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REVIEW
Metabonomics technologies and their applications in physiological monitoring, drug safety assessment and disease diagnosis

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In this review, metabonomics, a combination of data-rich analytical chemical measurements and chemometrics for profiling metabolism in complex systems, is described and its applications are reviewed. Metabonomics is typically carried out using biofluids or tissue samples. The relevance of the technique is reviewed in relation to other '-omics', and it is shown how the methods can be applied to physiological evaluation, drug safety assessment, characterization of genetically modified animal models of disease, diagnosis of human disease, and drug therapy monitoring. The different types of analytical data, mainly from nuclear magnetic resonance spectroscopy and mass spectrometry, are summarized. The outputs from a metabonomics study allow sample classification, for example according to phenotype, drug safety or disease diagnosis, and interpretation of the reasons for classification yields information on combination biomarkers of effect. Transcriptomic and metabonomic data is currently being further integrated into a holistic understanding of systems biology. An assessment of the possible future role and impact of metabonomics is presented.

Keywords: Metabonomics, proteomics, genomics, transcriptomics, nuclear magnetic resonance, mass spectrometry, physiology, genetic modification, diagnosis, drug safety, atherosclerosis.

Introduction

Given that the human and many other species genome sequences are now known, there has been surprisingly little impact on the numbers of new drug substances coming to the clinic, although there is now a greater understanding of 'druggable' targets (Stumm *et al.* 2002). Over the last few years, billions of dollars have been pumped into a huge industry built on measuring gene expression changes (transcriptomics), mostly involving the use of gene-chip technologies (Kahl 2001). Subsequently, a similar explosion has occurred for proteomics, which largely comprises mass spectrometry-based methods for characterizing the consequent changes in protein levels (Aebersold 2003). One possible explanation is that transcriptomics, in particular, and proteomics do not provide evidence of endpoint markers for disease diagnosis or evaluation of beneficial or adverse drug effects in the same way as altered biochemistry provides the ultimate diagnostic information. However, metabonomics hold out this possibility.

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In this review, metabonomics as a technology for completing the understanding of biological processes is introduced and explained. Its relationship to the other '-omics' – genomics and proteomics – is represented in figure 1. It is important to realize that factors other than changes in gene expression or single nucleotide polymorphisms (SNPs) can affect the systems biology view of an organism; environmental factors such as diet, age, ethnicity, lifestyle and gut microfloral populations have a large influence and these various factors need to be deconvolved. Metabonomics can be regarded as providing real biological endpoints and is defined as 'the quantitative measurement of the time-related multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification' (Nicholson *et al.* 1999, 2002). Metabonomics involves the generation of metabolic databases, based on tissue or biofluid samples, for control animals and humans, diseased patients, animals used in drug safety testing, etc., allowing the simultaneous acquisition of multiple biochemical parameters on biological samples.

Metabonomics is usually conducted on biofluids, many of which can usually be obtained non-invasively (e.g. urine) or relatively easily (e.g. blood), but other more exotic fluids such as cerebrospinal fluid, bile or seminal fluid can be used. It is also possible to use cell culture supernatants, tissue extracts and similar preparations, and in special cases, as described later, intact tissue biopsy samples.

A variety of analytical methods could in principle be used to generate metabonomic data sets so long as the approach provides information on the molecules that give rise to the experimental data. Thus ultraviolet spectroscopy and other forms of electronic spectroscopy are less than ideal since they only provide information on molecular fragments, such as different types of aromatic molecules giving rise to the chromophores, and the spectral line widths are so broad that signals from all species overlap considerably. Infrared (IR) spectroscopy provides more molecular information in the sense that any differences in spectra due to a

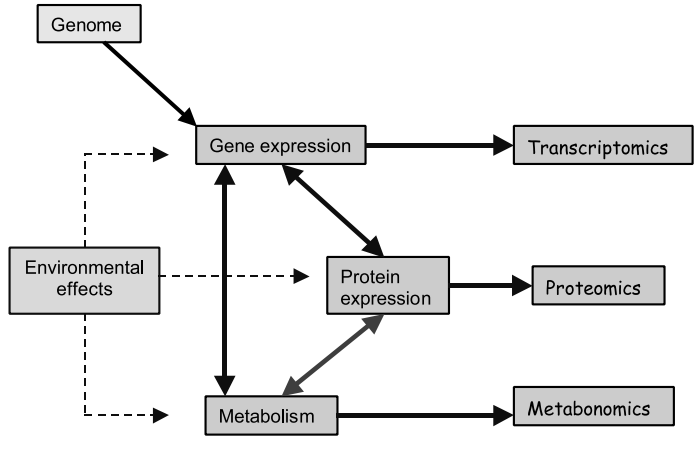

Figure 1. The relationship between genomics, proteomics and metabonomics. Metabonomics can operate at all levels of an organism, namely organelle, cell, tissue, organ, biofluid or organism. Environmental factors can also have a major influence at all levels of molecular biology.

perturbation can be interpreted crudely in terms of the functional groups of the substances involved. Again, resolution is limited, for example carbonyl stretch frequencies from all amides such as in different peptides appear overlapped, and molecular identification is generally only possible by IR spectroscopy for pure compounds by direct comparison with a database of authentic spectra.

The two most information-rich techniques that give atom-specific molecular structural information are mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy. Currently, for MS-based metabonomics, it is generally necessary to carry out a separation step, usually using liquid chromatography (LC) or chemical derivatization and gas chromatography (GC) before the MS stage (Niessen 1999). The use of Fourier transform MS with its exceptional resolution may remove the need for the separation step (Asamoto 1991). Moreover, MS can be more sensitive than NMR spectroscopy and can give lower detection limits. However, there are problems of non-uniform detection caused by variable ionization efficiency. Nevertheless, a few metabonomics studies using MS detection are now being published and are discussed here (Fiehn 2002, Plumb *et al.* 2003).

^{1}H NMR spectroscopy is especially suitable for metabonomics as it requires little or no sample preparation, is rapid and non-destructive, and uses small sample sizes (Nicholson and Wilson 1989). More recently, the technique of magic-angle spinning NMR spectroscopy has opened up the possibility of metabonomics applied to tissue samples (Moka *et al.* 1998). The NMR-detected metabolic response of an organism to a particular disease, toxin or pharmaceutical compound can then be extracted from the complex data sets, which are also subject to biological variation, by application of appropriate multivariate statistical analyses.

Metabonomics is a successful approach because disease, drugs or toxins cause perturbations of the concentrations and fluxes of endogenous metabolites involved in key cellular pathways. For example, the response of cells to toxic or other stressors generally results in an adjustment of their intra- and/or extracellular environment in order to maintain constancy of their internal environment (homeostasis). This metabolic adjustment is expressed as a fingerprint of biochemical perturbations that is characteristic of the nature or site of a toxic insult or disease process. Urine, in particular, often shows changes in its metabolite profile in response to toxic or disease-induced stress. This is because the body's cellular systems attempt to maintain homeostasis in the face of a challenge. One means of achieving this is to modulate the composition of biofluids and hence eliminate substances from the body. Hence, even when cellular homeostasis is maintained, subtle responses to toxicity or disease are expressed in altered biofluid composition.

The term metabonomics was devised by us. It involves the Greek roots '*meta*' meaning change and '*nomos*' meaning rules or laws (as used in economics), to describe the generation of pattern recognition-based models that have the ability to classify changes in metabolism. There has been a parallel set of developments in a subject called metabolomics (Raamsdonk *et al.* 2001). This is similar to metabonomics but is regarded as a subset of the topics covered by metabonomics. Metabolomics has arisen from metabolic control theory (Derr 1985) and was originally based on the metabolome, the metabolic analogy of the genome or proteome, which was defined as being the metabolic composition of a cell. In

metabonomics, not only are static cellular and biofluid concentrations of endogenous metabolites evaluated, but also the time courses of fluctuations in metabolites, exogenous species and molecules that arise from chemical rather than enzymatic processing (metabonates). In addition, as originally defined, metabonomics, as well as providing molecular concentrations, also covers the study of molecular dynamic information such as molecular reorientational correlation times and diffusion coefficients in intact tissues. Thus, metabonomics can be regarded as a full systems biology approach in that, when studying a whole organism with separate organs and many cell types, effects that are displaced not only in time but also in distance (e.g. the effects of one organ on another) can be integrated into a holistic view.

Metabonomics techniques

A variety of spectroscopic methods can be used to generate metabonomic data sets on complex biological samples so long as the data sets are rich in molecular information. A number of investigations, primarily in plant and microbiological contexts (Fiehn 2002), have used MS, mainly because of its overall greater sensitivity compared with NMR spectroscopy. This has usually been coupled either to LC or to GC after chemical derivatization. However, high resolution ^{1}H NMR spectroscopy has shown to be one of the most powerful technologies for biofluids and essentially the only one capable of studying intact tissues, producing a comprehensive profile of metabolite signals without the need for pre-selection of measurement parameters or the use of separation or derivatization procedures (Nicholson and Wilson 1989). Furthermore, variable detection responses, such as differential volatilization or ionization effects as in MS, are not an issue for NMR spectroscopy. However, it is clear that the two approaches are complementary, giving information on different sets of biomarkers, and integration of both technologies to provide more comprehensive classification and biomarker information is now occurring. As yet, there are few metabonomic studies on mammalian systems in the literature that have used MS as an experimental approach and even fewer that have identified novel biomarkers. Where such studies exist, they are discussed below, but at present, since most metabonomics studies are based on ^{1}H NMR spectroscopy, this review will concentrate on this methodology.

Typically, ^{1}H NMR spectra of biofluids such as urine and plasma contain thousands of signals arising from hundreds of endogenous molecules representing many biochemical pathways. Conventional measurement of the major NMR signals can be used to detect biochemical changes, but the complexity of the spectra and the presence of natural biological variation across a set of samples often make it difficult to detect meaningful patterns of change by eye. Generally it is necessary to use data reduction and pattern recognition (PR) techniques in order to access the latent biochemical information present in the spectra.

Biofluid samples are usually prepared for NMR spectroscopy into covered 96-well plates using proprietary robotics (the Bruker SampleTrack system (Beuker Biospin, Rheinstetten, Germany) and a Gilson 215 Preparation robot (obtained from Bruker) is used in our laboratory.). Approximately 500 µl of sample is injected into the flow-injection NMR probe (Spraul *et al.* 1997). For urine samples, some

stabilization of pH across samples can be achieved by the addition of buffer and subsequent centrifugation to remove insoluble salts.

Water is present at such high concentration in biofluids that its NMR peak is so huge that it can obscure other molecular information. It can also cause dynamic range problems in the NMR detector. For these reasons, ^{1}H NMR spectra of urine are measured using a standard water suppression pulse sequence resulting in a total acquisition time of about 4 min per sample. For serum or plasma, a suite of ^{1}H NMR spectra is usually measured including a spin-echo spectrum (for retaining mainly small molecule resonances) and a diffusion-edited spectrum (for macromolecule profiles). Additionally, it is possible to measure a two-dimensional (2-D) J-resolved spectrum, which enables the separation of NMR chemical shifts and homonuclear spin coupling constants into orthogonal dimensions. When these data are projected on to the chemical shift axis, a spectrum from only the small molecules is observed, with all coupling splittings removed. Examples of these different types of spectra are given in figure 2, showing the different patterns observed.

With developments in robotic sample preparation/ transfer systems and in NMR flow probes, the capacity for NMR analysis has increased enormously and up to 200–300 samples per day can now be measured. Although ^{1}H NMR spectra of urine and other biofluids are very complex, many resonances can be assigned directly based on their chemical shifts, signal multiplicities and by adding authentic material. However, further information can be obtained by using spectral editing techniques. Two-dimensional NMR spectroscopy (Croasmun and Carlson 1994)

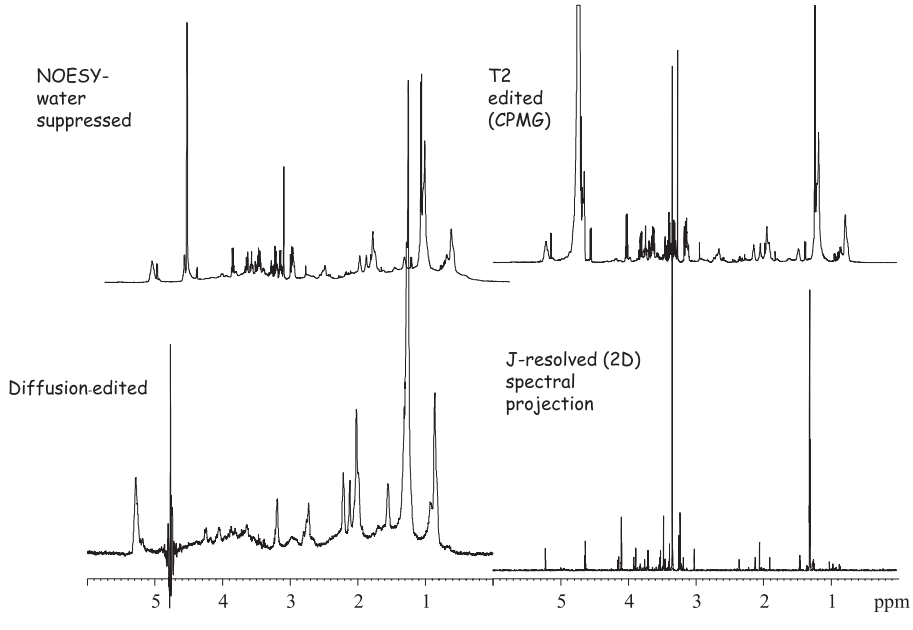

Figure 2. Standard (NOESY)-water suppressed, T2 edited (CPMG) spin-echo, diffusion-edited and 2-D J-resolved F2 projection NMR spectra of rat serum, showing the various NMR responses that are possible through the use of different pulse sequences that edit the spectral intensities based on differences in diffusion coefficients and spin relaxation times. Many of the peaks have been assigned (Lindon *et al.* 1999).

can also be useful for increasing signal dispersion and for working out the connectivities between signals, thereby enhancing the information content and helping to identify biochemical substances. These include the 2-D J-resolved experiment, which reduces the contribution of macromolecules and yields information on the multiplicity and coupling patterns of resonances. Other 2-D experiments such as correlation spectroscopy (COSY) and total correlation spectroscopy (TOCSY) provide $^{1}H-^{1}H$ spin–spin coupling connectivities. Use of other nuclei can be important to help assign NMR peaks; heteronuclear correlations, usually $^{1}H-^{13}C$, can also be obtained using sequences such as heteronuclear multiple quantum coherence (HMQC) experiments giving the chemical shifts of the carbon atoms to which the protons are attached. With the advent of NMR detectors cooled to near cryogenic temperatures (cryoprobes), a sensitivity gain of about 500% is achievable, making it possible to measure smaller samples or use less time. In addition, natural abundance ^{13}C NMR spectroscopy is now also feasible (Keun *et al.* 2002a).

Although identification of molecules is not necessary to achieve classification of samples, working out the identification of the molecules that differentiate spectra from different sample classes (biomarker combinations) can lead to insight into biochemical mechanisms of disease or drug effects. If all of the above methods fail to identify a given set of NMR peaks, then off-line chromatographic procedures such as solid phase extraction chromatography (SPEC) or high performance liquid chromatography (HPLC) can be used to simplify or clean up biofluid samples prior to NMR spectroscopy. In selected cases, directly coupled HPLC–NMR and HPLC–NMR–MS methods can be of value in determining endogenous metabolite structures (Lindon *et al.* 2000).

If tissue samples are available, then complementary information to that in biofluids can be obtained. Although *in vivo* NMR spectroscopy has been used to investigate abnormal tissue biochemistry, spectral quality is always severely compromised by the low magnetic fields used, leading to poor sensitivity and peak dispersion. Heterogeneity in the sample results in magnetic susceptibility differences causing magnetic field inhomogeneity and this, combined with constrained molecular motions of molecules in some tissue compartments, leads to poor resolution and lower sensitivity. Therefore, NMR spectral analysis of tissues has largely relied on tissue extraction methods. However, extraction processes can result in the loss of tissue components such as proteins and lipids.

Within the last few years, the development of high resolution ^{1}H magic-angle spinning (MAS) NMR spectroscopy has had a substantial impact on the ability to analyse intact tissues (Garrod *et al.* 1999). Rapid spinning of the sample (typically $\sim 4-6$ kHz) at an angle of 54.7° relative to the applied magnetic field serves to reduce line broadening effects due to magnetic field inhomogeneity caused by sample heterogeneity, dipolar couplings and chemical shift anisotropy. Thus, it is possible to obtain very high quality NMR spectra of whole tissue samples with no sample pretreatment using only about 20 mg of material. Typical high resolution ^{1}H MAS NMR spectra of a range of tissues are shown in figure 3. Such experiments indicate that diseased or toxin-affected tissues have substantially different metabolic profiles to those taken from healthy organs (Cheng *et al.* 1996,

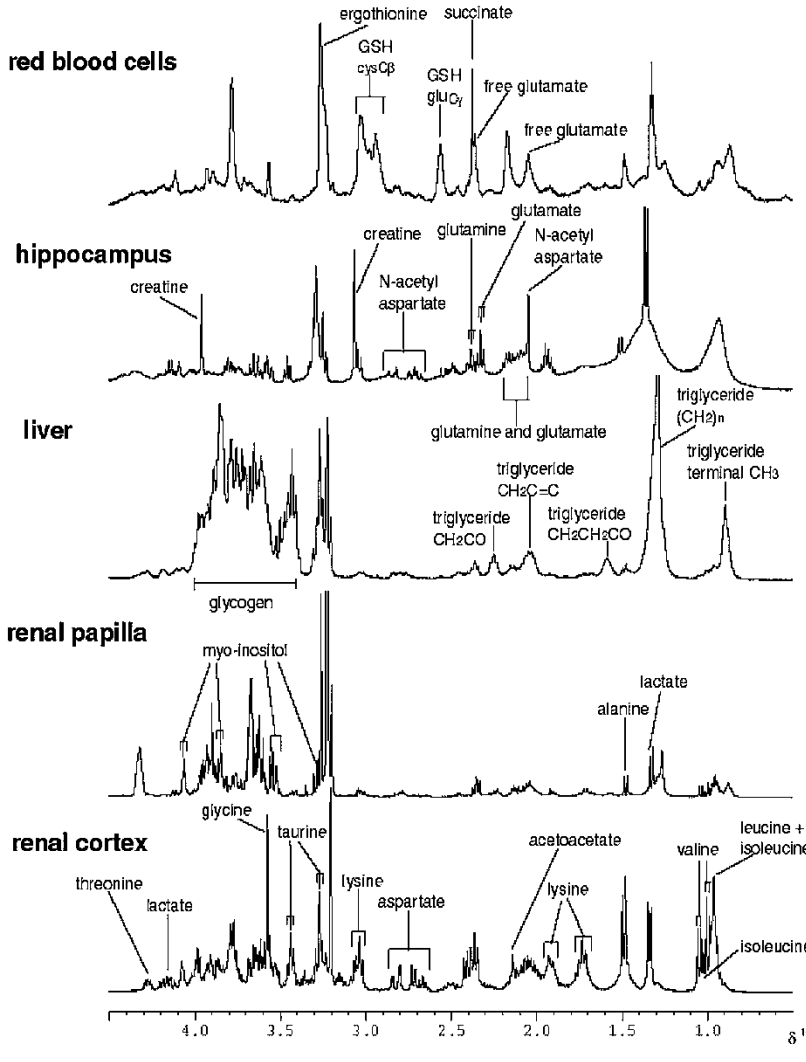

Figure 3. High resolution MAS ^{1}H NMR spectra (400 MHz CPMG spin-echo spectra at a rotation rate of 4.2 kHz) of different rat tissues, showing characteristic biochemical variation between tissues. GSH, glutathione.

Tomlins *et al.* 1998). In addition, MAS NMR spectroscopy has been used to access information regarding the compartmentalization of metabolites within cellular environments (Griffin *et al.* 2000a); this study is a demonstration that MAS NMR spectroscopy can yield information on molecular dynamics which hitherto would be very difficult to obtain.

^{1}H MAS NMR spectroscopic analysis of tissues has great potential within the pharmaceutical industry for the toxicological screening of novel compounds. Using this technology it is possible to 'bridge the gap' between biofluid analysis and histopathology and to gain real insight into the mechanisms of toxicity at a molecular level.

However, the problem of interpreting the data in metabonomics studies is the determination of significant changes in a large set of NMR spectra of a biofluid from a cohort of animals or humans demonstrating a variety of effects such as normal physiological variation that might obscure a drug-induced effect. This is achieved through the use of PR methods. In chemistry, the term chemometrics is generally applied to describe the use of PR and related multivariate statistical approaches to chemical numerical data (Lindon *et al.* 2001). The general aim of PR is to classify an object or to predict the origin of an object based on the identification of inherent patterns in a set of experimental measurements or descriptors. PR can be used for reducing the dimensionality of complex data sets, for example by 2-D or 3-D mapping procedures, thereby facilitating the visualization of inherent patterns in the data set. Alternatively, multiparametric data can be modelled using PR techniques so that the class of a separate sample can be predicted based on a series of mathematical models derived from the original data or 'training set' (Sharaf *et al.* 1986).

PR methods can be divided into two categories: 'unsupervised' and 'supervised' methods. Unsupervised multivariate techniques are used to establish whether any intrinsic clustering exists within a data set and include methods that map samples according to their properties without *a priori* knowledge of sample class. Examples of unsupervised methods include principal components analysis (PCA) and clustering methods such as hierarchical cluster analysis. Supervised methods of analysis use the class information given for a training set of sample data to optimize the separation between two or more sample classes. These techniques include soft independent modelling of class analogy (SIMCA), K-nearest neighbour analysis and neural networks. Supervised methods require a second independent data set to test or validate any class predictions made using the training set (Sharaf *et al.* 1986).

Subtle biochemical changes in ^{1}H NMR spectroscopic profiles of biofluids can be obscured in NMR PR analysis by interfering factors such as variations in pH, which can cause changes in NMR chemical shifts because of differences in the ionization state of some molecules. In NMR spectroscopy of urine, one means of limiting the effects of pH on the chemical shift of sensitive moieties is to add a standard amount of buffer to the sample prior to NMR spectroscopic analysis (Holmes *et al.* 1998a). Alternatively, mathematical algorithms can be used to realign the chemical shifts of resonances from protons near ionizable groups displaced by pH effects (Brown and Stoyanova 1996). The signal intensity in some spectral regions, such as those containing water or urea, is very variable due to water NMR peak suppression effects. In addition, many drug compounds or their metabolites are excreted in biofluids and these can obscure significant changes in the concentration of endogenous components. Therefore, it is usual to remove these redundant spectral regions prior to PR analysis.

For situations where large numbers of samples need to be processed, there is a need for automatic data reduction and PR analysis. One example of a robust automatic data reduction method, which has been widely used, is the division of the NMR spectrum into regions of equal chemical shift ranges followed by signal integration within those ranges (Farrant *et al.* 1992). Automatic data reduction of

2-D NMR spectra can be performed using a procedure similar to that for one-dimensional (1-D) spectra, in which the spectrum is divided by a grid containing squares or rectangles of equal size and the spectral integral in each volume element is calculated. This is not a universal solution and other approaches are possible and have been used, including shifting peak positions to take into account small pH-dependent variations in chemical shift, in which case the full NMR spectrum can be used for PR. However, it should be remembered that, although the initial pattern recognition methods may have used segmented data, having identified regions of interest that are changed in some pathological situation it is always possible to return to the real NMR spectra for peak assignment and metabolite identification.

PCA, which has been used extensively in metabonomics, is a well-known approach that allows the expression of most of the variance within a data set in a small number of factors or principal components (PCs). The properties of PCs are such that each PC is a linear combination of the original data parameters whereby each successive PC explains the maximum amount of variance possible not accounted for by the previous PCs. Each PC is orthogonal and therefore independent of the other PCs. The output of the method results in two matrices known as scores and loadings. Scores are the co-ordinates for the samples in the established model and may be regarded as the new variables. Each point represents a single NMR spectrum. The PC loadings define the way in which the old variables are linearly combined to form the new variables. The loadings define the orientation of the computed PC plane with respect to the original variables and indicate which variables carry the greatest weight in transforming the position of the original samples from the data matrix into their new position in the scores matrix. In the loadings plot, each point represents a different NMR spectral region.

Unsupervised methods such as PCA are useful for comparing pathological samples with control samples, but supervised analyses that model each class individually are preferred where the number of classes is large. Two common supervised methods are SIMCA and partial least squares (PLS) (Sharaf *et al.* 1986).

SIMCA operates by establishing the multivariate boundaries for each class in a data set. Models are formed from a defined set of samples with known class identity referred to as a 'training set'. A separate PCA is performed for each class of data within the training set, and an independent or 'test' set of samples is then used to assess the predictive ability of the models. Each sample in the test set is fitted to every class model and predictions are made on the goodness of fit. SIMCA models have advantages over some other supervised techniques in that a sample may be assigned to a single category, to more than one category or to no category, thereby avoiding forcing the sample into a given category.

PLS is a method that relates a data matrix containing independent variables from a set of samples to a matrix containing dependent variables (or measurements of response) for those samples. PLS can be used to examine the influence of time on a data set, which is particularly useful for biofluid NMR data collected from samples taken over the course of a disease, therapy or toxic effect. PLS can be combined with discriminant analysis (DA) to establish the optimal position to place a discriminant surface that best separates classes. An example of a PLS–DA

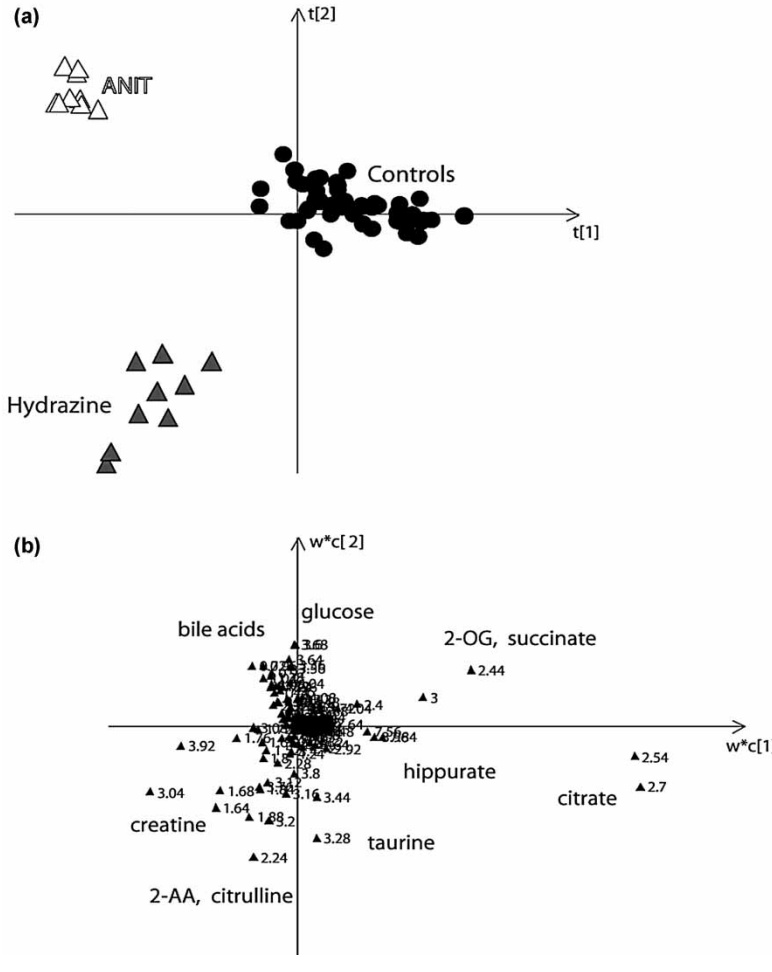

Figure 4. Visualization of xenobiotic toxicity using PLS–DA. (a) PLS scores plot based on NMR spectra of rat urine from control animals and those dosed with the liver toxins α-naphthyl isothiocyanate (ANIT) and hydrazine. (b) Corresponding loadings plot indicating those NMR spectral regions (annotated by ^{1}H NMR chemical shift) responsible for the separation. Some assignments have been marked.

analysis is shown in figure 4. This is based on NMR spectra of urine from three predefined classes: control animals and those dosed with two liver toxins, α-naphthyl isothiocyanate (ANIT) and hydrazine. The PLS scores plot in figure 4a shows clear distinction between the three classes, and the corresponding loadings plot in figure 4b can be interpreted in terms of the biomarkers that cause this separation.

Neural networks are a non-linear method of modelling data. A training set of data is used to develop algorithms, which 'learn' the structure of the data and can cope with complex functions. The basic network consists of three or more layers, including an input level of neurons (spectral descriptors or other variables), one or more hidden layers of neurons that adjust the weighting functions for each variable, and an output layer that designates the class of the object or sample. Several types

of neural network have been successfully applied to predicting toxicity or disease from NMR spectral information (Holmes *et al.* 2001).

A range of different chemometric approaches have been applied to evaluating xenobiotic toxicity based on NMR spectroscopy of urine (Holmes *et al.* 1998a, b).

Application to physiological variation in animals and humans

A wide range of physiological and environmental effects can have an influence on the metabolic composition of human and animal biofluid samples. In particular, changes visible in the composition of urine may be substantial since a system's response to its attempt to maintain homeostasis results in changes in composition of the excreted fluids. Changes seen can include the appearance of previously absent metabolites following the ingestion of food, but many are subtle variations in the relative concentrations of multiple components. NMR-based metabonomics has been used to distinguish between various normal physiological states in humans and animals, including evaluating temporal effects (Holmes *et al.* 1994). The scale of changes seen varies according to the type of physiological effect, and a hierarchy of metabolic patterns of different magnitudes is seen. Clearly such effects have to be taken into account prior to attempting to establish the effects of pathological events such as xenobiotic toxicity.

Control urine samples from different species can be differentiated using metabonomic analysis and there are several well-characterized specific metabolic differences. Figure 5 shows a PCA scores plot of urinary NMR data from 'healthy'

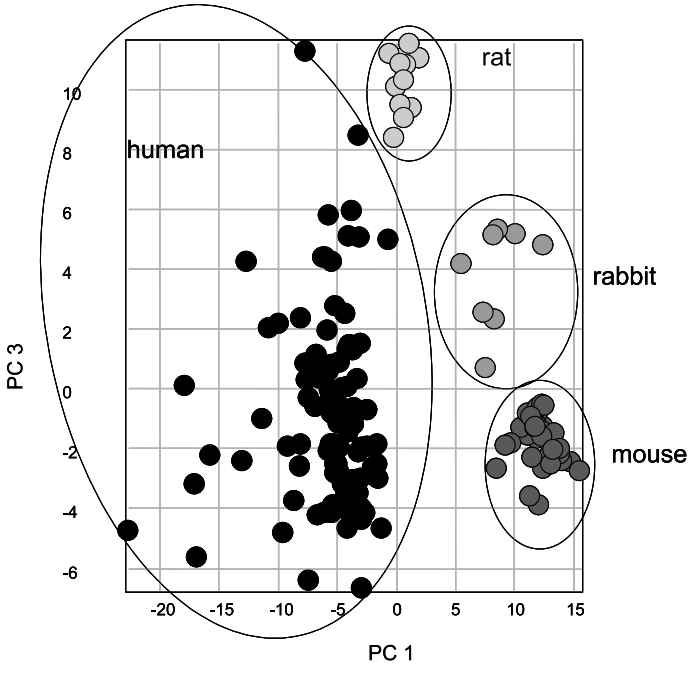

Figure 5. Species differences in the biochemical composition of urine. Plot of the scores (PC1 versus PC3) from a PCA study based on ^{1}H NMR spectra of urine samples from humans and other species. Reproduced with permission from Robertson *et al.* (2002).

humans, rats, rabbits and mice, illustrating considerable differences in urinary profiles between species (Robertson *et al.* 2002).

The pharmaceutical industry requires animal species or strains that are appropriate models for toxicological screening or for disease evaluation. Studies of inter-species differences in urinary metabolite profiles between the Sprague Dawley (SD) and F344 rat and the multi-mammate mouse (*Mastomys natalensis*) have been carried out (Holmes *et al.* 1996, 1997); the multi-mammate desert mouse, as expected and like most desert species, is more efficient at concentrating urine. The ^{1}H NMR spectra of control urine samples from *Mastomys* and F344 rats indicated higher levels of glycine, creatine, succinate, *N*-acetyl glycoproteins, pyruvate, betaine and other amino acids in *Mastomys*, whilst the F344 rat excreted greater amounts of 2-oxoglutarate and trimethylamine-*N*-oxide. The urinary profiles of SD and F344 rats were similar, but SD rats had slightly higher glucose and amino acids than the F344 rats.

Distinct species variations in the metabolic adaptation of small animals according to diet and growth strategy are known to affect the metabolic composition of urine. The metabolic profiles of three wild mammals (bank vole, shrew and wood mouse) with different natural diets were investigated using ^{1}H NMR and pattern recognition methods and compared with that from the SD rat (Griffin *et al.* 2000b). The four species had distinct urinary metabolite profiles; for instance, the bank vole excreted higher amounts of aromatic amino acids in its urine than the laboratory rat, but the rat urine contained more hippurate. Rat urine was more homogeneous in composition, containing lower levels of amino acids and tricarboxylic acid cycle intermediates, and appeared to have less individual variation. This is not surprising, as the laboratory strains of rat are inbred to increase physiological homogeneity. In addition, all three wild animals had higher concentrations of plasma triglyceride compared with the laboratory rat.

Metabonomics has been utilized to determine the metabolic urinary differences between Han Wistar (HW) and SD strains of rat (Holmes *et al.* 2001). In fact, the two strains are very similar metabolically and genetically, and are both routinely used for toxicity screening within the pharmaceutical industry. However, using PCA, NMR urinary profiles of the two strains could be partially separated and, despite the degree of overlap in the data, the strain of rat could be predicted correctly 86% of the time using a supervised classification method (SIMCA). From visual comparison of the ^{1}H NMR spectra of urine samples from the two strains, HW rats were determined to have higher levels of acetate, lactate and taurine, whilst SD rats had elevated levels of hippurate. Furthermore, using a different type of PR approach – probabilistic neural networks, distinct differences were observed in the metabolic profiles of control urine from SD and HW rats. Detailed statistical variations in specific spectral regions of the ^{1}H NMR spectra obtained from control SD and HW rat urine samples have also been defined (Tate *et al.* 2001).

Metabonomic techniques have been used to successfully determine the difference between two types of mice, namely AlpK:ApfCD (white-haired) and C57BL107 (black-haired) mice, based on NMR spectra of urine (Gavaghan *et al.* 2000). Using PCA it was possible to separate the two types and, furthermore, in a supervised PR approach, it was possible to predict the strain of the mouse in 98%

of cases using PLS–DA. From comparison of the ^{1}H NMR spectra, white mice had elevated levels of 2-oxoglutarate, citrate, trimethylamine-*N*-oxide and guanidinoacetic acid in their urine, whilst black mice had higher levels of taurine, creatinine, dimethylamine and trimethylamine. This area of application has been amplified using the same strains of mouse together with a strain of nude mouse, studied with LC–MS (Plumb *et al.* 2003). Each strain of mouse was well separated using PCA of the LC–MS data.

In addition, metabonomic techniques have been used to differentiate morphologically indistinguishable but phenotypically differentiated species of earthworm by analysis of tissue extracts and coelomic fluid using ^{1}H NMR spectroscopy and multivariate statistics (Bundy *et al.* 2002a).

Using NMR spectroscopy, together with PR analysis, it is possible to derive metabolic profiles from biofluids or tissue to compare wild-type animals and genetically modified animal models of disease. One example is the investigation of the *mdx* mouse, a model of Duchenne muscular dystrophy (Griffin *et al.* 2001a, b). Samples of cardiac and brain tissue from the *mdx* mouse could be separated from controls using PCA based on MAS NMR spectra of the tissue. This was due to differences in the levels of creatine and phosphocholine relative to taurine. In these tissues, taurine was elevated; this has previously been reported to be a biomarker for dystrophic tissue in skeletal muscle (McIntosh *et al.* 1998) and is thought to be an adaptive response to a loss of dystrophin. More detailed spectroscopic analysis using 1-D and 2-D high resolution MAS NMR showed changes in lipid resonances together with increases in lactate and threonine (Griffin *et al.* 2001b).

In a study examining physiological variation in female SD rats, urine from a group of 10 animals was sampled over a 10 day period (Bollard *et al.* 2001). In PCA of the ^{1}H NMR spectra, the scores from the first two PCs showed that samples from individual animals generally overlapped within one standard deviation, but some degree of dissimilarity could be distinguished. For instance, one animal excreted relatively low concentrations of tricarboxylic acid cycle intermediates and hippurate and higher concentrations of taurine, dimethylglycine, creatinine and glucose compared with the other rats. In other studies, citrate, taurine, hippurate and the renal osmolytes have been shown to have a high interindividual variation in control animals (Temellini *et al.* 1993, Zuppi *et al.* 1997, Phipps *et al.* 1998, Holmes *et al.* 2001).

Gender-related differences in the urinary profiles of AlpK:ApfCD (white-haired) and C57BL107 (black-haired) mice have recently been investigated using LC–MS (Plumb *et al.* 2003). Male and female examples from each strain of mouse showed good separation of clusters using PCA of the LC–MS data.

In rats, the levels of aromatic molecules, citrate, 2-oxoglutarate, taurine and creatinine in the urine have been shown to vary according to the age of the animal as well as being affected by diet and gut microflora (Bell *et al.* 1991, Phipps *et al.* 1998). In addition, young rats ($\leq$1 month old) excrete significantly increased amounts of betaine and trimethylamine-*N*-oxide compared with older rats. The age-related differences in urinary profiles are illustrated in the PC plot given in

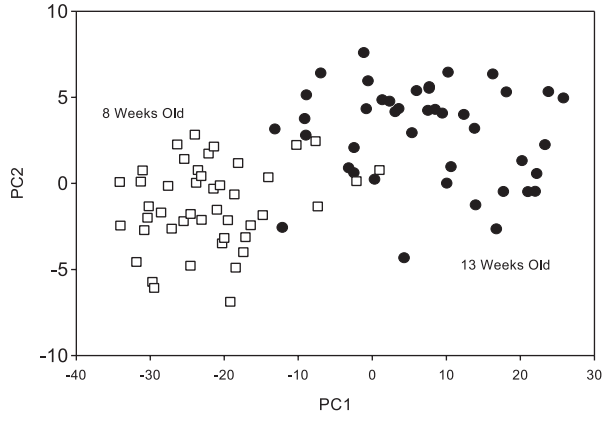

Figure 6. The effect of age on the biochemical composition of urine. Plot of the scores (PC1 versus PC2) from a PCA study based on ^{1}H NMR spectra of rat urine samples. The open squares represent data from urine of 8 week old rats and the solid circles are data from urine of 13 week old rats. Reproduced with permission from Robertson *et al.* (2002).

figure 6, where rats aged 13 weeks could be clearly separated from 8 week old rats based on NMR spectra of urine (Robertson *et al.* 2002).

Oestrus cycle-related perturbations in metabolic urinary profiles have been investigated using female SD rats sampled twice daily for 10 days (Bollard *et al.* 2001), with the stage of the oestrus cycle at each urine collection point being determined using vaginal cytology. Rats progress through one complete oestrus cycle every 3–4 days, moving through pro-oestrus, oestrus, metoestrus and dioestrus. The metabolic perturbations related to each stage of the oestrus cycle were investigated using NMR spectroscopy and the partial separation of the different stages of the cycle observed by PCA. The principal changes were to the levels of citrate, trimethylamine-*N*-oxide, creatine, creatinine, taurine, glucose and *N*-acetyl glycoproteins. The changes were consistent with other studies that have linked citrate changes to oestrogen levels (Zuppi *et al.* 1997), and the trimethylamine-*N*-oxide/trimethylamine ratio to menstruation (Zhang *et al.* 1996).

Rats are generally nocturnal, and there is thus a distinct diurnal variation to their activity. This is reflected in their urinary profiles and has to be taken into account when considering sampling periods for any type of toxicological or biochemical analysis. The simplest method of averaging out diurnal variation in metabolite profiles is to collect 24 h urine samples.

The diurnal effects on the metabolic composition of SD rat urine have been investigated using samples obtained from female rats during light (6 a.m. to 6 p.m.) and dark (6 p.m. to 6 a.m.) cycle periods for 10 days (Bollard *et al.* 2001, Solanky *et al.* 2003). NMR spectra of the light and dark period samples were easily separated in a PC scores plot. Urine samples collected during the day were found to have lower levels of taurine, hippurate and creatinine, together with elevated levels of glucose, succinate, dimethylglycine, glycine, creatine and betaine. Similar results have been found for the HW rat (Tate *et al.* 2001).

Two similar studies in AlpK:ApfCD (white-haired) and C57BL107 (black-haired) mice using both NMR spectroscopy and MS have also been reported

(Gavaghan *et al.* 2002, Plumb *et al.* 2003). Samples collected during daylight hours contained higher levels of creatine, hippurate, trimethylamine, succinate, citrate and 2-oxoglutarate and lower levels of trimethylamine-*N*-oxide, taurine, spermine and 3-hydroxy-*iso*-valerate relative to samples collected during the night, many of these effects being comparable in the rat and mouse.

As expected, dietary changes can influence the biochemical composition of biofluids, particularly urine (Griffin *et al.* 2000b). For example, dietary changes in the rat alter the amount of hippurate and chlorogenic acid metabolites excreted in the urine (Phipps *et al.* 1998, Gavaghan *et al.* 2001). The balance between the two types of urinary metabolites has been attributed to variations in the composition of the diet, plus redistribution of gut microfloral populations. In an earlier study (Bell *et al.* 1991), rats fed a casein diet for 1 month post-weaning excreted lower levels of hippurate, succinate, citrate, trimethylamine-*N*-oxide and betaine and higher amounts of taurine and *N*-methylnicotinamide compared with rats fed chow diets.

The importance of gut microfloral effects on urine profiles has been highlighted in a study where germ-free (axenic) rats were compared before and after exposure to a 'normal' environment (Nicholls *et al.* 2003). Changes observed over the first 17 days included an increase in glucose, a decrease in tricarboxylic cycle intermediates and increases in trimethylamine-*N*-oxide, hippurate, phenylacetylglycine and *m*-(hydroxyphenyl)propionic acid. At 21 days, the urinary profile resembled that of control animals maintained under a normal non-sterile environment. Some typical ^{1}H NMR spectra are shown in figure 7, indicating the scale of the changes seen. The changes observed are indicative of the colonization and redistribution of gut microflora and the varying health of the animal, and this has implications for drug metabolism and toxicity investigations where some of the changes observed in the urine after drug treatment may be a result of metabolism by organisms in the gastrointestinal tract.

Application to drug adverse effects

The current drug safety assessment approaches used in the pharmaceutical industry can still fail to prevent molecules that have no realistic chance of reaching the market from entering development, and there is a need for methodologies that can pick up potential problems earlier, faster, more cheaply and more reliably. The later that a molecule or even a whole class of molecules is lost from a company drug development pipeline, the higher the financial cost to the pharmaceutical company involved. Worst of all is withdrawal from the clinical market because of unpredicted safety issues. Minimizing attrition is therefore one of the most important aims of pharmaceutical research and development. Novel technologies, such as metabonomics, that increase the probability of making the right choice early can save resources and promote safety, efficacy and profitability.

Although transcriptomic/genomic and proteomic measurements respond to the administration of toxic agents, it is difficult to relate findings to classical toxicological or disease endpoints, and hence to influence the drug attrition rate. Metabonomics offers a complementary approach that gives information on whole organism functional integrity over time following drug exposure. Target tissues or

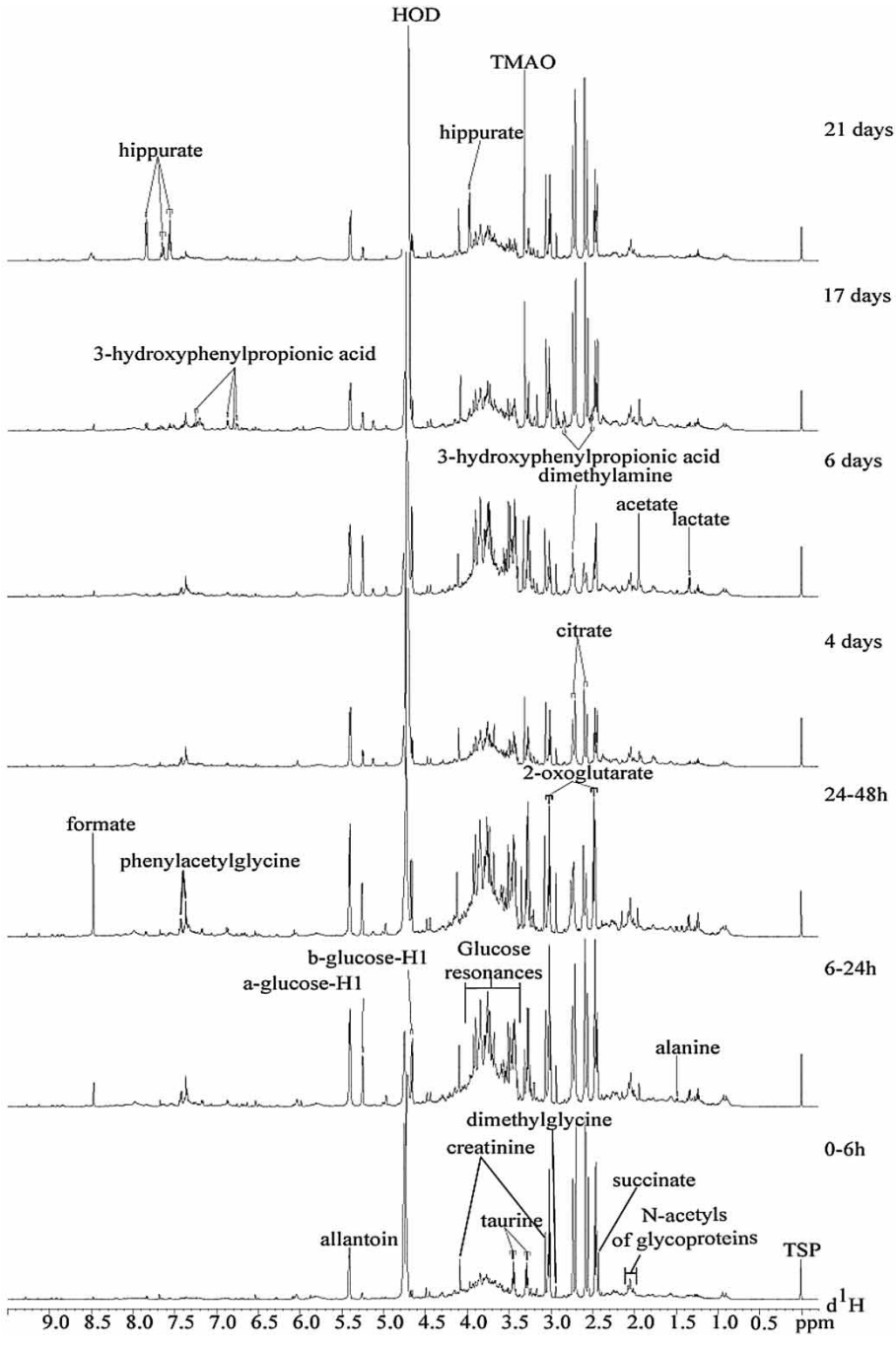

Figure 7. Stack plot of 500 MHz ^{1}H NMR spectra of whole rat urine at selected time points after release from a germ-free environment. The assignments were based on literature data and 2-D NMR experiments. HOD, residual water, partially deuterated; TMAO, trimethylamine-*N*-oxide. Reproduced with permission from Nicholls *et al.* (2003).

processes, and biomarkers, can be identified from characteristic changes in the pattern of concentrations of endogenous metabolites in biofluids that relate to the site and mechanism of toxicity.

The first attempt at using pattern recognition to classify NMR spectra of biofluid samples was on urine from rats in a variety of experimental toxicity states (Gartland *et al.* 1989a, b, 1990a). ^{1}H NMR signal intensities for 17 predefined endogenous urinary metabolites were regarded as coordinates in multidimensional space and analysed using PR methods, through which the dimensionality was reduced for display and categorization purposes by employing the unsupervised learning methods of hierarchical cluster analysis, 2-D non-linear map (NLM) analysis, and 2-D or 3-D PCA. Different types of toxin (hepatotoxins and cortical and papillary nephrotoxins) were classified according to NMR-detectable biochemical effects. Various refinements in the data analysis were investigated, including taking NMR urinalysis data at three time points after exposure of the rats to six different nephrotoxins and employing a dual-scoring system (time and magnitude of change). The maps generated from the time-course information produced the best discrimination between nephrotoxins from different classes. The robustness of the classification methods and the influence of the addition of new scored biochemical data reflecting dose–response situations, nutritional effects on toxicity, sex differences in biochemical response to toxins, the addition of a new toxin class (cadmium chloride, a testicular toxin and renal carbonic anhydrase inhibitor) and an additional metabolite descriptor (creatine) to the PR analysis were also evaluated.

The above approach was then extended by carrying out computer-based PR procedures on ^{1}H NMR spectra of rat urine to interpret the biochemical effects of 15 acute nephrotoxin treatments affecting the renal cortex and/or renal medulla (Gartland *et al.* 1990a). The ^{1}H NMR signal intensities for 16 urinary metabolites representative of several major intermediary biochemical pathways were estimated using either a simple semiquantitative scoring system or complete peak intensity quantification. A good separation of renal cortical toxin treatments from renal medullary toxin treatments was achieved. Clinical chemical data and NMR data were also combined for PR analysis, and it was shown that classifications based on NMR data alone were not improved by the inclusion of clinical chemistry results (Gartland *et al.* 1990b).

Rather than simply relying on mapping positions in PC scores plots, the concept of a metabolic trajectory in hyperspace has been proposed and exemplified using PC analysis of NMR spectra of rat urine from animals dosed with specific toxins (Holmes *et al.* 1992). Nephrotoxic lesions were induced in rats using two kidney toxins: $HgCl_2$, a proximal tubular toxin, and 2-bromoethanamine (BEA), a medullary toxin. The biochemical effects of these toxins on urinary composition were observed by ^{1}H NMR spectroscopy over 9 days following dosing. The onset of, progression of, and recovery from the induced toxic lesions were also followed histopathologically and related to the perturbed urinary biochemistry based on 20 endogenous substances at eight time points for each rat. The time-course trajectories showed characteristically different paths for each toxin, allowing the time points at which there are maximum metabolic differences to be determined

and providing visualization of the net movements of the treatment group populations in time in relation to inter-animal variation. The trajectory for BEA showed different routes for onset of and recovery from toxicity, whereas for $HgCl_2$ the outward trajectory (onset of the kidney lesion) mapped a space similar to the inward trajectory (recovery phase). This indicates that the mechanisms involved in the generation of and recovery from the toxic lesion are different for the two toxins. For $HgCl_2$, it appears that the process is a directly reversible, single type of lesion. However, BEA causes two types of lesion: initially a mitchondrial effect and then a change to renal osmolytes caused by renal papillary damage. Because the recovery from the first effect takes place after onset of the second lesion, the trajectory will not show a simple reversibility. The metabolites that discriminated between the two toxins included valine, taurine, trimethylamine-*N*-oxide and glucose for $HgCl_2$, and acetate, methylamine, dimethylamine, lactate and creatine for BEA, whereas citrate, succinate, *N*-acetyl resonances from as yet unidentified metabolites, hippurate, alanine and 2-oxoglutarate played an important role in defining the biochemically perturbed trajectory of both toxins.

The same approach has also been used to investigate metabolic trajectories for the liver toxins ANIT, D-(+)-galactosamine (GalN) and butylated hydroxytoluene (BHT) (Beckwith-Hall *et al.* 1998). NMR spectra of the urine samples showed a number of time-dependent perturbations of endogenous metabolite levels that are characteristic for each hepatotoxin. Biochemical changes common to all three hepatotoxins include a reduction in the urinary excretion of citrate and 2-oxoglutarate and increased excretion of taurine, acetate and creatine. Increased urinary excretion of betaine, urocanic acid, tyrosine, threonine and glutamate was found to be characteristic of GalN toxicity. Both GalN and ANIT caused increased urinary excretion of bile acids, while glycosuria was evident in BHT- and ANIT-treated rats. Each toxin gave a unique time-related metabolic trajectory that could be visualized in 2-D PC maps and in which the maximum distance from the control point corresponded to the time of greatest cellular injury (as confirmed by conventional toxicological tests). Thereafter, the metabolic trajectories changed direction and moved back toward the control region of the PC map during the recovery phase. The combination of urinary metabolites that were significantly altered at various time points allowed for differentiation between different types of liver damage. This approach to the non-invasive detection of liver lesions should be of value in furthering the understanding of hepatotoxic mechanisms and assisting in the discovery of novel biomarkers of hepatotoxicity, given that the commonest cause of candidate drug failure in the safety assessment phase of development of a new pharmaceutical is liver toxicity.

The whole approach has been extended to cover both kidney and liver toxins and to generate supervised PR models for toxicity prediction (Holmes *et al.* 1998a, b). PR approaches were developed and applied to the classification of 600 MHz ^{1}H NMR spectra of urine from male rats dosed with compounds that induce organ-specific damage in either the liver or kidney. Data corresponding to the periods 8–24 h, 24–32 h and 32–56 h post-dose were first studied using PC analysis. In addition, samples obtained 120–144 h following the administration of adriamycin and puromycin were included in one analysis in order to encompass the later onset

of the kidney glomerular toxicity caused by these agents. Having established that toxin-related clustering behaviour can be detected in the first three PCs, some of the data were used to construct a SIMCA model. The remainder of the data were used as a test set of the model. Only three out of 61 samples in the test set were misclassified. Finally, as a further test of the model, data from the ^{1}H NMR spectra of urine from rats that had been treated with a kidney toxin not included in the training set, uranyl nitrate, were used. In addition, it was possible to demonstrate distinct metabolic trajectories in PC maps for the toxins.

Extending the idea of using supervised methods, NMR spectra of urine from rats treated with a range of liver, kidney and testicular toxins at various doses have been classified using neural network methods (Anthony *et al.* 1995). Toxin-induced changes in the levels of 18 low molecular weight endogenous urinary metabolites were assessed using a simple semiquantitative scoring system. These scores were input into an artificial neural network for exploration of its ability to predict the class of toxin. With this limited data set, based only on the level of the maximal changes in these 18 metabolites, the network was able to predict the class and hence the target organ of the toxins.

The neural network method has been extended by the application of newer probabilistic methods (Holmes *et al.* 2001). ^{1}H NMR spectroscopy was used to characterize the time-related changes in the urinary metabolite profiles of rats treated with 13 toxic compounds that predominantly target the liver or kidney. These ^{1}H NMR spectra were data-reduced and subsequently analysed using a probabilistic neural network (PNN) approach. A database of 1310 spectra was used, of which 583 comprised a training set for the neural network, with the remaining 727 (independent cases) being employed as a test set for validation. Using PNN techniques, characterization of control samples and four defined classes of toxicity (liver, kidney, combined liver and kidney and mitochondrial), together with differentiation of rat strain for each type of toxicity (two types were used – HW and SD), were distinguishable to greater than 90%. Analysis of the ^{1}H NMR spectral data by conventional multilayer neural networks and PCA gave similar but less accurate classification than PNN analysis, which was particularly effective for small classes where PC-based methods can fail.

Toxic effects in tissues themselves can also be studied using ^{1}H MAS NMR spectroscopy; for example, renal and liver toxins have been investigated in this way (Garrod *et al.* 2001, Waters *et al.* 2001, Coen *et al.* 2003).

Metabonomics expert systems for toxicity prediction based on supervised PR methods could operate at one or more of three levels. The simplest type of classification is whether a sample or organism is 'normal' or 'abnormal'. Classification as abnormal indicates a deviation from the control population and can be caused by numerous factors including toxicity, disease, dietary differences, genetic modification and contamination. This selection of abnormal samples can be achieved automatically 'on line', and any sample defined as abnormal can then be used for further NMR measurements or multivariate statistical analysis with a view to ascertaining the nature of the abnormality. The second level of decision is the classification of toxicity. Samples identified as being dissimilar to matched control samples can be fitted to a series of mathematical models that define the

multivariate boundaries for known classes of toxicity. Therefore, biofluid or tissue samples from experimental animals treated with novel drugs can be tested to ascertain if the drug induces biochemical effects that would infer a particular organ site or mechanism of toxicity. Finally, a system can be generated to identify the biomarkers. The metabolites that differ between biofluid samples obtained from drug-treated and control animals can be elucidated, giving an insight into possible mechanisms of toxicity or dysfunction.

The interest in the use of metabonomics to evaluate drug safety is highlighted by the initiation of the Consortium on Metabonomic Toxicology (COMET). This has been formed between five pharmaceutical companies and Imperial College London to define and apply metabonomic data generated using ^{1}H NMR spectroscopy of urine, blood serum and tissues for preclinical toxicological screening of candidate drugs. This is being achieved by generating databases of metabonomic results for a wide range of model compounds, toxins and drugs linked to computer-based expert systems for toxicity prediction. The five industrial partners are Bristol-Meyers-Squibb, Eli Lilly, Hoffmann-La-Roche, NovoNordisk and Pfizer Inc. This project has been in operation for over 2 years and is generating comprehensive metabonomic databases and multivariate statistical models for the prediction of toxicity, and an overview paper has been published recently (Lindon *et al.* 2003).

Efforts have concentrated on liver and kidney toxicity in the rat and mouse. During the initial phase of the project, a detailed comparison was made of the ability of the six companies to provide consistent urine and serum samples from a study of the toxicity of hydrazine in the male rat. All samples were measured using NMR spectroscopy at Imperial College and this showed an exceptionally high degree of consistency between samples from the various companies in terms of spectral patterns and biochemical composition. A detailed statistical model was constructed based on the NMR spectra of urine from control rats that enabled identification of outlier samples and the metabolic reasons for the deviations. Chemometric models were constructed for the urine samples from rats dosed with hydrazine, allowing evaluation of dose effects and of the time course of the biochemical response to the toxin. Serum samples were also subjected to multivariate analysis based on four types of NMR spectra and excellent consistency between all the companies was demonstrated. Differences between samples from the various companies were small compared with the biochemical effects of hydrazine. Furthermore, a comparison has been made of NMR spectra measured at Imperial College and spectra obtained within two companies; again a high degree of robustness has been found for data compatibility (Keun *et al.* 2002b). Sample preparation and NMR data acquisition was duplicated at two sites using two identical sets of urine samples from an 8 day acute study of hydrazine toxicity in the rat. One site used an operating spectrometer field of 600 MHz, the other 500 MHz. Both 500 MHz and 600 MHz datasets generated strikingly similar distributions using PCA, giving near-identical descriptions of biomarker response to hydrazine treatment. The main consistent difference between the datasets was related to the extent of water resonance suppression in the spectra. In a four-PC model of both datasets combined, describing all systematic dose- and time-related variations

(88% of the total variation), differences between the two datasets accounted for only ~3% of the total modelled variance compared with ~15% for normal physiological variation. Furthermore, less than 3% of the spectra displayed distinct inter-site differences, and these were clearly identified as outliers in their respective dose-group PCA models. No samples produced clear outliers in both datasets, suggesting that the outliers observed do not reflect an unusual metabolic profile but rather sporadic differences in sample preparation or spectral artefacts. Estimations of the relative concentrations of citrate, hippurate and taurine were in >95% agreement between sites, with an analytical error comparable to normal physiological variation. The high analytical reproducibility and robustness of metabonomic techniques is highly competitive compared with the best proteomic analyses and is in significant contrast to genomic microarray platforms.

In order to assimilate and compare data from such large data collections, new chemometrics methods have been developed. One approach has been based on probabilistic theory and is termed classification of unknowns by density superposition (CLOUDS), a novel non-neural implementation of a classification technique developed from probabilistic neural networks (Ebbels *et al.* 2003). In this case, a subset of the COMET data was used comprising NMR spectra of urine from rats from 19 different treatment groups, collected over 8 days, and processed to produce a data matrix with 2844 samples and 205 spectral variables. The spectra were normalized to account for gross concentration differences in the urine, and regions corresponding to non-endogenous metabolites (0.4% of the data) were treated as missing values. Modelling the data according to the organ of effect (control, liver, kidney or other organ), with a 50/50 train/test set split, over 90% of the test samples were classified as belonging to the correct group. In particular, samples from liver and kidney treatments were classified with 77% and 90% success, respectively, with only a 2% misclassification rate between these classes. Further analysis of the data, counting each of the 19 treatment groups as separate classes, resulted in a mean success rate across the groups of 74%. Finally, as a severe test, the data were split into 88 classes, each representing a particular toxin at a particular time point; 54% of the spectra from non-control samples were classified correctly, particularly successful when compared with the null success rate of ~1% expected from random class assignment. The CLOUDS technique has advantages when modelling complex multidimensional distributions, giving a probabilistic rather than absolute class description of the data, and is particularly amenable to the inclusion of prior knowledge such as uncertainties in the data descriptors.

An integrated metabonomics study using high-resolution ^{1}H NMR spectroscopy has been applied to intact liver tissue (using MAS NMR spectroscopy), liver tissue extracts and blood plasma samples obtained from control and paracetamol-treated mice (Coen *et al.* 2003); typical NMR spectra from the various types of samples are shown in figure 8. PCA was used to visualize similarities and differences in the biochemical profiles. The time- and dose-dependent biochemical effects of paracetamol were related to the drug toxicity, as determined using histopathology. Metabolic effects in intact liver tissue and lipid-soluble liver tissue extracts from animals treated with high doses of paracetamol included an increase

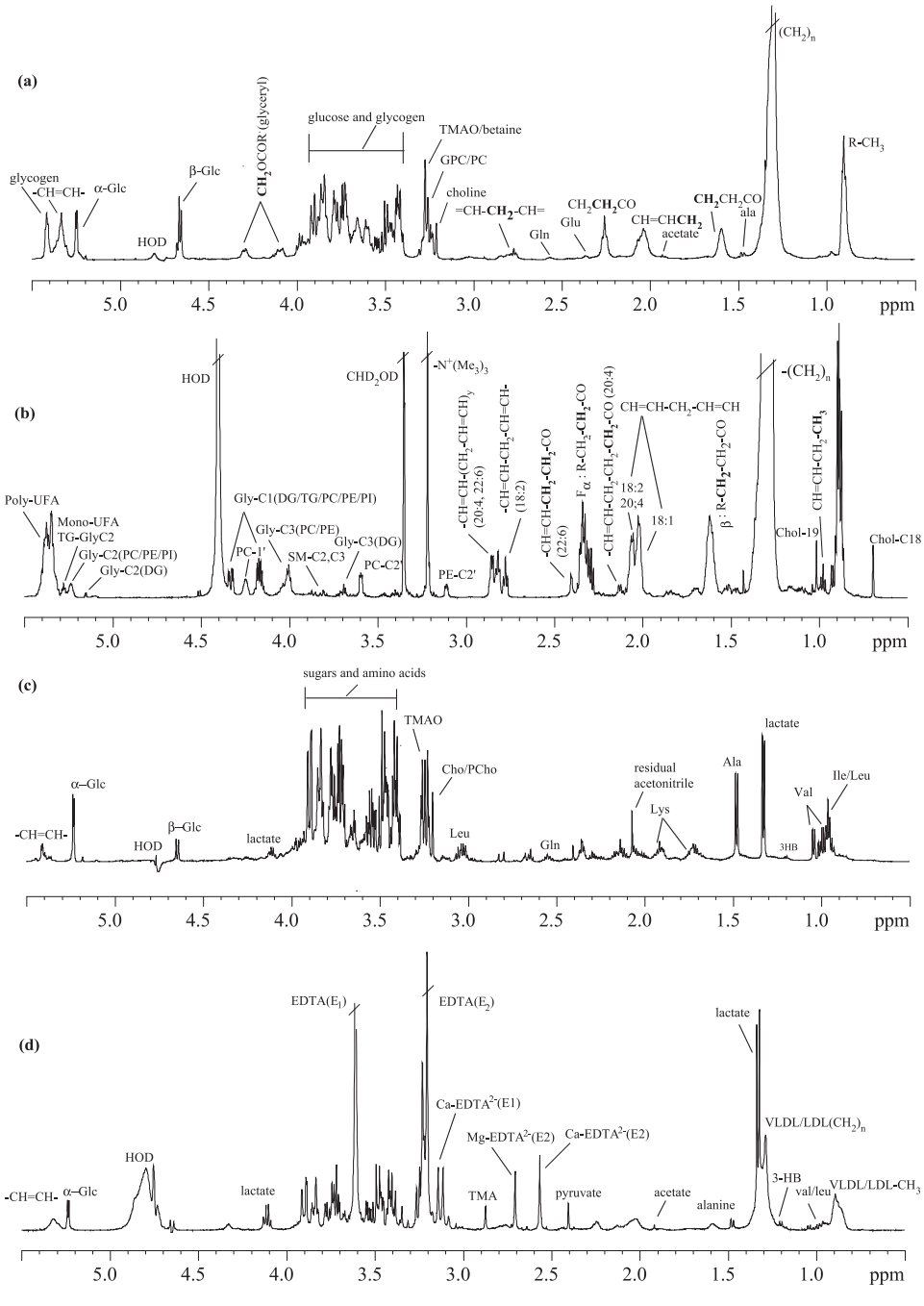

Figure 8 (*Continued*)

in triglycerides and mono-unsaturated fatty acids, together with a decrease in poly-unsaturated fatty acids, indicating mitochondrial malfunction with concomitant compensatory increase in peroxisomal activity. In addition, a depletion in phospholipids was observed in treated liver tissue, which suggested inhibition of

enzymes involved in phospholipid synthesis. There was also depletion in the levels of liver glucose and glycogen. In addition, the aqueous-soluble liver tissue extracts from high-dose animals also revealed an increase in lactate, alanine and other amino acids, together with a decrease in glucose. Plasma spectra showed increases in glucose, acetate, pyruvate and lactate. These observations all provide evidence for an increased rate of glycolysis. These findings could indicate mitochondrial inability to use pyruvate in the citric acid cycle and also reveal the impairment of fatty acid β-oxidation in liver mitochondria of treated mice.

This approach has been taken further by the integration of results from transcriptomics and metabonomics (Coen *et al.* 2004). Gene-chip array (Affymetrix) data from liver tissue and ^{1}H NMR spectra from liver tissue, tissue extracts and plasma from mice dosed with paracetamol have been analysed separately to identify biochemical changes arising from hepatotoxicity. These two sets of data were then interpreted together in terms of common metabolic pathways to show a shift in energy metabolism. The principal metabonomic changes comprised a decrease in hepatic glucose and glycogen in intact tissue, coupled with an increase in lipid content, plus increases in the levels of glucose, pyruvate, acetate and lactate in plasma and increases in alanine and lactate in the aqueous tissue extracts. This provided evidence for an increased rate of glycolysis. These observations were consistent with the altered levels of gene expression related to lipid and energy metabolism in liver that both preceded and occurred at the same time as the metabolic perturbations. The results show that these two technology platforms together offer a complementary view into cellular responses to toxic processes, providing new insight into the toxic consequences, even for a drug as well studied as paracetamol.

Metabonomics has already been applied in fields outside human and other mammalian systems. For example, studies in the environmental pollution field have highlighted the potential benefits of this approach in studies of caterpillar haemolymph (Phalaraksh *et al.* 1999) and earthworm biochemical changes as a result of soil pollution (Bundy *et al.* 2002b). In addition, a study of heavy metal toxicity in wild rodents living on polluted sites has been successfully conducted (Griffin *et al.* 2001c).

Disease diagnosis using metabonomics

Many examples exist in the literature of the use of NMR-based metabolic profiling to aid disease diagnosis. Most of the earlier studies have been reviewed (Lindon *et al.* 1999) and hence are only summarized here (references to the original

Figure 8. (a) 600 MHz ^{1}H MAS NMR CPMG spectrum of intact control liver tissue. (b) 600 MHz ^{1}H NMR spectrum of a control lipid-soluble liver tissue extract. (c) 600 MHz solvent presaturation ^{1}H NMR spectrum of a control aqueous-soluble liver tissue extract. (d) 500 MHz ^{1}H NMR CPMG spectrum of control blood plasma. Key: Ala, alanine; Cho, choline; Chol, cholesterol; DG, diglycerides; EDTA, ethylene diamine tetra-acetic acid; Glu, glucose; Gln, glutamine; Glu, glutamate; GPC, glycerophosphorylcholine; Glc, glucose; Gly, glycerol; 3-HB, 3-d-hydroxybutyrate; HOD, residual water; Ile, isoleucine; LDL, low density lipoprotein; Leu, leucine; Lys, lysine; PC, phosphatidylcholine; PCho, phosphocholine; PE, phosphatidylethanolamine; Phe – phenylalanine, PI, phosphatidylinositol; SM, sphingomyclin; TG, triglyceride; TMA, trimethylamine; TMAO, trimethylamine-*N*-oxide; UFA, unsaturated fatty acid; Val, valine; VLDL, very low density lipoprotein.

studies are given in Lindon *et al.* 1999). For example, diseases caused by inborn errors of metabolism have been described in medical writings for over 300 years since the reported case of a boy with a benign condition now known as alkaptonuria. However, not all inborn errors of metabolism are benign, and conventional diagnostic methods including specific enzyme assays and GC/MS are widely used and sensitive. These can be time-consuming and involve considerable sample preparation. However, NMR spectroscopy of biofluids has been shown to be a very powerful and general method for the detection of inborn errors of metabolism. Most of the work has involved ^{1}H NMR studies on human urine, and in some cases the disease diagnosis was confirmed by GC/MS or enzymology.

A variety of other diseases have been investigated using ^{1}H NMR spectroscopy. These include analysis of the composition of the urine and plasma of a number of diabetic patients. There are marked elevations in the plasma levels of ketone bodies and glucose, post-insulin withdrawal, and changes to lipoprotein composition. No other technique can provide a simultaneous assay on all three ketone bodies, glucose, lipoproteins and other important biochemicals in one fast and non-destructive test.

The levels of a variety of endogenous components in synovial fluid aspirated from the knees of patients with osteoarthritis, rheumatoid arthritis or traumatic effusions have been studied using ^{1}H and ^{13}C NMR spectroscopy. The NMR spectrum of synovial fluid shows the signals of a large number of endogenous components, although many potential markers of inflammation could not be monitored because of their low concentrations or because of their slow tumbling caused by the high viscosity of the fluid. Correlations were reported between the disease states and levels of the *N*-acetyl signals from acute-phase glycoproteins and the levels and type of triglyceride.

The chemical composition of cerebrospinal fluid (CSF) gives a good indication of the health of the central nervous system (CNS) and therefore biofluid NMR studies of CNS diseases have focused on the CSF. Studies in this area include investigation of drug overdose, Reye's syndrome and Alzheimer's disease

A number of investigations involving kidney disease have been reported, including chronic renal failure, the uraemic syndrome, glomerulonephritis, autosomal dominant polycystic kidney disease and renal transplantation outcome.

Identification of a considerable number of metabolites present in seminal fluid has been achieved based on ^{1}H NMR spectra of this biofluid, including its component secretions (prostatic and seminal vesicle fluids). The spectra derived from normal controls have been compared with those from patients with vasal aplasia (obstruction of the vas deferens leading to blockage of the seminal vesicles) or non-obstructive infertility.

There is a great need for a sensitive, general test for cancer within the asymptomatic population, and this remains an elusive goal despite much effort and false starts using metabonomics. Metabonomics has been used to address this need based on the NMR spectra of perchloric acid extracts of human brain tumour tissue (Howells *et al.* 1992). Tumours were classified as meningiomas, astrocytomas grades 1, 2 and 3, glioblastomas, other glial tumours, medulloblastomas, metastases and other tumours. Biopsy samples were taken at surgery, extracted

in a standard manner using perchloric acid, and ^{1}H NMR spectra were measured. The data were subjected to PCA to reduce the data dimensionality and classified using a neural network software approach. This gave 85% correct classification for meningiomas and non-meningiomas, but using specific NMR signals from creatine and glutamine gave a slightly improved value of 89%. Classification of gliomas gave 62% correct within one grade. A complementary approach has been used more recently using MAS NMR spectroscopy of involved and uninvolved tissue from renal cell carcinoma biopsies. Chemometric analysis allowed excellent distinction of the two types of tissue, but could not distinguish a metastatic tumour from the other, primary tumours (Moka *et al.* 1998).

Coronary heart disease (CHD) is a major cause of mortality in developed countries, and a range of risk factors based on lifestyle, diet and biochemical measurements have been identified. Whilst these are very useful for ensuring modifications to behaviour and have been helpful in unravelling the molecular basis of the disease, there remains no clear non-invasive diagnostic method for CHD. The main method requires the injection of X-ray opaque dye into the blood stream and visualization of the coronary arteries using X-ray angiography. This is both expensive and invasive, with an associated mortality of 0.1% and adverse effects in 1–3% of patients. Recently metabonomics has been applied to provide a method for the diagnosis of CHD non-invasively through analysis of a blood serum sample using NMR spectroscopy (Brindle *et al.* 2002). Patients were classified into two groups: those with normal coronary arteries and those with triple coronary vessel disease based on the gold-standard angiographic examination. Around 80% of the NMR spectra were used as a training set to provide a two-class model after appropriate data filtering techniques had been applied. Samples from the two classes were easily distinguished. The remaining 20% of the samples were used as a test set and their class was then predicted based on the derived model (figure 9),

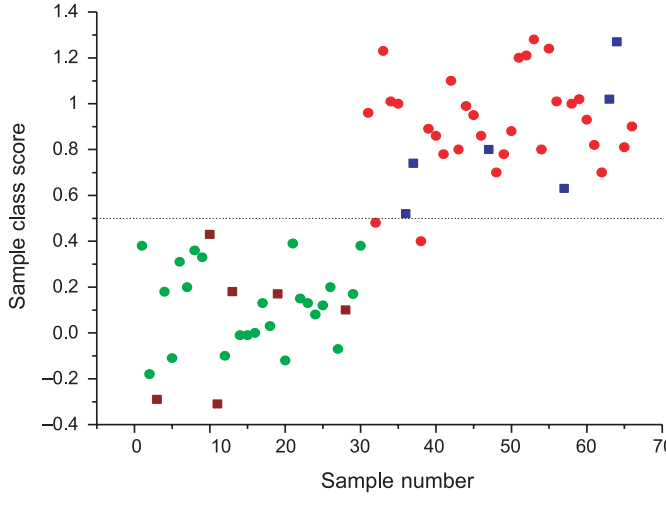

Figure 9. Prediction of coronary artery status based on a PLS–DA model using ^{1}H NMR spectra of blood serum. The green and red circles represent the training set samples from subjects with normal coronary arteries (NCA) and triple vessel disease (TVD), respectively. The magenta and blue squares correspond to the test set samples with known classes of NCA and TVD, respectively.

with a sensitivity of 92% and a specificity of 93% based on a 99% confidence limit for class membership.

The study was extended to investigate whether it was possible to diagnose the severity of the CHD that was present using serum samples from patients with stenosis of one, two or three of the coronary arteries. Although this is a simplistic indicator of disease severity, separation of the three sample classes was evident, and two class models were built based on mild-moderate, moderate-severe and mild-severe differences. None of the conventional clinical risk factors that had been measured (age, blood pressure, low density lipoprotein and high density lipoprotein cholesterol, total cholesterol, total triglycerides, fibrinogen, plasminogen activator inhibitor, white blood cell count, creatinine or history of smoking) was significantly different between the classes at the 0.05 level. The potential of this approach for diagnostic testing is currently being explored through a clinical trial known as MAGICAD (www.med.cam.ac.uk/magicad), with the aim of collecting samples and patient details for 2000 people, with currently > 800 already acquired.

Conclusions

Metabonomics is now recognized as an independent and widely used technique for evaluating the toxicity of drug candidate compounds, it has been adopted by a number of pharmaceutical companies into their drug development protocols, and has been the subject of a number of recent conferences. Using metabonomics it is possible to identify the target organ of toxicity, derive the biochemical mechanism of the toxicity, and determine the combination of biochemical biomarkers for the onset, progression and regression of the lesion. Additionally, the technique has been shown to be able to provide a metabolic fingerprint of an organism (metabotyping) as an adjunct to functional genomics and hence has applications in the design of drug clinical trials and for the evaluation of genetically modified animals as disease models. Within pharmaceutical companies there is a need for accepted validation tests and reliability tests for monitoring drug safety preclinically and clinically and for evaluating the effects of therapy in clinical trials and later when the drug is on the market. One key criterion will be acceptance in the USA by Food and Drug Administration (FDA) of safety assessment metabonomic data at the time of the investigational new drug application (IND) and of clinical efficacy metabonomic data in new drug application (NDA) submissions.

Finally, using metabonomics, it has proved possible to derive new biochemically based assays for disease diagnosis and to identify combination biomarkers for disease, for example in atherosclerosis (Brindle *et al*. 2002). Such biomarkers and methods could then be used to monitor the efficacy of drugs in clinical trials. Clearly, metabonomics is not a panacea for all future drug safety studies and clinical investigations, and will in some cases complement rather than entirely supplant conventional methods, particularly bearing in mind the needs of regulatory bodies. There are certainly particular areas within drug discovery where metabonomics will probably not prove useful because of sensitivity or specificity issues, and these require further exploration. One potential area of importance in

the future is the understanding of the reasons for the rare human toxicity ('idiosyncratic' toxicity) of certain drugs already on the market or in late stage development.

The metabonomics approach can also be readily adapted to investigate the functional consequences of genetic variation and transgenesis, which is potentially of great importance in the creation and validation of new models of human disease and efficacy. Additionally, there is considerable scope for the application of metabonomic approaches within the pharmaceutical industry from the discovery of potential therapeutic agents through clinical development and beyond. Within the discovery phase, applications include early *in vivo* toxicological testing, lead compound selection and pre-lead prioritization, and *in vivo* efficacy screening in animal models. Within the development phase, applications include finding novel preclinical safety biomarkers and mechanisms, metabotyping and the validation of animal models against human disease profiles, and the discovery of novel clinical safety and efficacy biomarkers. In human studies, metabonomics also has the potential for disease diagnosis and may even be able to provide prognostic information.

It is possible that within the next 5–10 years the world will see a revolution in health care, in that new pharmaceuticals will be targeted at specific populations who can best respond to these powerful drugs. The concept of personalized health care will become a reality. As a consequence, the need for personalized diagnostic methods will be absolute. Metabonomics offers the possibility of such individual health checks and of monitoring an individual's response to different types of therapy. It is possible to envisage future situations where metabonomics data, gene expression data and proteomics data are interrogated using multivariate approaches to provide a holistic picture of complex organisms undergoing physiological stress, using optimized biomarker combinations from all three platforms. Such integration of data types will also pave the way to understanding the relationships between gene function and metabolic control in health and disease.

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