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Biochemical classification of kidney carcinoma biopsy samples using magic-angle-spinning ^{1}H nuclear magnetic resonance spectroscopy

Detlef Moka [a], Roland Vorreuther [b], Harald Schicha [a], Manfred Spraul [c], Eberhard Humpfer [c], Marion Lipinski [a], Peta J.D. Foxall [d], Jeremy K. Nicholson [d], John C. Lindon [d,*]

[a] *Department of Nuclear Medicine, University of Cologne, D-50924 Cologne, Germany*
[b] *Department of Urology, University of Cologne, D-50924 Cologne, Germany*
[c] *Bruker Analytische Messtechnik, D-76287 Rheinstetten, Silberstreifen, Germany*
[d] *Department of Chemistry, Birkbeck College, University of London, Gordon House, 29 Gordon Square, London WC1H 0PP, UK*

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Abstract

High resolution ^{1}H nuclear magnetic resonance (NMR) spectra using spinning at the magic angle (^{1}H MAS NMR) have been obtained on intact normal and pathological kidney tissue samples from patients undergoing surgery for renal cell carcinoma (RCC). The spectra were measured on ca. 80 mg samples and provided high resolution ^{1}H NMR spectra in which effects of dipolar couplings, chemical shift anisotropy and magnetic susceptibility differences are minimised thus yielding high spectral resolution. Conventional one-dimensional and spin-echo spectra and two-dimensional J-resolved, TOCSY and $^{1}H-^{13}C$ HMQC spectra were also measured on selected samples and these allowed the assignment of resonances of endogenous substances comprising both cytosolic and membrane components. The tumour tissues were characterised principally by an increased lipid content. These are the first reported results on human tumour tissues using this technique and the approach offers potential for the rapid classification of different types of tumour tissue.

Keywords: Biochemical classification; Kidney carcinoma; Nuclear magnetic resonance

1. Introduction

High resolution nuclear magnetic resonance (NMR) spectra from solids and solid-like materials require specialised techniques and most studies on solids have been carried out using low natural abundance nuclei such as ^{13}C. In that case, a combination of cross-polarisation from ^{1}H magnetisation to the less sensitive ^{13}C nuclei and high power ^{1}H decoupling to remove $^{13}C-^{1}H$ dipolar couplings is used. Dipolar couplings are observed between all magnetic nuclei in both the same and different molecules and give rise to very broad

* Corresponding author. Fax: +44 171 3807464.

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NMR signals in solids and solid-like materials. Magic angle spinning (MAS) at 4–15 kHz is then also used to remove the residual asymmetric band shapes caused by chemical shift anisotropy [1]. The magic angle ($\theta = 54.7°$) is that which the rotation axis makes with the magnetic field where dipolar couplings and chemical shift anisotropies, which are scaled by $3(\cos^2\theta - 1)/2$, go to zero. The use of cross polarisation, dipolar decoupling and MAS techniques then allow high resolution ^{13}C NMR spectra to be obtained from solid samples.

To obtain high resolution ^1H spectra from solids, where decoupling of the strong ^1H–^1H dipolar interactions is not possible, it is necessary to resort to more technically difficult methods such as CRAMPS (combined rotation and multiple pulse sequence) [2]. It is known that for materials which are not rigid solids, it is possible to obtain high resolution ^1H NMR spectra simply by using MAS alone at modest speeds. The method works because of the relatively high degree of internal molecular motion of the materials under study and this has the effect of reducing the ^1H–^1H dipolar couplings so that they can be removed by spinning at the magic angle at speeds of about 4 kHz. Also ^1H chemical shift anisotropies are small and are probably only significant for aromatic or olefinic protons and these are also removed easily by the use of MAS. The first applications of high resolution ^1H MAS NMR spectroscopy were to the study of synthetic organic materials attached to resin synthesis beads used in the production of combinatorial chemistry libraries [3].

Recently, advances in high resolution MAS NMR spectroscopy have allowed very small (ca. 25–80 mg, according to the NMR detector used) biological samples to be analysed directly without sample pretreatment and, because MAS eliminates many of the line broadening effects, the resulting NMR spectra comprise sharp, well-resolved lines. Biological applications of this technique to various food products [4,5], lipid micelles [6,7], rat lymphoma samples [8] and to resolution of intracellular and extracellular water in red blood cells [9] have been published.

We have shown that control human kidney tissue samples give rise to well-resolved ^1H MAS NMR spectra [10] and in order to assess the usefulness and potential of MAS NMR spectroscopy as a rapid diagnostic method for human disease, we have examined a series ($n = 10$) of renal cell carcinomas (RCC) and paired non-pathological samples from the same kidney.

2. Experimental

Paired kidney tissue biopsy samples were obtained from ten patients during surgery and stored immediately at $-70°C$ until required for analysis. Samples were thawed and a piece of tissue of approximate weight 80 mg was placed in a 4 mm i.d. zirconia MAS rotor (Bruker, Rheinstetten, Germany) and NMR spectra acquired within 5 min using a Bruker DRX-400 spectrometer operating at 400.13 MHz for ^1H NMR observation. Typically, data were acquired for 256 transients using an acquisition time of 1.37 s into 16 384 data points using either a single pulse experiment with a 30° flip angle or a Carr–Purcell–Meiboom–Gill (CPMG) spin-echo sequence in which the total relaxation delay was 40 ms. The FIDs were multiplied by a linebroadening factor of 1.0 Hz and zero-filled by a factor of 2. The spin rate was typically 4000 Hz. Two-dimensional J-resolved (JRES) MAS NMR spectra were obtained by the acquisition of eight transients per increment for 64 increments using a spectral width of 9980 Hz on the chemical shift axis and 78 Hz on the spin coupling axis. The data were multiplied by sine-bell apodisation functions in both dimensions before Fourier transformation. Two-dimensional total correlation (TOCSY) MAS NMR spectra were measured using 48 transients covering a spectral width of 6410 Hz in both dimensions using the MLEV sequence for the spin-lock with a mixing time of 8 or 16 ms. The data were multiplied by sine-bell-squared apodisation functions in both dimensions before Fourier transformation. Two-dimensional inverse-detected ^1H–^{13}C heteronuclear correlation (HMQC) MAS NMR spectra were obtained using 48 transients per increment for 200 increments into 1024 data

points covering spectral widths of 4990 Hz and 18 100 Hz on the ^1H and ^{13}C axes, respectively with ^1H decoupling by the GARP method [11]. These data were zero-filled by a factor of two on the ^1H axis and to 1024 points on the ^{13}C axis. A shifted sine-bell squared apodisation function was applied in both dimensions. Chemical shifts were referenced internally to the methyl doublet of lactate at $\delta 1.33$ for ^1H and at 20.9 ppm for ^{13}C shifts.

3. Results

A series of CPMG spin-echo ^1H MAS NMR spectra ($\delta 0.5$–$\delta 5.5$) of renal cortical biopsy tissue obtained from two patients immediately after nephrectomy are shown in Fig. 1. Fig. 1A arises from tissue from a normal region from the first patient and Fig. 1C is from a similar sample from the second patient. Fig. 1B,D are from the first and second patients, respectively, and from the same kidneys, obtained at the same time and processed identically, but from regions of RCC. The assignments of the major NMR resonances are given on the figure. These are based on previous assignments in the literature [10,12,13] and through the two-dimensional NMR experiments described below. A typical result such as this can be obtained with ≈ 5 min of analysis time.

The two spectra from normal renal tissues (A and C) are very similar indicating the reproducibility of the technique. These tissues exhibit spectra with resonances mainly from the small molecule cellular components such as amino acids and sugars and there are only minor signals characteristic of the cell wall phospholipids. On the other hand, the samples of carcinoma tissue taken from the same kidneys (B and D) give MAS NMR spectra which are dominated by large signals from lipid components. These are mainly from the fatty acyl sidechains of such lipids which appear to exist at least partially as triglycerides as evidenced by the characteristic signals for the glyceryl fragment at $\delta 4.0$, 4.2 and 5.2. There also appear to be cholesteryl lipids present as deduced from the signal at $\delta 0.7$ which arises from the C18 axial methyl group of cholesterol. It is also seen in the NMR spectrum of blood plasma where it has been assigned to the cholesteryl component of lipoproteins [13]. Previous studies of these types of RCC have only reported in general terms on the increased lipid content [14]. The carcinoma samples also show decreased levels of alanine, valine and lysine.

The assignment of the NMR resonances has been strengthened through the use of two-dimensional MAS NMR spectroscopy. These comprised: (i) MAS JRES NMR spectra in which the spin-coupled multiplets are rotated orthogonal to the chemical shift axis, thereby reducing spectral overlap; (ii) ^1H–^1H MAS TOCSY NMR spectra in which information on those protons which are close together in chemical bond terms is obtained; and (iii) ^1H–^{13}C HMQC MAS NMR spectra in which values for both ^1H and ^{13}C chemical shifts for CH_n groups in the metabolites are determined. These two-dimensional NMR experiments can all be applied to samples undergoing MAS.

The JRES MAS NMR spectrum ($\delta 0.7 - 4.4$) of a typical renal carcinoma biopsy sample is shown in Fig. 2. As the JRES experiment is based on a spin-echo method, most of the signals arise from species with long T_2 relaxation times and hence this approach discriminates against the membrane components in favour of the free solution molecules. For example, the large resonances from fatty acid side-chains of lipid components, visible in the one-dimensional NMR spectra, are absent in the JRES MAS NMR spectrum shown in Fig. 2. Many of the resonances can be assigned by inspection based on previous studies [12,13] and on known cellular cytosolic components. The assignments of the major peaks are marked. Resonances from the amino acids isoleucine, valine, alanine, lysine, asparagine, methionine, phenylalanine, histidine, tyrosine and glutamine are well resolved. In addition, resonances for the acids, lactate, acetate, acetoacetate and 3-hydroxybutyrate are also assigned. Several signals for glucose can be assigned as well as the $N(CH_3)_3^+$ and OCH_2 signals of choline. Due to the JRES experiment discriminating against components with short T_2 values, any differences between normal and carcinoma sample spectra based on the large lipid molecules present in the latter will be min-

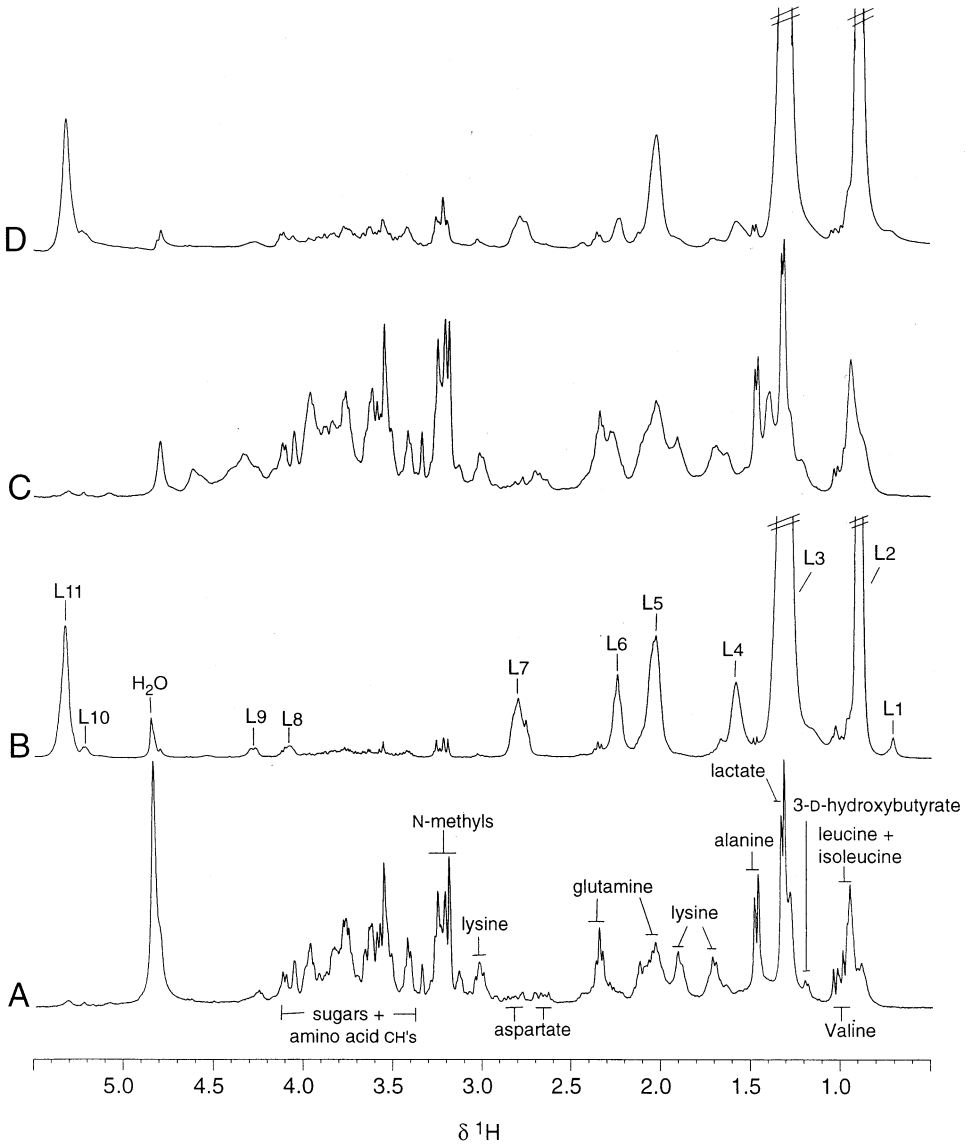

Fig. 1. Partial ($\delta 0.5-5.5$) ^{1}H CPMG MAS NMR spectra of renal cortex biopsy samples. (A,C) normal tissue, (B,D) carcinoma region. Samples (A, C and B, D) are paired specimens taken from each of two patients. Assignments are as marked. L1–L11 arise lipid components as follows: L1—axial C18 methyl of cholesterol; L2—$\mathbf{CH_3}(CH_2)n$; L3—$(\mathbf{CH_2})n$; L4—$\mathbf{CH_2}\cdot CH_2\cdot CO$; L5—$\mathbf{CH_2}\cdot CH{=}$; L6—$CH_2CO$; L7—$=CH\cdot\mathbf{CH_2}\cdot CH{=}$; L8, L9—$CH_2$ of glyceryl; L10—CH of glyceryl; L11=CH.

imised. It is probable that for the particular tumour type studied here, the MAS JRES experiment will have limited diagnostic value and its principal use will be to aid assignment of NMR resonances and hence characterise the biochemial changes associated with the carcinoma tissue.

The ^{1}H–^{1}H TOCSY MAS NMR spectra of the aliphatic region for both normal and carcinoma regions of the same kidney are shown in Fig. 3. In the spectrum of the normal renal tissue a number of resonances can be assigned. These include the amino acids valine lysine, arginine, glutamine,

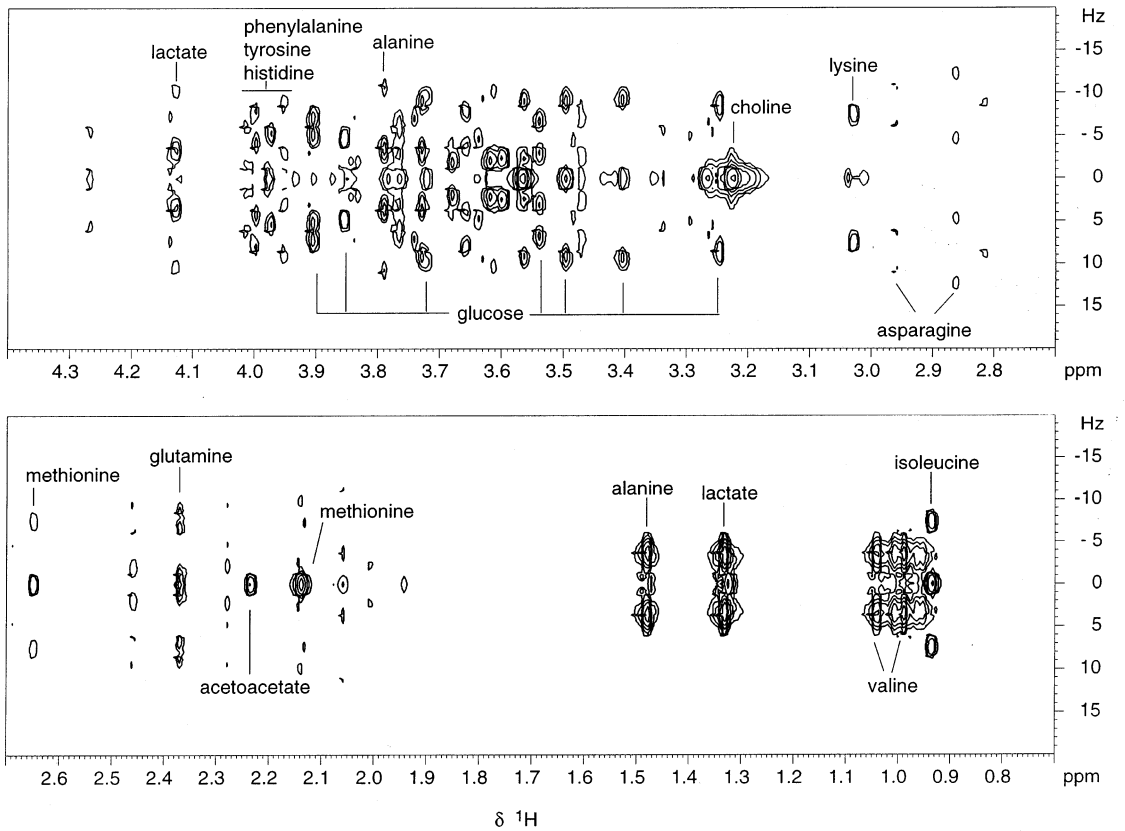

Fig. 2. JRES MAS ^{1}H NMR spectrum of RCC tissue (lower) $\delta 0.7-2.7$, (upper) $\delta 2.7-4.4$. Assignments are as marked.

leucine, isoleucine and the acid lactate. In addition, major cross-peaks now appear for the lipid components, mainly the fatty acyl side chains of phospholipids or triglycerides. Considerable differences are observed between the MAS NMR profiles of the two sample types. The spectra from the carcinoma tissue show strong peaks from lipid components from the cell membranes and reduced levels of some amino acids. Hence, TOCSY MAS NMR spectra provide evidence on both the cytosolic and membrane components of the cells. The assignments have again been based on earlier work on tissues [10], biofluids and tissue extracts [12,13] and are given in Fig. 3. It is therefore likely that TOCSY MAS NMR spectra might be useful in diagnosis especally if the data can be acquired rapidly using magnetic field gradients.

The ^{1}H–^{13}C HMQC MAS NMR spectrum for normal renal tissue is shown in Fig. 4. This experiment, like the TOCSY pulse sequence, provides information on both the small molecule cytosolic components and the macromolecular and supramolecular bodies. The HMQC spectrum of the control tissue has been used to confirm assignment mainly of the lipid moieties [13]. The HMQC MAS NMR spectra of carcinoma tissue samples are similar (not shown) and provide evidence for increased levels of fatty acyl groups.

By comparing all of the ten paired samples, there is a consistent finding of large increases in the lipid components in the tumour samples and these observations agree with the ultramicroscopic-detectable lipid droplets in RCC [14]. As well as major accumulations of lipids in this type of carcinoma, increases in other substances, which

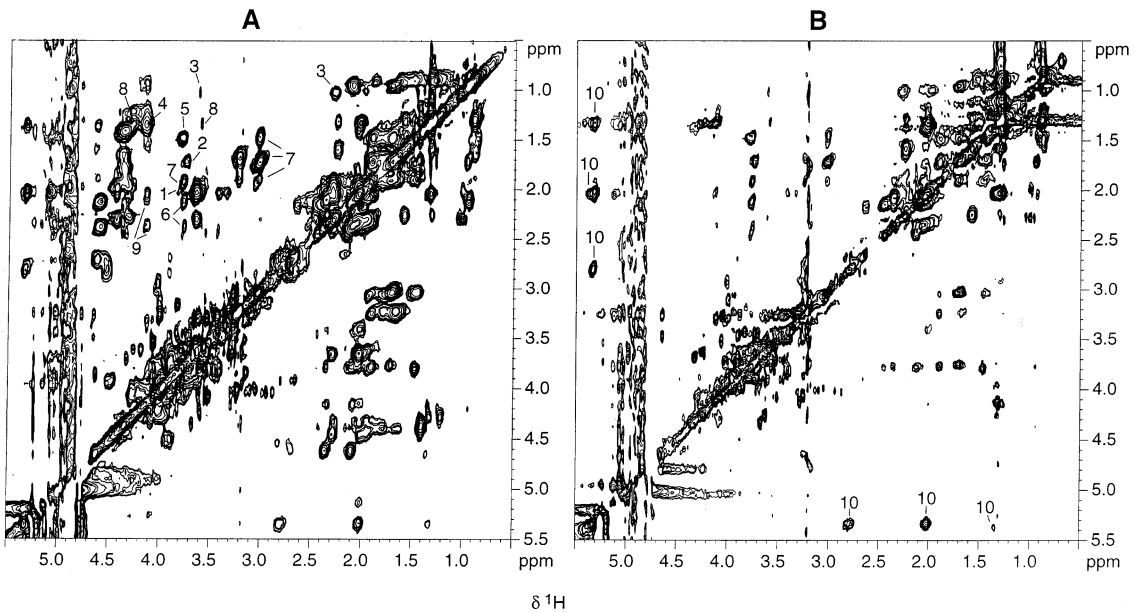

Fig. 3. $^{1}H-^{1}H$ TOCSY MAS NMR spectra ($\delta 0.5-5.5$) of (A) normal renal cortex tissue and (B) RCC tissue taken from the same kidney. Assignments are as follows: 1, isoleucine; 2, leucine; 3, valine; 4, lactate; 5, alanine; 6, glutamine; 7, lysine; 8, threonine; 9, proline; 10, lipid.

are normally only shown by large scale chromatographic or chemical methods, can be detected. These include *N*-acetylneuraminic acid, *N*-acetylglucosamine and various amino acids. The extent of the quantitative and qualitative differences between tumour and normal tissue are significantly greater than those observed with other NMR spectroscopic approaches, either in vivo or in vitro. This, together with the rapidity of analysis, on a timescale required for surgical pathological diagnosis, indicates that high resolution MAS NMR spectroscopy can be of great value as an additional diagnostic tool for tumours and other types of tissue pathology.

There have been a number of attempts to use NMR spectroscopy for diagnostic purposes. NMR spectrocopy of biofluids has proved useful for characterising inborn errors of metabolism [15–18] and when coupled with pattern recognition analysis, it has proved to be an efficient new method of investigating toxicity profiles of xenobiotics [19,20]. Specifically in cancer diagnosis, the first study attempted the classification of blood plasma samples based upon certain peak linewidths [21] and the use of ^{1}H and ^{31}P NMR spectra of tissue extracts [22,23] and ^{31}P NMR spectra obtained in vivo [24,25] have been used extensively to investigate abnormal tissue biochemistry. Results using ^{1}H NMR spectroscopy have proved less useful despite providing biochemical information [26] since spectral quality is often compromised by the high heterogeneity of the sample and relaxation problems due to constrained molecular motions in certain tissue compartments leading to broad lines and poor lineshapes. Within the last 2 years, with the development of high resolution MAS technology, it has become possible to obtain very high quality NMR spectra of whole tissue samples with no sample pre-treatment. This leads to many diagnostic possibilities as information on a variety of metabolites in different cellular environments can be screened rapidly. The preliminary data shown here indicates that diseased tissues have substantially different metabolic profiles to those of healthy tissues.

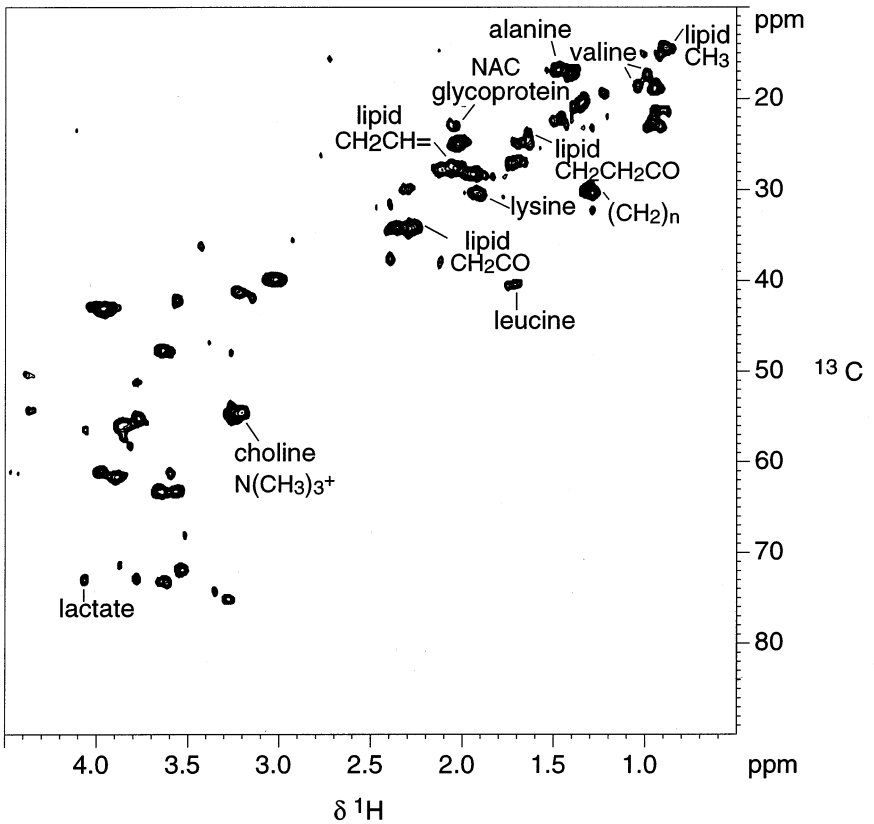

Fig. 4. $^{1}H-^{13}C$ HMQC MAS NMR spectrum of normal renal cortex tissue. Assignments are as marked.

From these preliminary studies, the MAS ^{1}H NMR spectroscopic technology, especially if harnessed to pattern recognition algorithms, offers much scope for studying non-rigid biological samples such as tissues and for producing diagnostic information rapidly.

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