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HIGH RESOLUTION NMR IN METABONOMIC ANALYSIS

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NMR spectroscopy is a powerful tool for providing comprehensive metabolic profile of components in biomatrices. Concentrations and fluxes of endogenous metabolites in key intermediary cellular pathways thus determined provide information on organisms' physiological or pathophysiological status. Such studies are finding increasing applications in clinical and medical sciences. ^{1}H and ^{31}P NMR provide noninvasive probes of high-energy compounds, amino acids, and compounds of phospholipid metabolism in cells and tissues. ^{13}C NMR has been used to measure and characterize molecules involved in metabolic pathways. ^{13}C-labelled substrates allow one to follow metabolism in a selective fashion. In this review, we describe one-dimensional inverse detection, and various two dimensional homonuclear and heteronuclear methods, water suppression, spectral editing and quantification techniques with special emphasis on methods for cell studies. Application of NMR in metabonomic analysis of various cells and tissues has been discussed with illustrative examples, to highlight the type of information that NMR can provide.

Key Words: NMR; Metabonomics; Cellular NMR; Water Suppression; Spectral Editing

Introduction

Genomics, proteomics and metabonomics play major roles in pharmaceutical industry. These technologies have the potential to generate megavariate data sets containing information relating to an organism's response to drugs at the levels of gene expression, cellular, molecular or metabolic level. Metabonomic analysis involves quantitation of the dynamic multivibrate metabolic response of an organism to a pathological event or genetic modification.

Applications of high resolution NMR in life sciences range from characterization of pure compounds to diagnosis from biomolecules to humans[1-6]. Recent improvements in gradient technology and the coupling of NMR to various chromatographic methods have opened new doors to the quantitative and qualitative analysis of complex metabolites extracted from body fluids and various natural products. The interaction of pharmacological agents with cells and tissues can also be monitored using high resolution magic-angle spinning (HRMAS) NMR. Several NMR nuclei have been used to probe *in vivo* biochemical reactions and metabolism. The non-invasive nature of measurements offers several advantages over conventional analytical techniques. NMR can simultaneously detect molecules which are expected, unexpected or which are difficult to assay using standard biochemical methods. It is cost effective and typically takes few minutes per sample. It requires little or no sample pretreatment or reagents and is therefore bioanalytically more efficient than the biochemical methods used to characterize genetic or proteomic composition of samples. Therefore, NMR has been extensively applied to study biochemistry, and physiology in living tissues and cells[1,2].

NMR spectroscopic information includes the resonance position (δ), spin-spin coupling constants (J), nuclear Overhauser effect (NOE), peak intensities and spin lattice (T_1) and spin-spin (T_2) relaxation times. The most suitable nuclei for studies of cells and tissues are ^{1}H, ^{13}C and ^{31}P, and we restrict our discussion to these. Multinuclear NMR has the advantage that it does not require precise selection of analytical conditions to obtain quantitative and qualitative information from endogenous metabolites. This ability has been successfully exploited in *in vivo* metabolism as well as in studies of isolated organs, cell cultures and extracts.

In *in vivo* samples, the information available is limited by the system heterogeneity, sensitivity, dynamic range problems, peak overlap, resolution, resonance line broadening due to macromolecular binding and chemical exchange. Some of the problems can be overcome by signal averaging, use of surface coils, adequate water suppression and use of larger volumes. High magnetic fields are necessary, to improve both spectral resolution and sensitivity. Resonances that extensively overlap in the one dimensional (1D) spectrum can be resolved and identified by two dimensional (2D) NMR.

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Main emphasis of this review is on role of NMR methodologies in metabonomic analysis of biological matrices such as cells and body fluids. This is followed by methods for data processing, assignment strategies and solvent suppression. Illustrative examples of applications of NMR including our own work on spermatozoa have been provided. In the rapidly expanding area of cellular NMR, it is not possible to give a comprehensive review, but we have tried to cover major developments on metabonomic analysis in quality control of food & chemical products, evaluation of environmental contamination, diagnosis of metabolic diseases, in pharmacy, clinical biopsies, evaluation of drug efficacy and monitoring physiological variations.

NMR Techniques

One Dimensional (1D) Techniques

Although much of recent efforts have been directed to the development of powerful 2D NMR experiments, there have also been significant developments in 1D methods, which offer important advantages. For example, indirect detection of resonances of insensitive nuclei through resonances of the attached ^{1}H (insensitive nuclei enhanced by polarization transfer - INEPT) has become an important method for cell studies. Techniques related to heteronuclear coherence transfer include normal INEPT, broad -band INEPT and various spin echo sequences. The INEPT sequence can also be performed in reverse, in which the population of the ^{13}C for example is transferred to ^{1}H.

Two Dimensional (2D) Techniques

The basic idea of the 2D NMR technique is to display interactions (correlations) between various protons in a given molecule onto a plane, thereby aiding assignment of individual resonances to specific nuclei. This concept has been extended to higher dimensions involving other nuclei, (^{13}C, ^{15}N and ^{31}P) and is thus used to disperse spectra on the basis of their resonance frequencies. For sensitivity reasons, the detected nucleus is invariably chosen as ^{1}H. Thus the frequency information of heteronuclei is transferred to ^{1}H. The number of approaches for such transfers is large and depends upon the sequence of pulses used. The information content of a multidimensional NMR spectrum is determined by the pathway of magnetization transfer among various spins. The transfers between various spins may be mediated either through J-couplings (coherence transfer) or through dipolar couplings (NOE transfer). One of the major applications of J-correlated spectra is in the identification of various molecules in a cellular system from their characteristic patterns.

In heteronuclear correlation spectroscopy (hetero-COSY), the resonance frequencies of scalar coupled ^{1}H and ^{13}C, ^{15}N, or ^{31}P are correlated and cross peaks with the coordinates $\delta(^{1}H)$ and $\delta(^{13}C)$, $\delta(^{15}N)$ or $\delta(^{31}P)$ are obtained. These are mostly based on one bond (J) coupling between the ^{1}H and the heteronucleus. However, sensitivity of the conventional hetero-COSY experiments is low. In heteronuclear multiple-quantum coherence (HMQC) experiments[7,8], optimum sensitivity can be obtained starting with ^{1}H polarization and ending up with ^{1}H detection. A related pulse sequence is the heteronuclear single quantum coherence (HSQC) experiment[9], which employs two INEPT type transfers to transfer magnetization from ^{1}H to the low-gyromagnetic ratio (γ) nucleus and back to protons. The enhancement in sensitivity is much greater than that obtained by NOE in simple heteronuclear correlated experiments. Development of the pulsed-field gradient (PFG) technology[10] has provided a great impetus to heteronuclear NMR spectroscopy, in particular, for ^{13}C-^{1}H COSY experiments for natural abundant ^{13}C samples, where the suppression of the large, unwanted signals from ^{12}C bound protons is important to obtain spectra free from t_1-noise artifacts[11].

A comprehensive assignment of blood plasma using 2D correlation methods at 750 MHz has been reported[12]. Assignments of signals from 43 low Mw. metabolites, including many with complex or strongly coupled spin systems have been achieved. New assignments are also provided from macromolecular species i.e. lipoproteins, albumin, and alpha 1-acid glycoprotein. ^{1}H-^{13}C HMQC at variable temperature allowed the detection of cholesterol and choline species bound in high-density lipoprotein and also confirmed the assignment of most of the lipoprotein resonances.

High-Resolution Magic Angle Spinning Technique

Magic angle spinning (MAS) of tissue specimens reduces the effects of dipolar coupling interaction on neighbouring spins, and averages the susceptibility inhomogeniety to zero. This improves NMR sensitivity, resolution, and spectral quality. Its application to the analysis of intact biological tissues has made a major contribution to directly correlate NMR-detected biomarkers in biofluids. High resolution MAS-^{1}H NMR has been used to characterize the low MW composition of a range of biochemical tissues and organelles

including liver, kidney, brain, heart, adipose and mitochondria and to evaluate the biochemical consequences of several disease processes[13-15]. It provides a reliable, quantitative biochemical profile of human soft tissue specimens and may be used to distinguish principle forms of liposarcoma[16-18]. A double-pulsed field gradient selective echo technique is employed together with MAS to selectively excite a region of the proton spectra, which contains important less abundant nontriglyceride metabolites such as phosphatidylcholine (PTC) and phosphocholine (PC). A quantitative analysis of PTC and PC is then used to distinguish well-differentiated liposarcoma from normal fat[19]. In addition to 'bridging the gap' between histopathology and biofluid analysis, MAS can be used to visualize dynamic processes and to gain insight into the compartmentalization of metabolites within cellular environments[20].

^{1}H- NMR

A narrow range of chemical shifts (~ 10 ppm), together with presence of a large number of proton containing compounds in the cell and slowly tumbling macromolecules, give rise to complex spectra of overlapping broad peaks. Broad and poorly resolved signals from proteins also overlap with the signals from metabolites of interest. An additional problem is one of dynamic range, for it is necessary to observe ^{1}H signals from compounds with concentrations below 1mM, in the presence of large intense signal from H_2O. Techniques have been devised to minimize these problems. Using different techniques on red cells and a variety of gland storage systems, signals from small molecules and some parts of proteins have been assigned. Complete ^{1}H resonance assignments have been carried out in spermatozoa using TOCSY and DQF COSY spectra of intact cell and cell free extract (Fig. 1)[21].

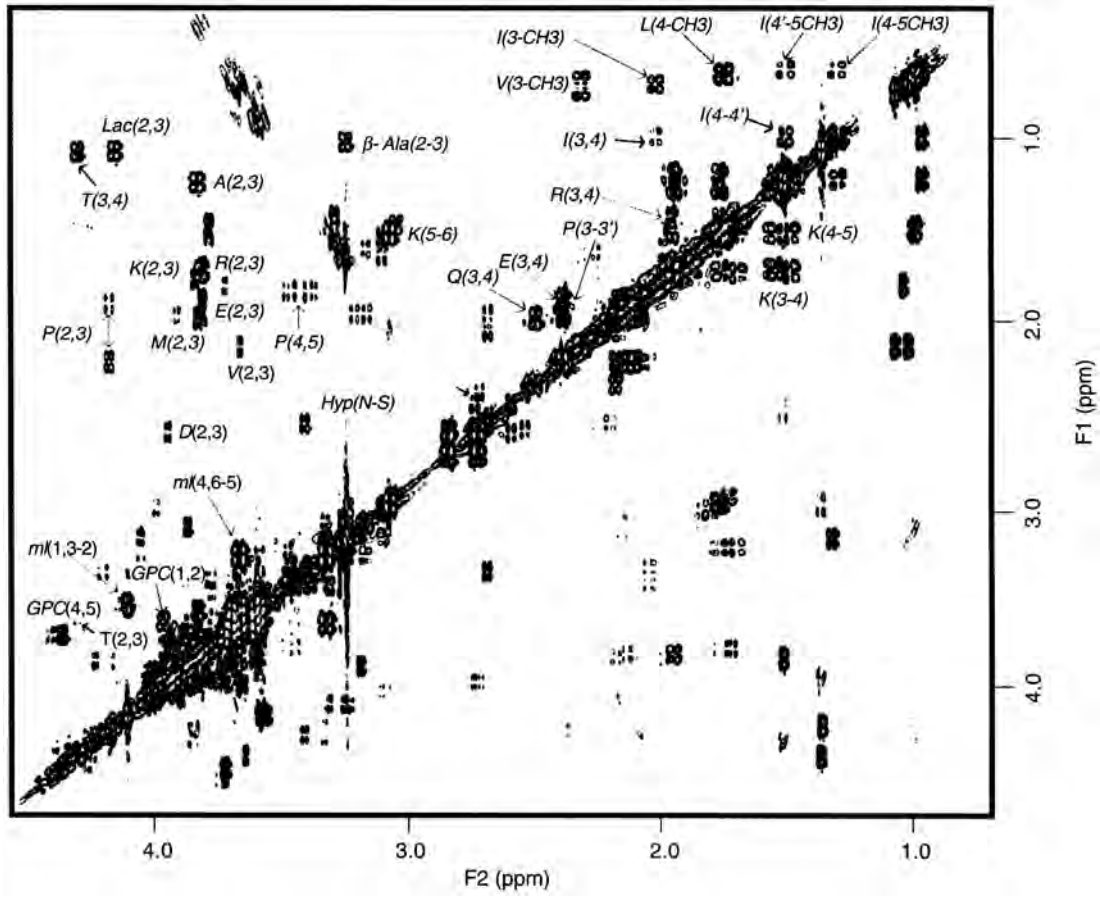


Fig. 1 Upfield region of the DQF-COSY spectrum of CFE of spermatozoa from caput region of goat epididymis. The peaks are marked with one letter code and appropriate numbering scheme for amino acids and for other compounds, Lac, Lactate, mI, m-Inositol, Hyp, Hypotaurine and GPC, Glycerophosphocholine. Experimental parameters are: spectral width 7 KHz, 32 scans, relaxation delay 1.5 s and 2048 & 512 data points. The data were zero filled to 4K and 2K data points and apodized with a sine shifted window function of $\pi/3$ and $\pi/2$ for the F2 and F1 dimensions respectively.

^{31}P NMR

^{31}P is a nucleus of choice for studies of *in vitro* and *in vivo* metabolism. In general, assignment of ^{31}P resonances is relatively simple, since the chemical shift range for biologically occurring phosphates is large (~ 40 ppm). The major signals are from ATP, ADP, phosphocreatine, Pi, sugar phosphates (mainly glucose phosphates), 2, 3-diphosphoglycerate, AMP and various phosphodiesters. ^{31}P NMR therefore serves as a valuable tool for studying physiology and pathology of tissues. It is possible to monitor a variety of biochemical processes including energy metabolism, pH balance and phospholipid pathways. The energy metabolism in semen and spermatozoa isolated from boars, rams, goats and bulls has been examined[22] and *in vitro* capacitation has been monitored. Progressive build up of phosphorous metabolites with time indicates a good correlation with the progressive maturation, metabolism and capacitation of these cells (Figs. 2 & 3)[23]. From the chemical shift calibration of the Pi resonance it is

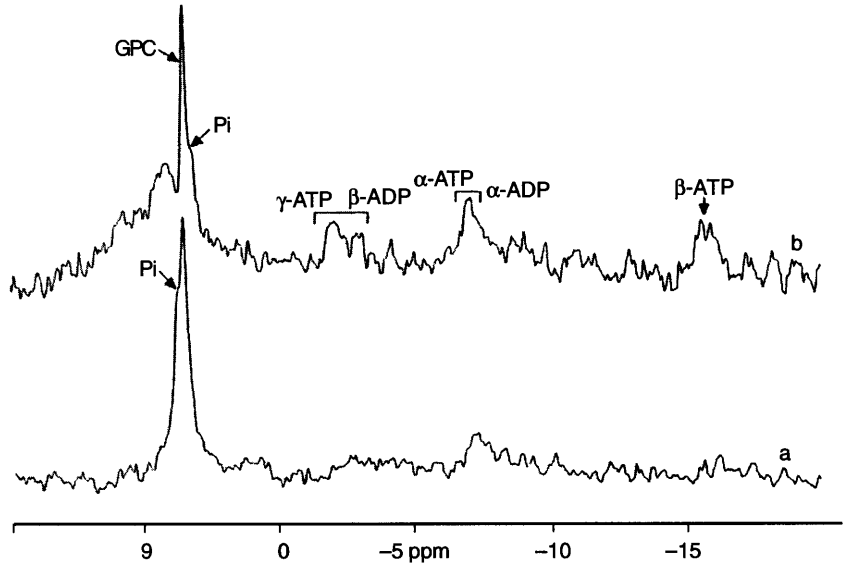


Fig. 2 202.5 MHz ^{31}P NMR spectrum of spermatozoa obtained from the cauda region of epididymis of sacrificed goat (a) cells suspended in Dulbecco medium (initial pH 7.2) without glucose, (b) cells undergoing glycolysis after incubation with 10% (w/v) glucose for 30 minutes. Spectral parameters used are: 2s relaxation delay, 10 ms pulse width corresponding to 60^0 flip angle and 10 KHz spectral width. Chemical shifts have been reported with respect to phosphocreatine signal. Resonance signal at -2.30 ppm, arises from the γ-P-ATP (with a small contribution from β-P-ADP). Peaks from α-P-ATP and the β-P-ATP can be observed at -6.78 ppm and -15.44 ppm, respectively. A signal at 3.0 ppm corresponds to inorganic phosphate (Pi). The Pi resonance is a composite signal having contributions from intra- and extra-cellular inorganic phosphate which are not significantly different at normal pH and therefore overlap. A strong signal slightly up-field to Pi corresponds to GPC.

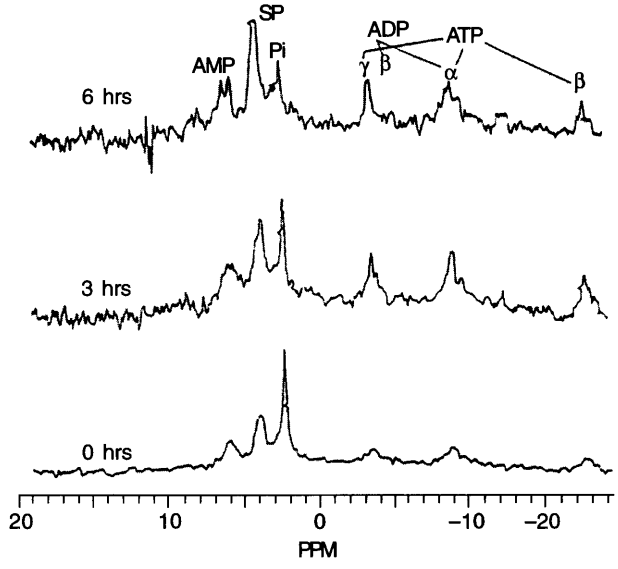


Fig. 3 202.5 MHz ^{31}P NMR spectrum of spermatozoa obtained from the cauda region of epididymis of sacrificed goat at different time intervals under *in vitro* capacitation condition.

possible to determine the intracellular pH. An NMR pH calibration for this range of temperatures, a summary of calibration data, and a discussion of the factors influencing pH standardization has been reviewed[24].

2D ^{31}P-^{1}H correlation spectroscopy is useful for the assignment of ^{31}P-NMR spectra. The peaks in the 2D spectra are only due to protons coupled to ^{31}P nuclei leading to simplification of the spectrum. Moreover, the absence of water signal, is an added advantage. Whenever new compounds are anticipated, especially in deranged metabolic status of diseases, it is necessary to obtain an unequivocal assignment of the signals, and 2D ^{31}P-^{1}H-correlation spectroscopy can provide such data[25]. Experiments such as ^{1}H-^{31}P HSQC and ^{31}P COSY, have been used to assign the resonances from low-molecular-weight phosphorylated compounds in yeast[26].

^{13}C NMR

Natural abundance ^{13}C NMR has been used to study *in vivo* metabolites and structural components in intact organelles, cells and tissues. This includes measurement and characterization of high-concentration components such as lipids and glycogen. Using ^{13}C-enriched molecules it is possible to do tracer experiments and follow the course of metabolism and monitor several related aspects. ^{13}C spectra are better resolved due to larger spectral range (~ 200 ppm). The resolution is further enhanced as line widths are narrower than the corresponding ^{31}P lines, leading to an increase in sensitivity, NOE from proton decoupling can improve signal to noise by a factor of 3. Development of selective ^{13}C decoupling methods can also lead to the resolution enhancement. ^{13}C NMR can provide information about the architecture and dynamics of structural components, the nature of the intracellular environment; and metabolic pathways and relative fluxes of individual carbon atoms. Using ^{13}C enriched substrates, a large number of resonances have been identified in isolated cells[27]. *In-vivo* metabolism of drug in perfused rat liver was studied using ^{13}C continuous flow NMR[28].

Using ^{13}C selectively enriched glucose as substrate, lactate production and glucose consumption has been monitored in real time. A study on spermatozoa explored the mechanism underlying action of L-arginine (which is administered in oligospermic and asthenospermic patients) on the metabolic activity of spermatozoa. The effect of L-arginine, L-lysine and L-ornithine on the glycolysis of epididymal goat spermatozoa has been elucidated. At optimal concentration of L-arginine, the forward metabolic rates have been found to increase by seven to eight times over control experiments (Fig. 4)[29].

NMR studies of Cells

Practical Considerations

Cell Perfusion

NMR can be used in a nondestructive manner to study suspension of cells. However, most cells require a well-regulated medium that includes buffered pH, nutrients and a continuous supply of oxygen. NMR studies can be performed under controlled conditions for extended periods of time, under sterile conditions.

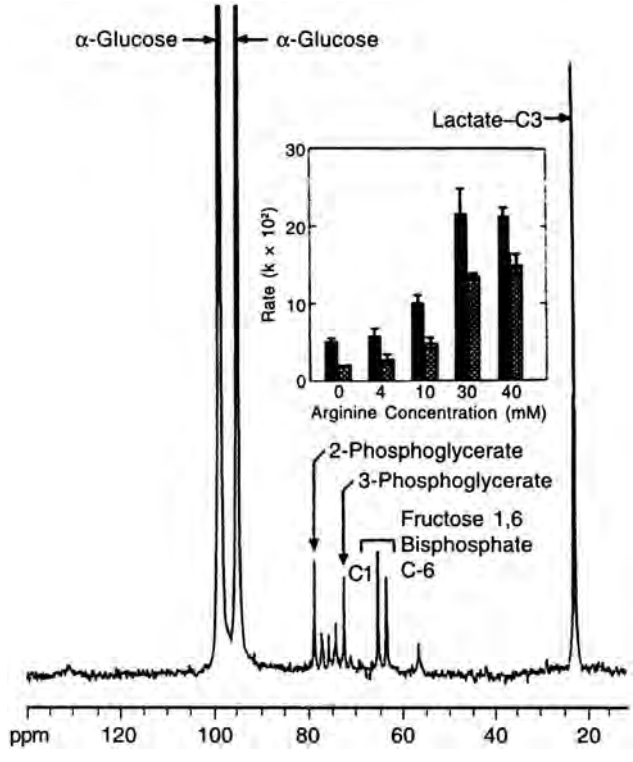


Fig. 4 ^{13}C NMR spectrum of goat spermatozoa obtained from the cauda region of the epididymis incubated with 20 mM [1-^{13}C] glucose. Inset figure shows rate constant of glucose consumption and that of lactate production for different concentrations of L-arginine. 1-^{13}C Glucose was added and ^{13}C NMR spectra were recorded with time. Glucose and lactate signals of subsequent spectra were integrated and expressed with respect to the glucose signal of the first spectrum, which is assigned as 100.The data was fitted to a first order rate equation (decaying) to determine the rate constant for substrate consumption whereas for lactate generation a rising exponential equation was used

Although several perfusion set-ups involving dialysis fibres and beads have been described, these could not be applied successfully to cellular studies. Gel thread methods are the simplest and closely approximate biological situation[30, 31]. Its application to metabolic studies by NMR has been demonstrated by following gluconeogenesis from (2-^{13}C-pyruvate in the perfused, isolated mouse liver, lipogenesis from (2-^{13}C) acetate in perfused *Acanthamoeba castellanii* cells embedded in agarose filaments[32] and in perfused MMQ cells[33]. It has been shown that rat hepatocytes immobilized in agarose threads continuously perfused with oxygenated Krebs-Henseleit solution are capable of maintaining the cell viability with excellent metabolic activity for more than 6 hours. Immobilization in Ba-alginate-coated agarose threads has been shown to be an efficient way of trapping human erythrocytes for whole cell investigations[34]. Noninvasive quantification of intracellular sodium in immobilized and perfused cancer cells using ^{23}Na NMR has been reported[94] . The mechanism of action of the antineoplastic drug lonidamine (LND) on MCF-7 human breast cancer cells embedded in alginate microcapsules, perfused with growth media and LND at physiological conditions has been studied using ^{31}P and ^{13}C NMR[35]. Recently, monitoring metabolism of growing cell line in the perfusion system has been reported[36]. In such a system, viable cells have been recovered from the perfusion system after NMR measurements for further biochemical studies. Thus this system can be useful for studying the physiology and biochemistry of exponentially growing cells for at least two days in NMR tube culture. A series of methods, which involve immobilization of cells, followed by perfusion of the immobilized cell sample, have been reviewed with erythrocytes and thymocytes as specific examples[37].

Cell Free Extracts

High-resolution ^{1}H spectra of biological matrices, such as urine, blood plasma tissue or cell samples, generate complex spectral profiles. The simplest procedure to solve the problem of peak broadening and overlapping lines during resonance assignments is to compare the spectrum with that of protein free extract. The cell free extracts (CFE) give better resolved spectrum with narrower lines than intact tissues or cell suspensions. For certain unidentified peaks, which are pH sensitive, a pH titration on the extract can be carried out. The assignments can be further supported by using spiking method in which small amounts of the suspected compounds are added to the extract and the spectra compared before and after the addition. Sometimes release of paramagnetic ions during extraction broadens the spectrum. Therefore it is desirable to pass the extract through an ion exchange column or to add EDTA. Using pH titration, individual model compounds, a model mixture solution and by tracing the coupling networks, several metabolites have been identified in tissues and cell extracts. Multinuclear NMR studies have been performed on aqueous solutions of lyophilysates of CFE and epididymal fluid (EF) from caput and cauda regions of epididymis of sacrificed goats. Identification of low molecular weight compounds present in different maturation phases of spermatozoa has been carried out[21].

Assignments using CFE has some serious drawbacks. For example, in some cases, compounds in the cells can not be observed in the extracts either due to limited solubility in the extracting solution or because they undergo a chemical modification during extraction. New compounds may appear in the extracts due to degradation of metabolites or macromolecules. Thus, NMR assignments of small molecules in mixtures of substances, even at the highest magnetic fields are not straight forward as reported in the assignment of two major components hypotaurine (previously called substance X) and carnitine in boar seminal plasma using TOCSY and gradient-selected HSQC and HMBC experiments[38].

Solvent (H_2O) Suppression

Several approaches have been used to remove interfering signals, particularly that of water. A simple approach is to replace the H_2O present in the sample by D_2O. However, deuteration of living cells and tissues may lead to undesirable effects on metabolism. An easy method to remove H_2O resonance is by low power, selective presaturation of its signal[39, 40]. This method has disadvantages, as it requires good magnetic field homogeneity. Peaks close to solvent resonance may also get eliminated, and exchanging protons intensity will diminish if their chemical exchange rate with the solvent is comparable to the magnetic relaxation rate T_1^{-1}. Alternative methods are selective excitation of the resonance with a series of either long, weak (soft) pulses or short, strong (hard) pulses separated by delays.

Further problems arise from overlapping signals from macromolecules. When measuring low molecular weight components, in cell suspensions

or protein rich biofluids such as plasma, it is usually advisable to use spin echo methods to eliminate broad lines from macromolecules, which have short transverse relaxation times. This may also result in attenuation of the relatively broad water signal in cell suspensions[39, 40]. The oxidation/reduction chemistry of penicillamine in human erythrocytes has been characterized directly in intact erythrocytes by using CPMG sequence to selectively eliminate interfering resonances from hemoglobin and from intracellular water[41]. A novel method (Water Attenuation by T_2 relaxation, WATR) for water suppression, based on the reduction of the water T_2 relaxation time by ammonium chloride or guanidinium chloride which can exchange protons with the solvent water[42] has been used. The resulting broad water signal is then selectively attenuated in CPMG spin-echo spectra. The method is also effective where water suppression is required in 2D-COSY experiments, the water signal being attenuated by T_2 relaxation during the delay allowed for the evolution of long range coupling[43].

Double inversion recovery used for water suppression can be made much less sensitive to pulse imperfections and to variations in relaxation times than a single inversion recovery. This insensitivity results in up to 10-fold improvement in the water suppression *in vivo*. The excellent water suppression by double inversion recovery is demonstrated experimentally by *in vivo* proton spectra obtained from a rat brain[44].

Spectral Editing

Spectral editing techniques have been developed using double quantum filtration both on PCA extract and on homogenate. Extension of these techniques for improved S/N ratios, modulation characteristics, metabolite selectivity; B0 and B1 inhomogeneity and motion effects have been reported. For example, frequency selective read pulses have been used for signal enhancement and lipid suppression. The multiple quantum modulation can be used to obtain specific metabolite editing[45].

A technique based on F1 oversampled J-resolved spectroscopy, has been introduced for obtaining *in vivo* ^{1}H spectra with all the advantages of a full water signal. It enables to separate metabolite signals from unwanted baseline artifacts. The powerful specificity of this method has been demonstrated with model compounds, phantoms, and *in vivo* systems[46].

Spectral editing via ^{1}H-^{13}C scalar coupling was performed with twin spin-echo double resonance (T-SEDOR), a pulse sequence which combines chemical specificity and high sensitivity, requires no solvent pre-saturation, and can easily be adapted to imaging protocols. Suitability of the pulse sequence for monitoring 6-^{13}C-2dG uptake in living cells has been demonstrated[47].

Conventional double-quantum editing techniques recover only one metabolite at a time. A stimulated-echo-enhanced selective double-quantum coherence transfer (STE-SelDQC) sequence can monitor changes involving several metabolites. A frequency selective double-quantum filter designed for lactate editing suppresses fat and water resonances and a stimulated-echo window of adjustable frequency and bandwidth is incorporated for observation of other metabolites[48].

An approach for the simplification of ^{1}H NMR allows spectra of slowly diffusing large molecules in bio-fluids by diffusion editing, the slowly relaxing small molecules by spin relaxation editing, or by a combination of the two methods[49]. These methods bring about considerable spectral simplification for assignment. Using ^{1}H-^{1}H diffusion-edited total-correlation (DETOCSY) spectra of human blood plasma, it has been possible to obtain signals from only the macromolecular components. The intensity of ^{1}H NMR signals depends on diffusion coefficients, which opens up new possibilities for pattern recognition of samples based on molecular mobility[49].

Quantification

There is an increasing use of NMR to examine quantitative variations in cell metabolism and/or cell components in response to physical, chemical, and biological stresses. Relative quantitative information can be obtained by comparison of signal intensities with control samples. Some of the methods use area ratios, external capillary, internal endogenous or exogenous marker, external marker in bath, tissue extracts, reference to internal ^{1}H water concentration, etc. The biological and technical aspects associated with quantification of metabolite concentrations have been reviewed[50]. A new method for quantitatively comparing the spectra of cell samples depends on a normalization algorithm which takes into consideration all cell metabolites. The algorithm has been applied to real spectra of cell samples to get qualitative and quantitative biological informations[51].

Applications

In Pharmaceutical Technology

NMR spectroscopy coupled with advanced chemometric data analysis has been the dominant analytical platform for acquiring metabonomic data on metabolic disorders, investigations of the biochemical basis of drug and xenobiotic metabolism, toxicological processes, and vertebrate biological fluids, such as, sweat, aqueous humour, amniotic fluid, seminal plasma, cerebrospinal fluid, synovial fluid and blood plasma[52-54]. NMR is used in pharmacy to determine the impurity profile of a drug, to characterize the composition of drug products, and to investigate metabolites of drugs in body fluids. For pharmaceutical technologists, solid-state measurements can provide information about polymorphism of drug powders, conformation of drugs in tablets etc. Micro imaging has been used to study dissolution of tablets. Whole-body imaging is a powerful tool in clinical diagnostics. The applications of NMR spectroscopy in drug analysis, in particular, methods of international pharmacopoeiae, pharmaceutics and pharmacokinetics have been reviewed[55].

In Identifying Biomarkers

Metabolites that differentiate between biofluid samples from drug treated and control samples can be elucidated giving an insight into possible mechanism of drug action and toxicity or dysfunction. Identification of biomarkers can be achieved by various means. For many compounds, chemical structure can be identified from 1D NMR and the structures verified by spiking biofluids with authentic solutions of proposed metabolites. For more challenging cases one may use 2D NMR such as Total Correlation Spectroscopy.

NMR has been used as a fast, simple method for "fingerprint" identification of urinary compounds. Numerous low-Mw. metabolites including creatinine, citrate, hippurate, glucose, ketone bodies, and various amino acids, which are related to the different physiological states and pathological conditions, have been identified in intact human urine[56, 57]. Resonances arising from major acetaminophen metabolites, including the L-cysteinyl conjugate in urine have been unambiguously assigned using 2D COSY spectrum[58]. Paraquat in urine from acutely poisoned patients has been characterized & quantitated[59]. ^{13}C NMR has helped in direct and simultaneous detection of phase I and phase II metabolites of antipyrine in rat urine[60].

Two metabolites of notopterol ((2E)-5-hydroxy-3,7-dimethyl-2,6-octadienyloxy) psoralen) which is an inhibitor of aminopyrine N-demethylase in liver microsomes and is metabolized by cytochrome P450, have been established by ^{1}H-NMR and liquid chromatography-mass spectrometry[61]. Acetaminophen and its glucuronide, sulfate, N-acetyl-L-cysteinyl, and L-cysteinyl metabolites have been detected in intact, untreated human urine. The time course of excretion of these metabolites after ingestion of the therapeutic dose of the drug has been followed. Excretion of some other metabolites in urine, including creatinine, citrate, hippurate, and sarcosine was measured[57]. Biosynthesis of polyamines in cell culture was examined using the labelled substrates. Semi-selective HSQC experiments were used to assign ^{1}H and ^{13}C resonances of polyamines such as putrescine, spermidine and spermine in PCA extracts of F98 glioma cells[62].

The intracellular metabolites in three types of human brain and nervous system tumors have been compared. Spectra from meningiomas show relatively high signals from alanine. Intense signals from creatine are present in neuroblastoma spectra. Significant differences are observed in the amounts of alanine, glutamate, creatine, phosphorylcholine and threonine in different types of tumors. These results have potential relevance for the development of non-invasive diagnosis of tumor lineage[63]. Benign and malignant tumor samples from brain surgery were treated for dual extraction of lipidic and aqueous phases. A highly significant variation of the spectral pattern was observed between benign and malignant tumors. The information may be statistically analyzed to elucidate tumor-specific biochemical pathways and improve interpretation of *in vivo* spectra[64].

Spectra of PCA extracts of cultured Schwann cells, perineural fibroblasts, dorsal root ganglion neurons, and cortical neurons derived from nervous tissues have been compared. The significant differences are that N-acetylaspartate was present in dorsal root ganglion neurons and cortical neurons, gamma-aminobutyric acid was present in large amounts in cortical neurons, and Schwann cell spectra displayed a large signal from glycine. Thus different cell types exhibit highly characteristic metabolite profiles[65].

In Quality Control

NMR in combination with pattern recognition techniques has been used in detecting adulteration of orange juices and apple juices. It has been used in

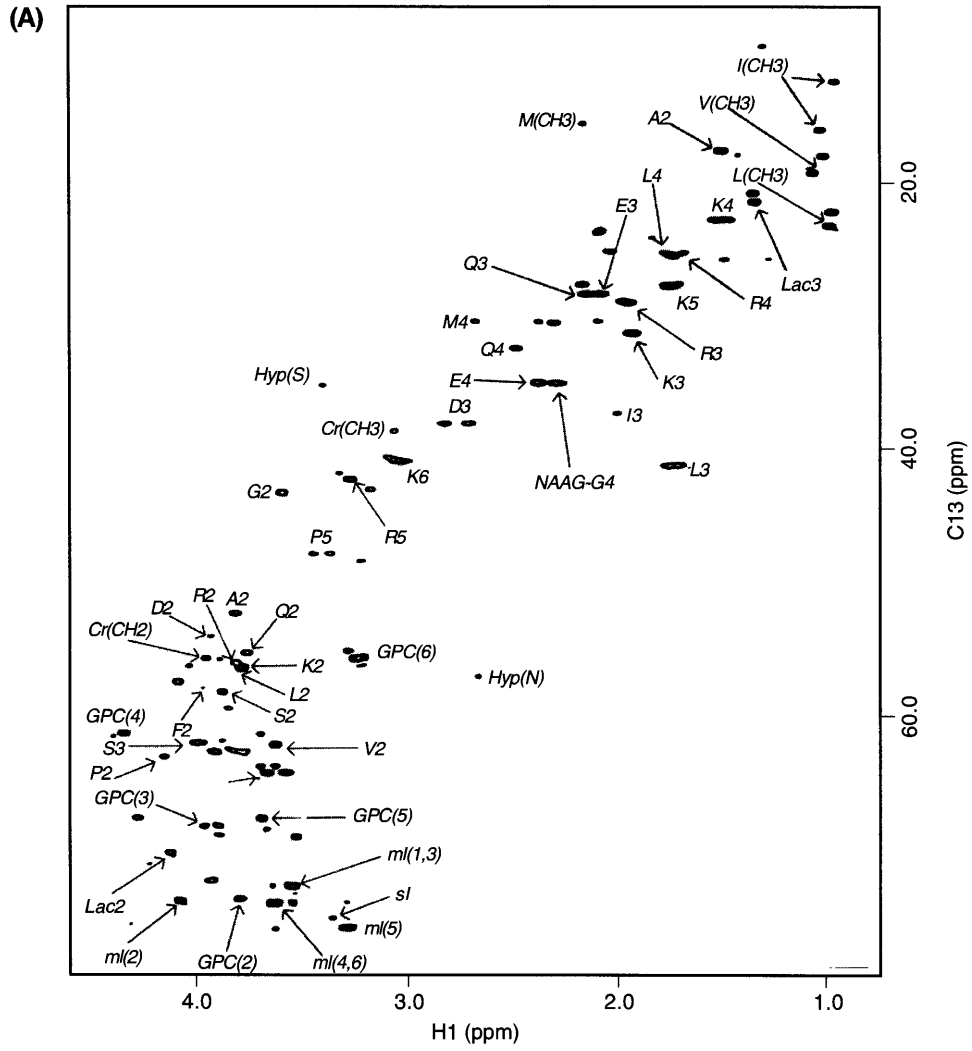
screening of virgin olive oils and in determination of geographical origin of oils[66-68]. Comparison of NMR spectra of milk from various species indicates that the rabbit milk contains unexpectedly large amounts of phosphate esters and low levels of alkaline phosphatase activity. In addition unknown compounds were detected in baboon milk and in some samples of sheep and goat colostrum. Thus ^{31}P NMR has been used to follow the changes during lactation and for the authentification of milk samples[69].

In Energy Metabolism

Infantile hydrocephalus is often caused by an obstruction in the cerebrospinal fluid flow pathway and results in ventricular dilatation and chronic trauma to the surrounding brain. Progressive changes in cortical (extracts) metabolites at three stages of infantile hydrocephalus were quantified. The results have been discussed in relation to hydrocephalus pathophysiology and prevention and reversibility with shunt treatment[70]. Metabolic changes in the cerebral cortex of treated and untreated infant hydrocephalic rats were studied *in vitro*. The effect of hydrocephalus on cerebral energy metabolites and on intermediates of membrane phospholipid metabolism has been studied in H-Tx rats with inherited infantile hydrocephalus[71]. Aminooxyacetic acid, a potent inhibitor of the mitochondrial malate-aspartate shunt, was used to assess the role of mitochondrial energy metabolism in damaged brain of rats by *in vitro* quantitative analysis of metabolites[72]. The ability of cultured astrocytes to metabolize (U-^{13}C) glutamate during hypoglycemia and hypoxia was investigated by ^{13}C NMR. The results indicate that glutamate-to-aspartate conversion is preferentially utilized by astrocytes when oxygen is available but glycolysis is impaired[73].

In Clinical Biopsy Tests

Pattern recognition techniques have been used to investigate and classify human brain tumors based on the ^{1}H spectra of chemically extracted biopsies[74]. Genetic programming (GP) is used to classify tumors



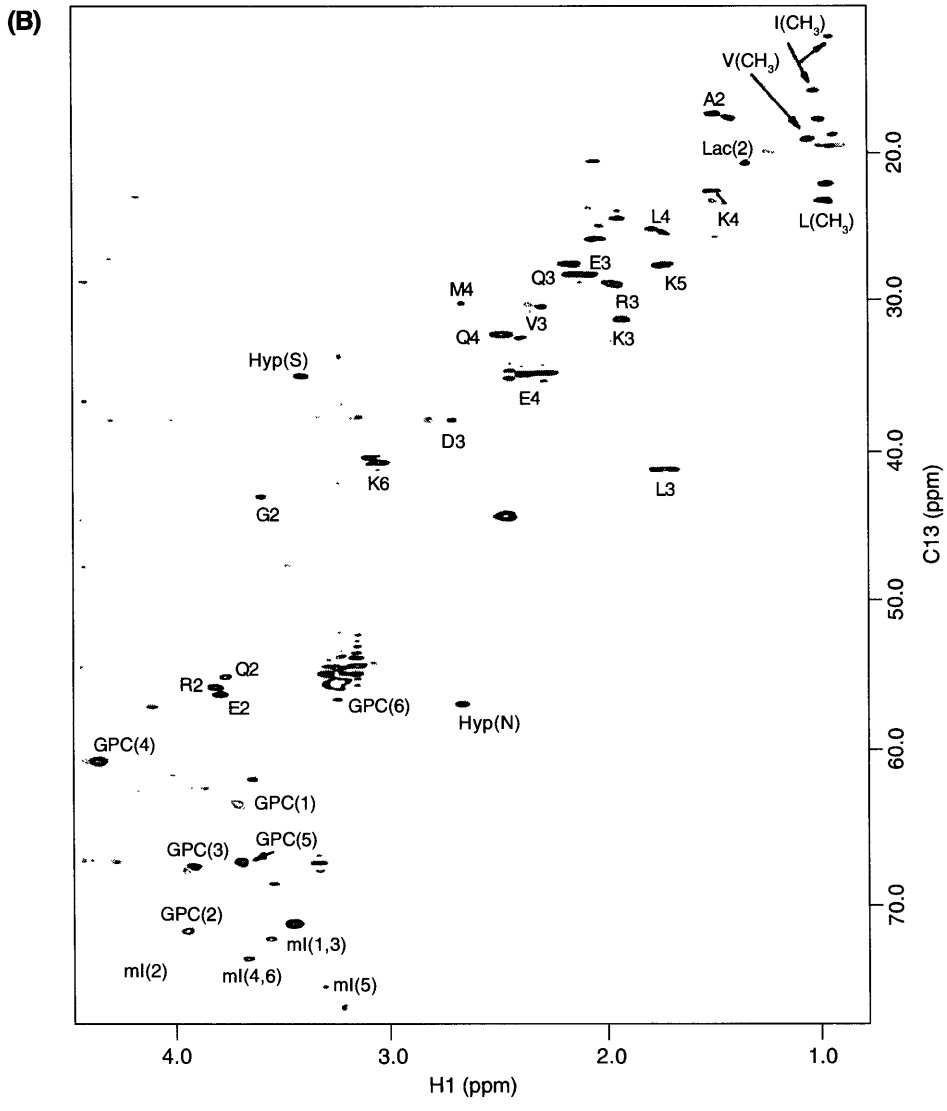


Fig. 5 (A) The upfield region of the Gradient enhanced HSQC spectrum of CFE of spermatozoa from caput region of goat epididymis. Peaks are marked with the corresponding one letter code of amino acid and with appropriate number as per numbering scheme for amino acids and for other compounds. Experimental parameters are: relaxation delay 1.5s, spectral width ^{1}H dimension 7 KHz, ^{13}C dimension 13 KHz, 64 scans, 2048 and 400 data points in respective ^{1}H and ^{13}C dimensions. The ^{13}C decoupling was carried out during detection; (B) The upfield region of the Gradient enhanced HSQC spectrum of intact spermatozoa from caput region of goat epididymis. Peaks are marked with the corresponding one letter code of amino acid and with appropriate number as per numbering scheme for amino acids and for other compounds. Experimental parameters are: relaxation delay 1.5s, spectral width ^{1}H dimension 7 KHz, ^{13}C dimension 13 KHz, 64 scans, 2048 and 512 data points in respective ^{1}H and ^{13}C dimensions. The ^{13}C decoupling was carried out during detection.

based on ^{1}H NMR spectra of biopsy extracts. Analysis of such data would ideally give not only a classification but also indicate which parts of the spectra are driving the classification. The advantage over the neural network method is that it makes use of simple combinations of a small group of metabolites, in particular, glutamine, glutamate and alanine. This may help in the choice of the most informative NMR methods for future non-invasive studies in patients[75].

In Reproductive Biology

There has been an active interest in the biochemistry of mammalian spermatozoa[72, 76-78]. It has been shown that the PE component of rabbit epididymal spermatozoa phospholipids differs from that of other cells in having the previously unreported diplasmalogen as its major constituent. NMR has been used to characterize the seminolipid from bovine spermatozoa[79]. The prominent constituents identified in the lyophilisate

and in the original seminal plasma are inositol (95% myo-inositol, 5% scyllo-inositol), citrate, lactate and glycerophosphorylcholine (GPC)[80]. Various phosphorous metabolites have been identified using proton detected 2D correlation experiments[81]. Several low molecular weight compounds, such as amino acids, carbohydrates and lipids have been identified in cell free extract (CFE), epididymal fluid (EF) and in intact cells from caput and cauda regions of epididymis of sacrificed goats with the help of DQF-COSY and HSQC (Fig. 5A & B). Presence of β-alanine and hypotaurine has been reported for the first time in goat epididymis. Relative concentrations of these metabolites have been compared in light of their function during maturation of these cells[21]. Inositol is widely distributed in the semen of the various species including man and boar. In boar spermatozoa, the ratio of m-inositol to s-inositol is reported to be 94:6[80]. This is in agreement with the observation in case of goat where the presence of m-inositol in CFE of both cauda and caput regions of epididymis has been observed. Relative concentration of GPC and amino acids arginine, glutamic acid and glutamine compared to other amino acids in cauda cells are more than those in caput cells. The unusual amino acid hypotaurine, which is an important compound for sperm survival, capacitation and fertilization processes, and embryonic development was too weak to be observed in caput as compared to cauda cells. These analysis help to understand the influence of different factors which play a role in the preservation of the sperms, to identify abnormal or diseased state of cells by qualitative or quantitative comparisons, to follow maturation of the cells and to design effectors for fertility control and control of related diseases[23, 78, 63, 82-85].

In Control of Oxidative Damage

Membrane metabolism is one of the key areas in understanding biological phenomena. Several studies have been undertaken to identify and define the detailed role of the phospholipid metabolites under different pathological conditions such as tumors and neurological disorders[86-91]. Relayed COSY, TOCSY, ^{1}H-^{13}C HMQC, and J-resolved techniques have been used to elucidate the molecular structures of lipid oxidation products (LOP) in diet. These techniques facilitate resolution of vinylic and aldehydic resonances of LOP which appear as complex overlapping patterns in conventional one-dimensional spectra[92]. Ability of antioxidant thiol drug N-acetylcysteine and exogenous cysteine to protect metabolites present in intact inflammatory synovial fluid samples against oxidative damage has been evaluated. Radiolytic products generated from the added thiols were simultaneously detected[93].

Future Perspective

NMR based metabonomic analysis offers a means for evaluating the direct effect of altered gene expression on the metabolic status of an organism. It can be used to address a large range of pharmacological, clinical and environmental problems. Studies on new metabolic pathways help developments in molecular cloning of labelled proteins and nucleic acids, which are essential in macromolecular structure determination. With the emergence of proteomics and genomics, the role of metabonomics in linking metabolic differences between organisms need not be over-emphasized. Ongoing developments in instrumentation, statistical techniques and user-friendly software would help to make metabonomics an integral component in pharmaceutical industry.

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