m/z 700–1300) chromatogram of rat urine, (b) mass spectrum of component "A" the metabolite eluting at 12.6 min., and (c) mass spectrum of metabolite reference standard*
(Source: adapted from L. Iavarone, M. Scandola, F. Pugnaghi and P. Grossi, Qualitative Analysis of potential metabolites and degradation products of a new antiinfective drug in rat urine, *J. Pharma. Biomed. Anal.*, 1995, **13**, 607–614, Figure 8)

These constituents were detected at concentrations as low as 10 parts-per-billion (ppb) with analyses conducted within 10 minutes.

The qualitative analysis of metabolites is also an important requirement of drug testing to evaluate both the half-life of a drug and the nature and potential toxicity of its metabolites.

LC-MS has been employed to follow the metabolic fate of a β-lactam antibiotic. The metabolic profiles of the antibiotic in rat urine were monitored by mass spectrometry in conjunction with ion exchange HPLC after a single intravenous administration of the drug. Two

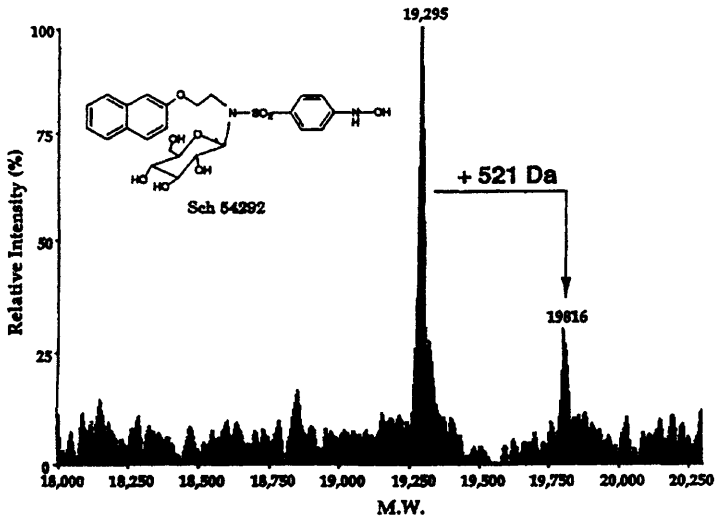

Figure 8.3 *Deconvoluted (molecular weight adjusted) ESI mass spectrum of a 1:1 ras GDP oncoprotein inhibitor (SCH 54292) complex with an average mass of 19,816 Da* (Source: A.K. Ganguly, B.N. Pramanik, E.C. Huang, S. Liberles, L. Heimark, *et al.*, Detection and structural characterisation of Ras oncoprotein-inhibitors complexes by electrospray mass spectrometry, *Bioorganic and Medicinal Chemistry*, 1997, **5(5)**, 817–820, Figure 1)

metabolites were detected, one of which corresponds to a ring-opened degradation product based upon its mass spectrum (Figure 8.2).

The association of drugs with protein targets can also be evaluated by mass spectrometry. As described in Chapter 7 (Section 7.5.2), ESI and to a lesser extent MALDI mass spectrometry are able to both preserve and detect specific solution state associations within a mass spectrometer. A 1:1 non-covalent complex between a ras GDP oncoprotein and a potential inhibitor is shown in Figure 8.3. Mutant ras proteins have been implicated in the growth of a wide range of human tumours and thus the inhibition of GDP could prevent continued tumour growth. The approach offers the opportunity to study protein complexes that cannot be investigated by other methods. In this case, crystals for the ras GDP protein could not be successfully prepared for X-ray crystallography.

The purity of a drug compound must also be thoroughly evaluated before it can be used in clinical trials. In some cases it is necessary that the drug be enantiomerically pure. Where a chiral drug has a particular potency and the drug is to be administered as a racemic mixture, it is necessary to establish that the inactive enantiomer affords no potential side effects.

The kinetic method developed by Cooks (Section 6.1.4) has been used to determine the enantiomeric composition of a drug mixture through the competitive dissociation of their copper-bound ion complexes. Copper(II)-bound ion complexes formed from seven model drugs together with a series of chiral reference compounds (L–amino acids only) were analysed by electrospray ionisation mass spectrometry. The complexes were found to undergo collisionally activated dissociation (CAD) by competitive loss of either the neutral drug molecule or the reference. The ratio of the two competitive dissociation rates allowed the composition of enantiomeric drugs in the mixture to be determined using a two-point calibration curve.

8.1.4 Quantitative Analysis of Drug Compounds and their Metabolites

Mass spectrometry plays a central role not just in confirming the presence or structure of a drug molecule, but also in measuring the absolute and relative levels of the compound or its metabolites in serum or plasma. A measurement of the concentration of the compound or its metabolites as a function of time, after the dose has been administered, provides a pharmacokinetic profile that is useful in establishing a drugs' bioavailability or its rate of metabolism throughout the body. Quantitation measurements are also necessary where the metabolite is toxic or pharmacologically active when it reaches a particular concentration.

In addition to selected ion monitoring (SIM) described above, *selected* or *multiple reaction monitoring* (SRM or MRM) is one of the most common approaches used for this purpose and is achieved within a tandem mass spectrometer. Here the metastable transition or conversion of a metabolic precursor to a product is monitored. Only selected precursor ions that decompose to product ions of a particular m/z ratio will be detected. This affords optimal sensitivities where compounds can be quantitated to the sub-ppb (part-per-billion) level. In a MRM experiment, a series of such reactions is monitored by rapid switching of the electric fields applied to the mass analyser to study each reaction in turn.

Figure 8.4 shows the MRM ion chromatograms for seven dosed compounds extracted from brain tissue plus an internal standard (top chromatogram).

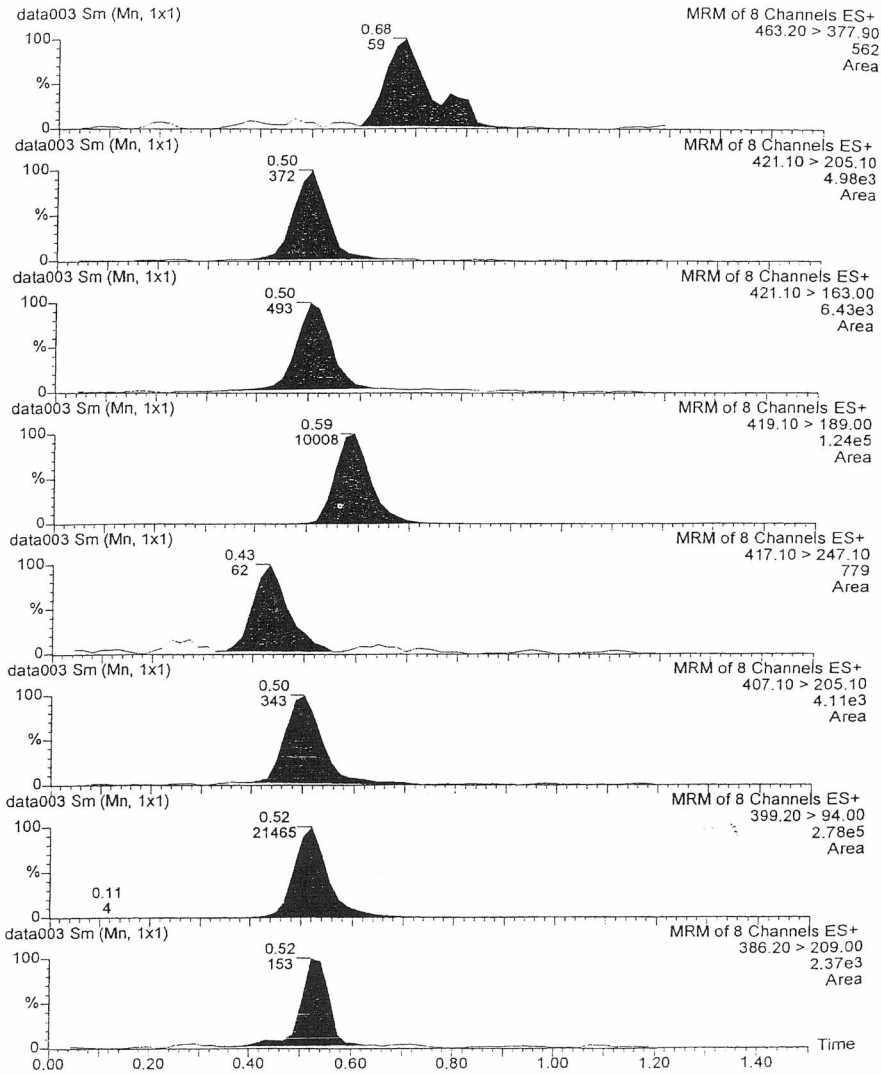

Figure 8.4 *ESI positive ion MRM chromatograms for seven compounds extracted from brain tissue plus an internal standard (top chromatogram)*
(Source: D.T. Rossi, Sample preparation and handling for LC/MS in drug discovery, in *Mass Spectrometry in Drug Discovery*, D.T. Rossi and M.W. Sinz (ed), Marcel Dekker, New York, 2002, Ch. 6, p. 206, Fig. 15)

8.2 DEFINING METABOLIC PATHWAYS WITH MASS SPECTROMETRY

Where the pharmacokinetic profile of a metabolite exists, it is often desirable to establish the reaction pathway through which the meta-

bolite is produced. Common metabolic processes include oxidation, methylation, acetylation and epoxidation as well as degradation reactions. These consequently cause changes in the molecular weight of the product in each step of a metabolic pathway.

Heavy isotopes (*e.g.* ^{13}C, ^{15}N) are of use for tracking the decomposition or reaction of a drug as it is metabolised. The isotopic enrichment of a drug changes its molecular weight and isotopic profile (Chapter 1, Section 1.2). If the heavy isotope is retained by the metabolite its ion will have a *m/z* ratio and isotopic distribution that will differ in appearance from that of its unlabelled form. Thus the drug and metabolite can be associated. The use of a series of labels at different positions throughout the drug enables a range of metabolites to be identified from which their dissociation pathways can be deduced. The placement of the heavy isotope should be decided upon with some care and it must be placed in a metabolically inert position since if the isotope "lost" early during its metabolism, the reaction pathway can no longer be followed.

8.3 CHARACTERISATION OF DRUG LIBRARIES BY MASS SPECTROMETRY

As mentioned at the introduction to this chapter, drugs and drug targets are usually constructed as part of a chemical library. These libraries are pooled and split and subsequent sets of compounds screened for activity in an assay. Library components can be characterised in these sets by reverse phase HPLC or capillary electrophoresis coupled to an ESI or APCI mass spectrometer (Chapter 3, Section 3.2.10). It is common to pass a portion of the eluant from the column to a secondary detector, such as an ultraviolet (UV) absorbance detector preferably operating over a range of wavelengths. The purpose of the secondary detector is to assist with the detection and quantitation of the components.

Stable isotopes can also be used to encode particular components of drug libraries to assist with their identification in what is termed *stable isotope encoding*. In many instances, libraries are constructed of structurally similar compounds whose molecular weights may coincidentally be identical or indistinguishable by mass spectrometry. If stable heavy isotopes are incorporated into some compounds during their synthesis, these can be mixed in series at varying molar ratios with their non-labelled counterparts, such that each component can be distinguished based upon the ratio of the ion signals for the labelled and unlabelled forms.

Establishing the chemical components in each library enables screening of the activity of these components to begin. For the most part, this

is performed without the aid of a mass spectrometer but a number of mass spectrometric-based assays have now been developed that are of use in drug discovery investigations.

8.4 DRUG SCREENING USING MASS SPECTROMETRY

The immobilisation of a drug target to a surface or membrane provides a means with which to screen drug libraries in an automated manner. A solution of mixtures of library compounds can be passed across the bound target and the surface washed after an appropriate incubation time. The bound drugs can then be chemically released and detected.

Mass spectrometry has been employed in such assays to detect the released drug including the use of MALDI to catalyse that release and subsequently ionise the bound drug. A multi-sample MALDI target or miniaturised chip format enables a series of drug interactions to be studied simultaneously where each position on the target or chip characterises a unique association. Such an approach has been used to screen protein associations in what has been described as *biomolecular interaction analysis mass spectrometry* (BIA-MS) (Figure 8.5).

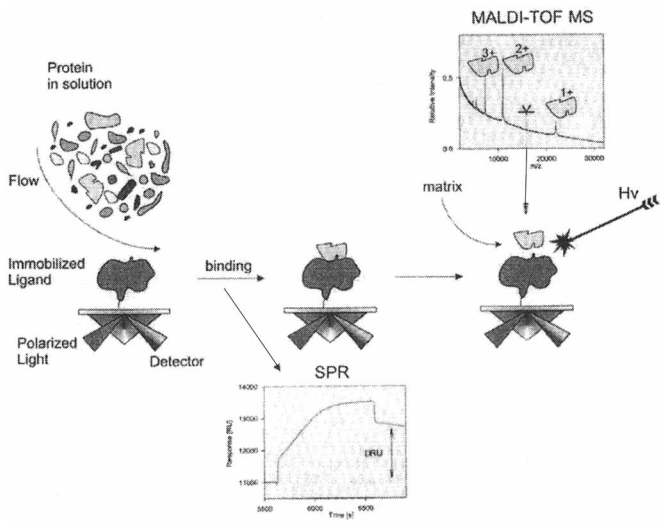

Figure 8.5 *Schematic representation of the BIA-MS method in which surface-immobilised ligands with affinities toward a protein of interest are used to retrieve the protein from a complex biological mixture. Surface plasma resonance is used to monitor the interaction and quantify the amount of retrieved protein(s). MALDI-MS releases the bound protein enabling it to be identified by molecular weight* (Source: D. Nedelkov and R.W. Nelson, Biomolecular interaction analysis mass spectrometry: A comprehensive microscale proteomics approach, *American Laboratory*, 2001, 22–25, Figure 1)

In an extension of this approach, Williams, Nelson and co-workers have used a 96-well robotic workstation to preferentially isolate proteins in parallel from human blood and plasma using tips filled with immobilised antibody to each protein target. The isolated proteins were subsequently transferred to a MALDI sample target for their detection by mass spectrometry (Figure 8.6). The affinity isolation of proteins from such biological matrices in combination with MALDI-TOF MS holds promise in proteomics for the detection of protein markers or mutants associated with genetic abnormalities and disease.

8.5 TRACE ELEMENT ANALYSIS IN NUTRITION

Just as isotopic labelling techniques are used to follow the absorption and metabolism of a man-made drug administered orally or intravenously, mass spectrometry can also be applied to follow the ingestion and absorption of essential vitamins and minerals. Because not all nutrients and minerals in a diet are retained and available for physiological function, the uptake of dietary elements can be followed using stable heavy isotopes.

Dietary iron is vital for correct physiological function and a lack of iron leads to anemia, impaired mental and motor development, and a reduced resistance to infection. The absorption of iron by red blood cells can be followed using test meals isotopically-enriched with ^{57}Fe or ^{58}Fe. Subjects are fed meals containing either ^{57}Fe or ^{58}Fe and the amount of isotope retained in the blood is quantified by mass spectrometry. *Inductively coupled plasma mass spectrometry* (ICP-MS) is used widely for such an analysis (see Section 9.1.1) and employs a high resolution mass analyser and multiple ion detectors.

ICP-MS has also been employed to study calcium uptake in bone. During bone growth, calcium is transferred into bone at rates greater than is lost. The balance alters with age resulting in a reduction in bone mineral density and the onset of osteoperosis. Women are at particular risk of osteoperosis and the resultant bone fractures. Stable isotope studies using diets containing ^{40}Ca and ^{41}Ca allow the absorption of calcium to be followed by monitoring calcium levels excreted in fecal matter or urine, or present in blood plasma.

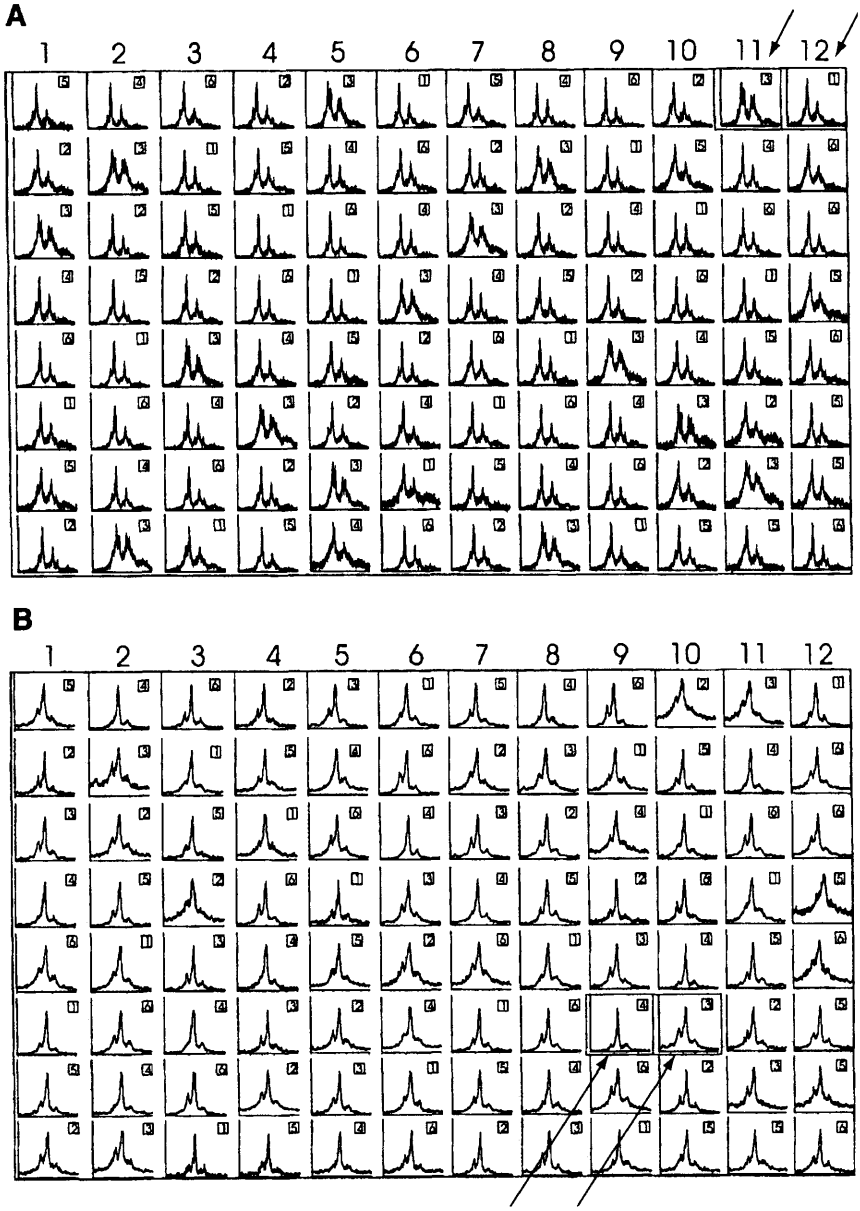

Figure 8.6

FURTHER READING

D.T. Rossi and M.W. Sinz (ed) *Mass Spectrometry in Drug Discovery*, Marcel Dekker, New York, 2002.

D.I. Papac and Z. Shahrokh, Mass spectrometry innovations in drug discovery and development, *Pharmaceutical Research*, 2001, **18(2)**, 131–145.

S.J. Gaskell and D.S. Millington, Selected metastable peak monitoring: A new specific technique in quantitative gas chromatography mass spectrometry, *Biomed. Mass Spectrom.*, 1978, **5**, 557–558.

U.A. Kiernan, K.A. Tubbs, K. Gruber, D. Nedelkov, E.E. Niederkofler, P. Williams and R.W. Nelson, High-throughput protein characterization using a mass spectrometric immunoassay, *Anal. Biochem.* 2002, **301**, 49–56.

F. Mellon, R. Self and J.R. Sartin, *Mass Spectrometry of Natural Substances in Food*, Royal Society of Chemistry, Cambridge, UK.

Figure 8.6 *(opposite) High-throughput Mass Spectrometric Immunoassay (MSIA) analysis of transthyretin (TTR) and retinol binding protein (RBP) using human plasma samples from six individuals randomly arranged in a 96-well titer plate. (A) Mass spectra result from MSIA analysis utilising anti-TTR derivatised pipette tips. Shown is the region of the singly charged TTR signals. Highlighted cells show spectra with resolvable parent ion differences between each other. (B) Mass spectra result from the MSIA analysis of the same samples utilising anti-RBP derivatised pipette tips. Shown is the region of the doubly charged RBP signals*
(Source: U.A. Kiernan, K.A. Tubbs, K. Gruber, D. Nedelkov, E.E. Niederkofler, P. Williams and R.W. Nelson, High-Throughput Protein Characterization Using Mass Spectrometric Immunoassay, *Anal. Biochem.*, 2002, **301**, 49–56, Figure 3)