The final publication is available at

http://dx.doi.org/10.1007/s00216-012-6001-z

Additional Information

Authors

Vicent Esteve[1,2], Bernardo Celda[1,2], M.Carmen Martínez-Bisbal[1,2]

Title

Quantitative alterations of metabolite concentration related with tissue features in human brain tumour biopsies evaluated by ^{1}H and ^{31}P HRMAS

 (1) Networking Research Center on Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN) Burjassot, Spain

(2) University of Valencia, Physical-Chemistry, Burjassot, Spain

ABSTRACT

Quantitative multinuclear HRMAS was performed in order to determine the tissue pH and the absolute metabolic concentration of 33 samples of human brain tumour tissue. Metabolite concentration quantification was obtained by 1D ^{1}H and ^{31}P HRMAS using the ERETIC synthetic signal. ^{1}H-^{1}H homonuclear and ^{1}H-^{31}P heteronuclear correlation experiments enabled the direct assessment of the ^{1}H-^{31}P spin systems of those signals overlapped in the ^{1}H 1D spectra, and connected the information among the ^{1}H and ^{31}P 1D spectra. Afterwards, the main histological features were determined and a high heterogeneity in the content of tumour, necrosis and not affected tissue proportions was observed. The metabolic profiles obtained by HRMAS showed the typical characteristics found in tumoral tissues, a depletion of energetic molecules and increment of concentrations of protective metabolites. Nevertheless, these conditions correlated with the total amount of living tissue better than only with tumoral cells content of the samples which could indicate an important contribution of the sampling conditions respect to the tumoral effect developed in vivo. The use of MDPA as a chemical shift and concentration reference for ^{31}P HRMAS spectra of tissues presented important drawbacks due to the interaction with the tissue. Moreover, the pH data obtained from ^{31}P enabled to establish a correlation between the pH and the distance of the $N(CH_3)_3$ signals of Phosphocholine and Choline in ^{1}H spectra in the tissue of these tumour samples.

KEYWORDS

^{1}H and ^{31}P spectroscopy, human tumour biopsies, metabolite concentration quantification

ABBREVIATIONS

HRMAS, high-resolution magic angle spinning; GBM, Glioblastoma Multiforme; PME, phosphomonoesters; PDE, phosphodiesters, PE, phosphoethanolamine; PC phospocholine; GPE glycerophosphorylethanolamine; GPC, glycerophosphocholine; PCr, phosphocreatine; MDPA, methylene diphosphonic acid; Cho, Choline; Cr, Creatine; Ala, Alanine; Gln, Glutamine; Glu, Glutamate; ERETIC, electronic reference to in vivo concentrations; GSH, Glutathione; NAA, N-Acetylaspartate; GABA, γ-aminobutiric acid; Tau, taurine; mI, myo-inositol; Gly, Glycine; FA, fatty acids; NAT, non affected tissue.

INTRODUCTION

High-resolution magic angle spinning (HRMAS) NMR has become in recent years a valuable technique to obtain detailed metabolic profiles of human brain tumours [1-6]. There is a widespread use of ^{1}H 1D spectra to quantify the metabolites [1-3, 6, 7]. 2D ^{1}H -^{1}H homonuclear or ^{1}H -^{13}C heteronuclear experiments have improved the assignments in HRMAS spectra [3-5, 8] but the quantitative metabolic information comes mainly from the ^{1}H 1D spectra. The use of ^{31}P NMR spectroscopy has also been demonstrated to be useful in the metabolomic study of brain cancer since there are ^{31}P-containing metabolites that can provide valuable information complementary to ^{1}H NMR in metabolic studies [9-11]. In this context, ^{31}P NMR spectra can inform about typical membrane components as the phosphomonoesters (PME) phosphoethanolamine (PE) and phospocholine (PC), mobile phosphodiesters (PDE) as glycerophosphorylethanolamine (GPE) and glycerophosphocholine (GPC) and less mobile phospholipids, as well as important energetic metabolism intermediates as phosphocreatine (PCr) and nucleotides-phosphate [9, 10, 12]. Moreover, the pH can be inferred from the changes in the chemical shift of some ^{31}P-metabolites [9, 10, 13]. In vivo, the pH is usually determined using the chemical shift of Pi signal relative to PCr, whose chemical shift is pH-independent in the physiologic range of pH [9, 14]. The in vivo pH$_i$ in brain tumours has been observed slightly more alkaline than that of normal brain [9, 10, 15].

HRMAS allows the study ^{1}H and ^{31}P-metabolites in the tissue and may provide information about the tissue pH. But the number of ^{31}P HRMAS studies of human cancer tissue is scarce. The suitability of this technique to detect ^{31}P metabolites and to determine the pH in tumour tissue has been demonstrated in experimental tumours in mice and mouse brain [16]. In humans, ^{1}H and ^{31}P 1D HRMAS experiments have been used to study the changes in cervical cancer and have showed a higher content of PC and PE in cancer tissue compared to normal tissue [17]. The combined use of ^{1}H and ^{31}P 1D HRMAS experiments has been also reported in human samples of gut biopsies [18] to reveal changes between different tissue microenvironment using 1D ^{1}H and ^{31}P spectra to construct virtual 2D heteronuclear correlation spectra that connect all protons on the molecule to the heteroatom [18]. It has been observed that in the ex vivo ^{31}P spectra of tissues there are no significant signals from the high-energy phosphates (ATP or PCr) due to the rapid metabolism either during the sample preparation or during the spinning [16, 17]. Thus, the use of PCr as chemical shift reference as usually performed in vivo ^{31}P NMR spectroscopy is not possible in ^{31}P-HRMAS experiments, and the changes in chemical shift with pH for several ^{31}P-metabolites make sometimes challenging the assignment of the signals and hence the determination of the tissue pH. This fact motivates the use of ^{31}P reference compounds (as 3-aminopropyl phosphonate [16], phosphono-methyl-glycerol [19], dimethyl phosphonic acid [20] or methylene-diphosphonic acid [16] (MDPA)) for the chemical shift that could be also a reference for the concentration. This might be useful in the NMR studies of liquid extracts since the external reference can be introduced in a capillary in the NMR tube, isolated from the sample. Nevertheless, a similar strategy in HRMAS is limited by the possible interaction between the reference and the sample (both, the tissue sample and the exogenous reference are in the same compartment inside the rotor) which in terms of quantization produces large variation in the measured peak areas for the reference compound [16].

The study of human brain tumour tissue by [1]H 1D and [1]H-decoupled ^{31}P HRMAS and posterior histological study will be here presented, including the evaluation of the pH for each sample and the acquisition of real 2D [1]H-^{1}H homonuclear and [1]H-^{31}P heteronuclear HRMAS experiments for the direct assessment of overlapped signals in the [1]H 1D spectra. Moreover, the use of MDPA as a reference for chemical shift and quantification will be here discussed and the influence in the pH of the biopsy sample. The ERETIC signal as a reference for NMR spectra quantification[21] has been already tested in ^{1}H spectra of human tissues either using either an external electronically generated reference [7, 22] or using a synthetic signal [23], but the use of the ERETIC signal has never been tested to evaluate ^{31}P metabolite concentrations in tissues. The use of ERETIC synthetic signal [23] will be here proposed as a reference to quantify the metabolites in ^{1}H and also in ^{31}P spectra of tissues. The quantitative information of ^{1}H and [31]P 1D will enable, on one hand confirm the robustness of the ERETIC method comparing the calculated concentration for those metabolites whose signals can be properly integrated in both ^{1}H and [31]P 1D experiments, and on the other hand, will complete the quantitative information for those metabolites whose signals are challenging to integrate in ^{1}H and can be properly integrated in ^{31}P as Phosphoethanolamine and Glycerophosphorylethanolamine. The correlation among the metabolites and between the metabolites, the pH and the histological features will be also discussed. Finally, preliminary considerations of slight changes in ^{1}H chemical shift with the pH observed in these spectra will be also presented.

EXPERIMENTAL:

This study involves the use of human brain tumour biopsy samples. It was reviewed and approved by the ethics committee of the Hospital de la Ribera, Alzira, Spain. Thirty-three patients underwent surgical resection of a brain tumour. After surgery, part of the resected tissue was sent for routine histological analysis and the remaining was stored at -80ºC until the time of spectroscopic analysis. In the histological analysis 27 tumours were identified as glial tumours and 6 as non glial tumours. The glial tumours group included 19 grade IV glioma (GBM), 2 grade III gliomas (1 anaplastic astrocytoma, and 1 anaplastic oligodendroastrocytoma), 2 grade II gliomas (1 oligodendroglioma and 1 oligoastrocytoma) and 4 grade I astrocytoma. Non glial tumours included 2 metastases (1 bowel adenocarcinoma, and 1 breast cancer), 3 meningeomas and one teratoma grade III. The Table S1 (see Electronic Supplementary Material) shows a list of the samples and the tumour type according to the histology classification for tumour diagnosis.

HRMAS of tissue, sample preparation and spectra acquisition

The samples were studied by HRMAS inside 4mm/50μL ZrO_2 rotors in a 11.7 T magnet (Bruker Avance DRX 500 spectrometer) operating at 500.13 MHz for ^{1}H and at 202 MHz for [31]P. The instrument was equipped with a 4 mm triple resonance ^{1}H/^{31}P/^{13}C HRMAS probe. The amount of tumour tissue studied for each patient ranged from 8.0 to 34.1 mg. The Table 1 shows the weight average and standard deviation for each group of tumours. The Table S1 (see Electronic Supplementary Material) shows in detail the weight for each one of the samples. The samples were introduced in the rotor without flushing, thus avoiding the leakage of metabolites reported in the washing of tissues [24, 25]. In 14 samples, 10 μL of methylene diphosphonic acid (MDPA) 10 mM in D_2O were added. In all the samples (33 samples) the volume of the rotor was completed with cold D_2O as a field frequency lock. The samples were spun at 4000 Hz in order to keep the rotation sidebands out of the acquisition window. A Bruker cooling unit was used to control the temperature by cooling down the bearing air flowing into the probe. In all the

3

experiments, the sample temperature was 6ºC as determined by internal measurement using a 100% methanol sample in a 4mm/50μL ZrO_2 rotor spinning at the same frequency. The shimming was optimized checking the quality of the signals and the resolution of the alanine (Ala) doublet (doublet from CH_3 protons at 1.465 ppm) in the ^{1}H spectra, present in all the samples analyzed.

For each sample, one-dimensional (1D) ^{1}H spectra with presaturation in the water signal (from now on ^{1}H spectra) and 1D [1]H-decoupled ^{31}P HRMAS spectra (from now on ^{31}P spectra) were acquired. Water presaturation ^{1}H spectra using a 1.36 s acquisition time, 256 transients, a 6000 Hz spectral width, and a relaxation delay of 1 s were acquired in 10 min. [1]H-decoupled ^{31}P spectra were acquired in 1 hour, using a 1.61 s acquisition time, 1024 transients, a 10162 Hz spectral width, a relaxation delay of 2 s and proton decoupling.

Additional [1]H-[1]H 2D homonuclear spectra Hartman-Hahn transfer using DIPSI2 sequence for mixing (from now on TOCSY spectrum) was acquired in 22 samples in order to identify the spin systems and to assess the ^{1}H assignments [26]. The TOCSY spectra were obtained in 2 hours, with 2 s water presaturation, 16 transients. The TOCSY mixing time was 50 ms. [1]H-[31]P 2D heteronuclear correlation experiments (from now on ^{1}H-^{31}P HSQC spectra) were also acquired in 24 of the samples. These experiments were acquired with double inept transfer and TPPI for phase-sensitive detection using a 500 ms acquisition time with decoupling and a 2 s relaxation delay. A total of 128 transients were averaged for each of 128 increments during t1, corresponding to a total acquisition time of 13 h.

HRMAS spectra processing

The ^{1}H spectra were processed with 2-Hz exponential apodisation, Fourier transformation and phase correction. The ^{31}P spectra were processed with 3-Hz exponential apodisation, Fourier transformation and phase correction. TOCSY and ^{1}H ^{31}P HSQC spectra were 2D transformed, phase and baseline corrected. In order to compare the metabolic content in the different samples and to obtain absolute concentration values of ^{1}H and ^{31}P metabolites, a synthetic digital ERETIC signal was added in the last step of processing of the ^{1}H and ^{31}P spectra at 10 ppm [23]. Finally, the baseline of the spectra was corrected using ABios program [27].

Standard solution for quantification and referencing

To establish the value of the synthetic signal of ERETIC for ^{1}H and ^{31}P spectra and to assess the assignment of ^{31}P signals and pH calculation, 2 solutions 20 mM in PCr and 12 mM in PC were prepared with pH 6.34 and 7.19. The PCr started to decompose in Cr and Pi in some extent before NMR measurement, so the solutions contained PC 12 mM, PCr and Pi, with [PCr+Pi] = 20 mM. The same experimental conditions of probe, rotor, spinning and temperature used for the tissue studies were applied for these standard solution samples at these 2 pH.

HRMAS spectra referencing

The chemical shifts in ^{1}H spectra were referenced to the Creatine (Cr) CH_3 singlet at 3.027 ppm and to the Ala CH_3 doublet at 1.465 ppm. The chemical shifts in the ^{31}P spectra of tissue were referenced to the GPC peak at 0.48 ppm, when detectable. The ^{31}P chemical shift of this compound is independent of the pH [14], thus, using this reference, the same offset was applied for all the ^{31}P spectra. This strategy was

checked with all the spectra that contained GPC. The ^1H and ^{31}P spectra in the Figure 1 show the assignments for the metabolites studied in the tissues for three samples with different kind of tissue.

The chemical shifts in the TOCSY spectra were referenced to the Ala CH_3/CH crosspeak at 1.47-3.77 ppm. The chemical shifts in the ^1H-^{31}P HSQC spectra were referenced to the GPC CH_2/P crosspeak at 4.32-0.48. This CH_2 is the closest CH_2 to the Phosphate group in the choline moiety of GPC molecule. The assignment of the homo and heteronuclear crosspeaks of GPC, GPE, PC and PE are displayed in the Figure 2, together with the structure of each of these molecules.

Spectra quantification and analysis

The ^1H and ^{31}P spectra were analysed with ABios [27]. Then, the peaks were finally assigned accounting for the deviation in chemical shifts and peaks widths. The resonances for the most common metabolites, lipids and macromolecules, and for ERETIC synthetic signal were integrated adjusting them to a Gaussian or Lorentzian function and the overlapping was corrected by using a least square minimization algorithm incorporated in the ABios program [27].

Histology post HRMAS

In order to determine the proportion of tumour %Tum, necrosis %Nec or normal appearing tissue %NAT the samples underwent histological analysis after the HRMAS experiments. The samples were immersed in 10% formalin. Once fixed, the tissue specimens were embedded in paraffin and the tissue pieces were serially sectioned in slices of 5 μm and H & E stained. The image analysis and measurements were carried out using ImageJ software [28]. The Figure 1 shows the slices of three biopsy sample tumours with different proportion of Tum, Nec and NAT (sample 1: 4% Tum, 96.0% Nec, 0.0% NAT, sample 7: 94.2% Tum, 5.8% Nec, 0.0% NAT, sample 10: 46.1% Tum, 1.0% Nec, 52.8% NAT) together with the ^1H and ^{31}P spectra. The Table 1 shows the average and the standard deviation of the content of Tum, Nec and NAT for each group of tumours. The histological data for each sample is detailed in Table S1 (see Electronic Supplementary Material).

Statistics

ANOVA analysis and correlation among metabolite concentration, pH and histological data was performed using ABios [27] which implemented MATLAB statistical libraries.

RESULTS

^1H and ^{31}P spectra assignation and pH calculation

The metabolites in ^1H spectra were assigned using the data in the literature [4, 29, 30] and the TOCSY and ^1H-^{31}P HSQC spectra acquired for the same samples. The metabolites identified in the ^1H spectra were GPC, PC, choline (Cho), Cr, lactate (Lac), Ala, Glutamate (Glu), Glutamine (Gln), Glutathione (GSH), N-Acetylaspartate (NAA), γ-aminobutiric acid (GABA), taurine (Tau), myo-inositol (mI) and Glycine (Gly) in the aliphatic region. Between 6 and 10 ppm the signals were assigned to the NH_2 groups of Gln, the NH group of Cr and to the resonances H1' of ribose, and the protons H2 and H8 in the ring of adenine in adenosine-3-phosphate (Ade). The assignments of adenosine-3-phosphate were confirmed by comparison with the spectra of the same compound downloaded from BMRB [31]. The broad resonances

of macromolecules and fatty acids in ^{1}H spectra were assigned using the data in the literature [32-35]. The ^{1}H assignments for the metabolites and FA are shown in the Figure 1.

In the ^{31}P spectra, once assigned GPC signal at 0.48 ppm, the pH was calculated using the chemical shift distance between the P_i signal and the GPC peak [14, 16]. According to this, the calculated pH of each sample is shown in the Table S1 (see Electronic Supplementary Material). The pH average and standard deviation for each group of tumours is shown in the Table 1. Once estimated the pH, the expected ^{31}P chemical shifts of PE, PC and GPE were calculated for each sample. Then, the signals in the ^{31}P spectra were assigned using these calculated chemical shifts. The ^{31}P spectra assignments of metabolites are shown in the Figure 1.

This strategy for pH and chemical shift calculation in ^{31}P spectra was tested in the standard solutions with PC, PCr and Pi, prepared at pH 7.19 and 6.34. The pH for each solution was calculated using the ppm distance between Pi and PCr [36], and the chemical shifts of PCr and PC were calculated depending on the pH, following the same procedures used for the tissue samples [14]. According to the pH determined by the electrode, the pH calculated using the chemical shifts showed an error lower than 5% (7.24 calculated for pH=7.19, error 0.7%, 6.77 calculated for pH=6.34, error 4.7%), validating thus this procedure to calculate the pH and the ^{31}P chemical shifts.

In the tissue spectra, the signals of PE and GPE clearly observed in some of the ^{31}P spectra were not always evident in the ^{1}H spectra for the same samples, due to the overlapping of the signals in this crowded region of the spectra. TOCSY and ^{1}H-^{31}P HSQC spectra were especially useful to assess the presence of PE and GPE in ^{1}H spectra given the presence of these signals in ^{31}P spectra. Moreover, ^{1}H-^{31}P HSQC helped to assess the ^{1}H and ^{31}P spectra assignments and to link the information from ^{1}H and ^{31}P spectra, combining the information of both nuclei for the same metabolite in the tissue. The path for the assignment of 'hidden' signals in the ^{1}H spectra is shown in the Figure 2 for GPC, GPE, PC and GPE.

Evaluation of MDPA signal

The signal of MDPA in the ^{31}P spectra showed a remarkable variation not only in the chemical shift but also the in the intensity and in the width. The theoretical concentration of MDPA introduced in each rotor was $1x10^{-7}$ mol. Given that the volume inside the rotor was 50 μL, the expected concentration of MDPA was 2.00 μM. The average experimental concentration calculated for MDPA in the samples was 1.98 ± 0.31 μM, ranging from 1.35 μM (sample 5) to 2.49 μM (sample 2) as shown in the Table S2A (see Electronic Supplementary Material). Despite the fact that the average concentration of MDPA was very close to the expected value, these data clearly showed a high deviation to consider MDPA as a concentration reference. The changes in the concentration of MDPA did not show any correlation with the pH or the mass of the tissue inside the rotor, but there was a significant correlation (-0.59 p = 0.032) between the detected concentration of MDPA and the amount of tumour (% of tumour x sample weight). Regarding to the chemical shift of MDPA, the average chemical shift of this signal was 16.63 ± 0.05 ppm ranging from 16.55 ppm for the sample 9 to 16.72 ppm for the sample 8 (data not shown). The changes in the chemical shift of MDPA showed a strong correlation with the pH (0.94 p < 0.001). The width of the peak of MDPA ranged between 7.12 Hz (sample 24) and 11.66 Hz (sample 5) with an average value of 9.72 ± 1.31 Hz (data not shown). The changes in the width were highly correlated with the pH (0.57 p = 0.035) and also with the mass of the tissue sample inside the rotor (0.68 p=0.007).

Histological features after HRMAS

The areas of tumour content, necrosis and not apparently tumour were identified in the tissue slices, and were quantified yielding a final percentage of each of them per sample. A broad distribution of tumor and necrotic areas was found in the histological inspection, as seen in the data of % of viable tumor content (%Tum), necrosis (%Nec) and not apparently tumor (%NAT) in the Table 1for each tumor type (the data for each sample is shown in the Table S1 in the Electronic Supplementary Material).

Evaluation of metabolites concentration

The digital ERETIC signal equivalence in number of mol was calculated for ^{1}H and ^{31}P spectra according to the signal intensity in the solution 12 mM in PC at pH 7.19. According to this, the integral of the tissue metabolites in ^{1}H and ^{31}P spectra was translated into number of micro moles per gram of tissue (μmol/g) as a measure of absolute concentration for the comparison among the different samples, accounting for the number of ^{1}H or ^{31}P atoms integrated in each resonance. The average concentration in each tumour type is shown in the Table 2 for each metabolite. The calculated concentrations for each sample are shown in Table S2A (see Electronic Supplementary Material). For some metabolites (as Cr, Lac, Glu, Gln and Ade) more than one signal was assigned and hence integrated. As a proof of the robustness of the ERETIC digital synthetic signal calibration for ^{1}H and ^{31}P spectra, the correlation between the intensities of the different resonances for the same metabolite was very high, as it is shown in the Figure 3 for the CH$_3$ and CH$_2$ peaks in the Cr (0.95 p < 0.001). These correlations have been found when all nearby overlapped peaks have been fitted in the integration as shown in the Figure 3 for the Cr CH$_2$ resonance. Therefore, given the equivalence between all the signals in one metabolite, only one signal per metabolite was included in the Table 2.

The broad peaks corresponding to fatty acids (FA) were integrated and the tabulated values reflect an estimation of the number of protons implied in each peak (for FA integration in each sample see Table S2B in Electronic Supplementary Material). The average intensity of each FA resonance for the different tumour types is shown in the Table 2.

The agreement between ^{31}P and ^{1}H spectra concentration estimations was checked for GPC and PC, which showed correlation coefficients of 0.81 (p < 0.001) for GPC and 0.94 (p < 0.001) for PC in the whole set of tumours (see the Figure 3).

Changes in the concentration of metabolites, the pH and the histological content

A great variation in the metabolite concentrations was observed over the whole set of samples (the coefficient of variation was always greater than 50%). The lowest variations were found for P$_i$, Lac and Tau. The metabolites with the highest variations were FA, NAA and GPE, followed by GABA, GPC and GSH. FA labelled with number 4 in the Tables 2, and S2B (-**CH$_2$**-CH=CH-) showed a variation much lower than the other FA signals, probably due to other contributing signals at the same chemical shift.

The correlations among the metabolite concentrations, the pH and the histological data post-HRMAS with p<0.01 are shown in the Table 3 for the whole set of tumours (for the complete set of significant correlations with r coefficients and p values lower than 0.05 see Table 3A in Electronic Supplementary Material) . The concentrations of NAA, Cr, GABA, GPE, GPC, Glu and Ade were positively correlated among them. The concentrations of NAA, GPE, GABA, Cr (p<0.01) and Glu (p<0.05) were highly

correlated to the %NAT. The concentrations of the group of fatty acids correlated highly among them, as it was expected (for the complete set of significant correlations with r coefficients and p values lower than 0.05 for fatty acids see Table 3B in Electronic Supplementary Material). Moreover, there was a positive correlation of fatty acids with the %Nec ($p<0.01$) and negative with the %Tum (see data in the Table 3B in Electronic Supplementary Material). On the contrary, Gln showed a positive correlation with %Tum (0.42 $p<0.05$) and a negative correlation with the %Nec (-0.49 $p<0.01$). The %Nec correlated negatively with Cr, Gln, GABA, Ade ($p<0.01$), and GPC ($p<0.05$) and positively only with the fatty acids ($p<0.01$).

The pH showed a negative correlation with the concentrations of Ade, Cr, NAA and GPE ($p<0.01$). No correlation was observed between the pH and the histological features in this group.

When the analysis was restricted to the GBM group the map of significant correlations was modified. The Table 4 shows the correlations among the metabolite concentrations, the pH and the histological data post-HRMAS with $p<0.01$ in the GBM group. The number of correlations between the tissue features and the metabolites was increased (see Table S4 in the Electronic Supplementary Material for the complete set of significant correlations with r coefficients and p values lower than 0.05). For instance, the %Tum for the whole group of biopsies correlated significantly only with Gln (0.42 $p<0.05$). However, restricting to the GBM group the %Tum correlated also positively with PC, GSH and mI (moreover Gln) ($p<0.05$), and negatively with FA ($p<0.05$). Likewise, the %Nec that was correlated negatively with Cr, Gln, GABA, Ade ($p<0.01$), and GPC ($p<0.05$) for all the biopsy samples, showed a general increment in the number of correlations for the GBM group and additional negative correlations aroused enclosing the intensity of PC, Lac, Glu, GSH, and mI ($p<0.05$) (see Table S4 in Electronic Supplementary Material). However, the %NAT lost the correlation with Glu, but it kept the correlation with GPE, Cr, NAA and GABA, and showed additionally significant positive correlation with GPC and Lac. The correlations of FA were in general improved. Regarding to the correlations among the metabolites, PE only kept the correlations with Pi and Gln, which were improved. Cho showed only significant correlation with Ala and Tau. GPC lost the correlation with Gln and GSH but increased the correlation with Lac, Cr, NAA, GABA and Ade. The Cr improved all the correlations (GPE, GPC, Lac, Glu, NAA, GABA, mI and Ade) but the correlations with Pi disappeared (see the Table 4 and the Table S4 in Electronic Supplementary Material). The pH showed a negative correlation with the concentrations of GPE, GPC, Lac, Ade, NAA, GABA and Cr and with the sum of %NAT and %Tum, but there was a positive correlation with %Nec. The whole map of significant correlations (r and p values) among the metabolites, the pH and the histological data post-HRMAS are shown in Table 4S for the GBM group.

No significant correlation was found between the grade of gliomas and the concentration of metabolites but the number of tumour samples other than GBM was too small to be considered. Nevertheless, slight differences between the means of low and high grade distributions were observed for some metabolites such as GPC and Cho/Cr ratio that were completely lost when the content of tumor or necrosis where considered (weighting by the corresponding percent values).

Additionally a series of ^{1}H spectra were recorded covering a time interval of several hours and checked for progressive changes in the metabolic contents. The concentration of some metabolites as Lac, Cr or Tau remained almost constant for several hours. Other metabolites as Ala, mI, Cho, GABA, Asp and Glu slowly rose, whereas others like GPC, NAA, Gln and Ade had the opposite tendency, slowly decreasing with time.

Changes in the ^{1}H chemical shift with pH

The average calculated pH values derived from ^{31}P chemical shift changes for each tumour type are gathered in the Table 1 (see the calculated pH value for each sample in the Table S1 in Electronic Supplementary Material). The assignment of the signals in ^{1}H showed slight changes for some metabolites (data not shown). Looking for some parallelism that can provide us with an estimation for the pH values based in the chemical shift change of these metabolites in ^{1}H spectra, a significant ($p < 0.001$) correlation of 0.86 between pH values and the difference in chemical shift of PC and Cho was found (Figure 4). A much lower correlation (0.41 $p = 0.02$) was found for the difference in ^{1}H chemical shift between GPC and PC and not significant correlation between GPC and Cho was observed. Also, not correlation was found in general with the absolute chemical shift value of PC, but a correlation of 0.90 ($p < 0.0001$) was found between pH and the absolute chemical shift of PC within the group of samples with MDPA.

Possible effect of MDPA

A significant ($p < 0.01$) drop of pH (-0.5 units) was found in the group of samples with MDPA compared to the rest of the samples prepared without MDPA. This decrease was observed even when MDPA was used at concentration as low as 2 μM, which is in the order of the concentration usually found in the literature [16].

Considering only those samples without MDPA, a correlation of 0.59 ($p < 0.01$) was found between the concentration of H^+ (calculated as 10^{-pH}) and the total content (number of moles) of Lac. When Lac and Glu amounts were added the correlation increased to 0.65 ($p < 0.01$). When all the samples were considered (with and without MDPA) these correlations were lost. Nevertheless, when the samples with the lowest pH (9, 12 and 24 in the Table 1) were removed from the analysis, there was a significant correlation of H^+ concentration and Lac (0.49 $p < 0.01$) and also for the sum of Lac and Glu (0.51 $p < 0.01$) lower than before. For MDPA + Lac + Glu the correlation with H^+ improves to 0.56 ($p < 0.01$).

No correlation was found between the pH values and the extension of necrosis or tumour content. The same can be said regarding to the tumoral type or grade in the gliomas group. Nevertheless, when only the samples without MDPA were considered, the values of pH showed a significant correlation with the values of %Tum (correlation of -0.74 $p<0.001$) and %Tum + %NAT (correlation -0.79 $p<0.001$).

DISCUSSION

The study of tissues by HRMAS can be considered an intermediate step between the in vivo study of the patient and the in vitro study of the extract of metabolites from the excised tissue [37]. The use of HRMAS improves the resolution, allowing the detection and quantification of a great number of metabolites and FA [1-4], but sometimes the 1D ^{1}H spectra are too crowded to allow an unequivocal identification of some overlapped resonances.

As it is well known, one advantage in ^{31}P spectra is the dispersion of the signals, greater than in ^{1}H spectra (as seen in the Figure 1 and the Figure 2). This enables, among other possibilities, the independent evaluation of PE and GPE with relevant biochemical information, and whose signals in the ^{1}H spectra are multiplets in areas crowded by signals from other metabolites, usually more intense, and are difficult to

9

detect only with 1D ^{1}H spectra [38]. Moreover, in this study, the acquisition of 2D ^{1}H-^{1}H TOCSY and ^{1}H-^{31}P HSQC correlation spectra has enabled the identification of the spin systems for the main metabolites in ^{31}P spectra and has confirmed the assignments with the homonuclear and the heteronuclear correlations. Previously it had been demonstrated that the metabolic information could be improved by the combination of 1D ^{1}H and ^{31}P spectra, constructing afterwards a kind of virtual 2D heteronuclear correlation [18, 39]. This 1D heteronuclear strategy has been applied in human to the study of gut biopsies to detect different tissue microenvironments [18] and in rat liver for the detection of drug-induced hepatotoxicity [39]. The study here presented, has shown the feasibility of ^{1}H-^{31}P HSQC spectra acquisition on human brain tumour tissue with signal to noise ratio adequate to observe the signals from the main metabolites identified in the ^{31}P 1D spectra, as shown in the Figure 2. The performance of ^{1}H-^{31}P HSQC spectra on these tissues has been very useful to directly and unequivocally confirm the ^{31}P assignments with the ^{1}H connected, following the whole set of ^{1}H and ^{31}P resonances in both ^{1}H-^{31}P HSQC and ^{1}H-^{1}H TOCSY, regardless of the pH which makes challenging the assignments in ^{1}D ^{31}P spectra. The ^{1}H-^{1}H TOCSY crosspeaks have contributed to confirm those resonances observed in the ^{31}P spectra but which are not properly observed in the 1D ^{1}H spectra since these 1D spectra are usually very crowded, as shown in the Figure 2.

Additionally, a remarkable advantage of the ^{31}P spectra is that allow an estimation of the pH inside the tissue samples, that relies in the change in the chemical shift of some phosphorylated metabolites present in the living tissues. Unfortunately, some of them, as for example PCr, are very labile and cannot be found in the samples from excised tissues [16]. In this work, GPC has been used as an alternative metabolite usually present in this kind of samples, since its chemical shift in ^{31}P spectra remains invariant with the pH. Furthermore, solutions of known concentration and pH have allowed here to crosscheck the consistency of the pH measurements.

The use of MDPA as a standard compound for reference in ^{31}P NMR studies in tumour tissue has been studied here and has shown some drawbacks that precluded its use as seen in this study and in the bibliography [16]. The MDPA signal should have been invariant for all the samples but, on the contrary, it showed changes in the intensity, the chemical shift and the width. The changes in the intensity and in the width indicated that the MDPA might be interacting with the sample since on one hand, the higher the quantity of tumour tissue sample was inside the rotor, the lower the amount of MDPA was detected, and on the other hand, the higher was the mass of the sample, the wider was the signal of MDPA. The experimental procedure of sample preparation might have introduced some minor error in the quantification since some small but undetermined quantity of the reference might have escaped from the rotor if the preparation was not performed carefully. However, the data in this study mainly points to the interaction between the MDPA and the sample, since both, the intensity and the width changed through the different samples studied with MDPA. According to this, the addition of MDPA as a concentration reference for tissue samples would not be convenient. Furthermore, the signal of MDPA also showed changes in the chemical shift and in the width related with the pH which makes it unsuitable as an absolute reference for chemical shift. In already published data a variability in the intensity of MDPA inside the rotor was detected [16] but the chemical shift of MDPA was assumed invariant. In fact, according to the data in the reference [16], the pH variability of these grown tumours was ± 0.09 pH units, which is not supposed to shift the MDPA signal in the whole set of samples. The samples on the

study of Payne et al. [16] were 15 samples from the same kind of homogeneous tumour and with little necrosis and few cystic regions and the authors did not mention pH changes along the series. On the contrary, in our study the 15 samples studied with MDPA were samples from real patients, mainly GBM (14/15), the highest glioma grade inherently heterogeneous [40], with very variable pH and proportions of tumour, necrosis and normal appearing tissue, as determined in the histological study whose results are shown in the Table 1 and Table S1. Finally, it deserves to be mentioned the effect of MDPA in the value of pH itself, as deduced from the changes in the pH (-0.5 pH units) in the samples with MDPA, compared to the samples without MDPA, and from the improvement of correlation of the pH with the concentration of metabolites that could account for the total concentration of H^+.

All these drawbacks for the MDPA prevent the use of standard compounds as a concentration reference, and suggest the use of other strategies, as ERETIC digital synthetic signal, that can be added in the post-processing of the spectra and that only requires the calibration with the data from a standard solution, acquired with the same experimental conditions and with the same sequence. This strategy had been previously presented in the study of human ovarian epithelial carcinomas tissue [23] with 1D ^1H Carr-Purcell-Meiboom-Gill pulse experiments. The electronically generated signal of ERETIC had already been presented in the quantification of 1D ^1H spectra of human brain tissues [7]. The results obtained using ERETIC digital synthetic signal clearly show that it can successfully be applied to the quantification of 1D ^{31}P and ^1H spectra of human brain tumour tissues. The high correlation observed between the quantification by ^1H and ^{31}P spectra, as shown in the Figure 3 and in the Table 3 for PC and GPC validates the use of ERETIC digital synthetic signal also for the ^{31}P spectra in tissues.

The pH has been a matter of interest in the study of biological samples since it is part of the physiological information that can be obtained from the tissue and can help in the characterization of a normal or a pathological state. The study of the pH in tissues in vivo or ex vivo has been usually relied upon ^{31}P NMR experiments, but the same metabolites that undergo changes in chemical shift in the ^{31}P resonances, may show changes in ^1H resonances near to this group or other resonances close to labile protons. There are several compounds seen in the ^1H spectra whose chemical shifts has been shown to change with pH (Cr, Gly, Acetate) [41] but the range in which these changes occur is out of the pH interval seen in the samples here studied. Previous studies on human brain tumour biopsies already reported slight changes on the chemical shift of metabolites in ^1H 1D spectra, For instances, the chemical shift of Gln was possibly related with the pH [2] but did not show any consistent pattern between or within the tumor types. Histidine has shown changes in the chemical shift of H4 and H2 [41, 42] that occur in the interval of pH observed in the samples here studied, but unfortunately, Histidine has not been detected among the metabolites here assigned. Several studies have dealt with the pH and ^1H spectra, either from the point of view of assessment of the pH [41] or with the objective of searching the pH in which the chemical shifts of the metabolites may be invariant [43]. In the first case, as before mentioned, the range of pH closer to the pH in these samples could be determined using the chemical shift of Histidine. The changes of δ PC vs Cho at pH interval between 5 and 7.5 observed in this study are in agreement with the changes in δ of PC in the ^{31}P and also with the pKa of PC that is 5.76 [14]. GPC, PC and Cho ^1H $N(CH_3)_3$ resonances did not change vs TSP at pH interval between 7 and 10 [43]. Nevertheless, the data here presented are in a pH interval closer to pKa of PC. The changes observed here are very slight, probably due to the relatively long distance between $N(CH_3)_3$ protons and the phosphate which undergoes protonation changes with the

pH. This is indeed a weak correlation for precise pH determination but it could provide a broad estimation, at least about if the sample is "better neutral" or "better acid". Another issue to be considered is the effect in the chemical shift of a metabolite inside of the tissue. It has been recently demonstrated tissue-specific differences between frequency distances of water and metabolites [44] comparing gray matter and white matter.

The number of tumours here studied with different histological grading is too reduced to found a statistical reliable correlation between the tumour grade and the metabolic changes detected. Nevertheless, the essential histological features of %Nec, %Tum and %NAT have been determined and several important correlations among the concentration of different metabolites and the histology data have been found.

Noteworthy, significant correlations have been found between %Tum and Gln, PE+PC, FA, Glu/Gln, and Cho/Cr. The fact that we obtained a higher correlation with GPC, PC, Cr, Gln, Ade, when it was considered the sum %Tum + %NAT suggested that the correlations observed with the %Tum sometimes may be due to the presence of viable cells (either tumour cells or normal cells, and after suffering some degree of damage) and not specific of the tumoral ones. It seems that a general agreement was found through a positive correlation between energetic metabolism intermediates and the %Tum + %NAT content as well as negative correlation with %Nec. The reverse situation was found respect to FA concentration, something commonly observed in the literature [35]. Actually, the proportions of these features are the responsible for the final tumour grade classification [40].

The absence of correlations between Lac or pH and %Tum led us to consider them as a consequence mainly of the anoxic conditions suffered after removal of the tissue sample. However, it is quite difficult to establish the contribution of the tumor effect in these parameters [45]. It is expected a combined contribution from both processes, the anoxic and the tumour. The observed correlation between the pH, in the group without MDPA, with %Tum + %NAT seems to indicate that the pH is mostly related to the total cell content under anoxic conditions more than to only tumour content.

Lac showed a high correlation with Ala and Tau, maybe because the period of anoxic metabolism. Moreover, Tau showed correlation with GSH, both of them are perhaps also related with protective properties (they have in common an antioxidant effect, and additionally Tau could be implied in the stabilization of the membrane and the inhibition of neurotransmission). PC showed correlation with P_i and GSH.

For the GBM subset of samples similar results were obtained but the correlations observed were increased. New positive correlations with %Tum appear for GSH, PC and mI. A negatively correlation was observed with GSH and %Nec (these could be the first effects of tissue deterioration due to tumor effect). There was observed a clear correlation among GPE, GPC, Cr, NAA and GABA. All of them are clearly connected with the content of non-affected tissue.

There was an evident correlation between %Nec and the decrease of some metabolites, particularly Cr but also with the decrease of Gln, GPC, GABA and Ade, and the raising in the concentration of signals from FA. When only GBM samples were considered the correlations were improved, and the set of metabolites which showed significant descent with %Nec included also PC, Lac, Glu, GSH and mI. This variation, in spite of showing a significant correlation, is not exactly linear: a broad lowering of Cr, Glu, GABA,

NAA, mI, and other metabolites was observed when %Nec increased. This is probably connected with a general decay in the tissue sample. Nevertheless, a low percentage of necrotic areas does not necessarily imply a high concentration of these metabolites which suggests that the deterioration in the cells is not entirely due to extensive necrosis, but also apoptosis or other cell deterioration processes that can take place. On one hand, changes in the FA signals have been observed following apoptosis, necrosis or lipid droplet formation [35, 46-48]. On the other hand it has been observed in vivo that MRS-visible lipids accumulate with apoptosis [49], suggesting that these signals of FA may serve as a surrogate marker for apoptosis detected in vivo by ^{1}H MRS[50].

There are different ways for tissue deterioration and it is difficult knowing which one is the most important and when it occurs. It could happen in vivo under the tumor growth effects, ex vivo during excision and freezing or during the NMR experiments. The series of HRMAS experiments acquired at 6ºC revealed that no appreciable variation of the concentration was observed in the first 40 minutes of acquisition and that only slow but appreciable variations were observed if extended for hours. Nevertheless, it is known that a time interval as short as 5 minutes is enough to develop anoxic conditions that produce important variations in the concentrations of metabolites [51]. In the data here obtained there were several indicatives of anoxic conditions: the high concentration of Lac, the low values of pH (that was not completely explained by the increase in Lac), the depletion of all energetic metabolic stores (ATP, PCr) and the presence of adenosine. Adenosine is an intermediate metabolite accumulated in stress conditions and has been recognized as implied in cytoprotection during ischemia or anoxia [52]. According to these observations, these conditions occurred previous to the NMR study, probably in the time between the surgery and the freezing of the sample. But some contribution from the tumoral growth cannot be discarded.

The great heterogeneity of the samples here analyzed, intrinsic by the tumour type, can be clearly observed in the histological examples of Figure 1, in the % of Tum, Nec and NAT in Table 1 and in the great variability of the concentrations of most of the metabolites detected. This is particularly evident in the GBM group, a type of tumour generally recognized as very heterogeneous [40]. The samples studied by HRMAS are frequently a small part of the original tumour piece and then can be considered as a partial representation when the tumor is not a homogeneous piece. Then, we must take into account both, the variable characteristics among different cases, and the internal heterogeneity of each one. These differences influence the conditions of the tissue sample during the HRMAS experiments after which considerable alterations and damage of the sample have been observed [25]. The metabolite profile of the tumor samples reflects the physiological state of the living tissue but can also reveal the conditions suffered by the sample during manipulation. In some circumstances the last can have a preponderant influence in the final profile even masking the original state. The changes in the metabolite concentrations can be very fast and then the metabolome is an instantaneous characterization of the state of a tissue when compared with the one provided by proteome or genome.

Conclusion

In this study the pH and the absolute concentrations of a set of metabolites in human brain tumor samples have been determined trough the analysis of ^{1}H and ^{31}P spectra by HRMAS, using the digital ERETIC

synthetic signal as a concentration reference, and homo and heteronuclear correlation experiments to follow the ^{1}H-^{1}H and ^{1}H-^{31}P spin systems and to confirm the assignments. The use of MDPA as a ^{31}P chemical shift and concentration reference has shown some drawbacks according to the demonstrated interaction between this compound and the tissue, considering a better approach the use of the ERETIC strategy for quantification either in ^{1}H or in ^{31}P experiments in tissues, and the use of endogenous substances whose chemical shift is known to be invariant with pH. Finally, the combination of ^{1}H and ^{31}P information has made possible to assess the correlation between the slight changes in chemical shift of some metabolites as PC and GPC with the pH.

The study of the main histological features of the samples has enabled to check the relation between the changes in the absolute concentration of the metabolites and the percentage of Tumour, Necrosis and not apparently tumour tissues. Two superposed effects have occurred in a manner that can be explained trough the physiological facts that may occur in the tumour or during the handling of the excised tissue. The alterations of the concentrations of characteristic brain metabolites that we have determined give some support to the idea that the mixture of tissues and deterioration on each type of tumour is what can be directly detected in the metabolic profiles by HRMAS, and the characterization of each type of tumour and degree relies on the characteristic contributions of such alterations.

Table 1 This table details the types of tumour studied. For each group the average and the standard deviation is provided for the pH and for the tissue percentage composition, evaluated in the histological analysis after HRMAS: tumoral tissue (%Tum), extensive necrosis (%Nec) and non affected tissue (%NAT). In the tumour type column, GBM means Glioblastoma Multiforme (grade IV Glioma)

Table 2 Concentrations (average±standard deviation µmol/g) of the more abundant metabolites evaluated by integration of the corresponding signals and using ERETIC digital as reference in each group of tumour. The quantification presented in this table is restricted to only one of the signals in each metabolite. Nevertheless, for GPC and PC we have included the quantification of the concentration obtained using both the ^{1}H and ^{31}P spectra for comparative purposes. For the fatty acids resonances, the values in this table represent the average concentration of these characteristics groups and not the concentration of independent molecules. FA 1, –CH$_2$-**CH$_3$**; FA 2, –CH$_2$-**CH$_2$**-CH$_2$-; FA 3, –CO-CH$_2$-

CH$_2$-; FA 4 –CH$_2$-CH=CH-; FA 5, –CO-CH$_2$-; FA6 and FA7, -CH=CH-CH$_2$-CH=CH-; FA8, -CH=CH-

Table 3 Significant correlations between the concentrations of metabolites, pH and proportions of different tissue types present in the samples analyzed. Only correlation coefficient for correlations with p<0.01 have been shown

[a] Data from ^{31}P spectra. Otherwise, data from ^{1}H spectra.

[b] Only data for FA1 is presented since the correlations of FA group with the metabolites, pH and tissue features is similar. For a complete set of FA data see Table S3B in Electronic Supplementary Material.

Table 4 Significant correlations between the concentrations of metabolites, FA signals, pH and proportions of different tissue types present in the samples analyzed restricted to the group of samples diagnosed as GBM. Only correlation coefficient for correlations with p<0.01 have been shown.

[a] Only data for FA1 is presented since the correlations of FA group with the metabolites, pH and tissue features is similar. For a complete set of FA data see Table S4 in Electronic Supplementary Material.

Tables in Electronic Supplementary Material:

Table S1 List of samples studied with specification of the type of tumor of origin, weight and pH evaluated using the ^{31}P chemical shifts as explained in materials and methods. For each sample there is also included three tissue percentage composition evaluated in the histological analysis after HRMAS: tumoral tissue (%Tum), extensive necrosis (%Nec) and non affected tissue (%NAT). In the tumour type column, GBM means Glioblastoma Multiforme; AA, anaplastic astrocytoma; ODAA, anaplastic oligodendroastrocytoma; OD, oligodendroglioma; ODA, oligoastrocytoma; AI, grade I astrocytoma; MET, metastases; MEN, meningeoma; TER, teratoma

Table S2A Concentrations (μmol/g) of the more abundant metabolites evaluated by integration of the corresponding signals and using ERETIC digital as reference. The quantification presented in this table is restricted to only one of the signals in each metabolite. Nevertheless, for GPC and PC we have included the quantification of the concentration obtained using both the ^{1}H and ^{31}P spectra for comparative purposes

[a] Data from ^{31}P spectra. Otherwise, data from ^{1}H spectra.

Table S2B Quantification of characteristic fragments of fatty acids deduced of the broad signals observed at typical chemical shift. The values represent the concentration of these characteristics groups and not the concentration of independent molecules. FA 1, –CH$_2$-CH$_3$; FA 2, –CH$_2$-CH$_2$-CH$_2$-; FA 3, –CO-CH$_2$-CH$_2$-; FA 4 –CH$_2$-CH=CH-; FA 5, –CO-CH$_2$-; FA6 and FA7, -CH=CH-CH$_2$-CH=CH-; FA8, -CH=CH-

Table S3A Significant correlations between the concentrations of metabolites, pH and proportions of different tissue types present in the samples analyzed. Normal font in half upper right part of the table expresses correlation coefficient, and data in italics in the lower left part of the table expresses the corresponding p value. Only data for correlation with p<0.05 have been shown

[a] Data from ^{31}P spectra. Otherwise, data from ^{1}H spectra.

Table S3B Significant correlations between the quantification of different FA signals, pH and proportions of different tissue types present in the samples analyzed. Data presentation follows the same criteria than table 3A

Table S4 Significant correlations between the concentrations of metabolites, FA signals, pH and proportions of different tissue types present in the samples analyzed restricted to the group of samples diagnosed as GBM. Data presentation follows the same criteria than table 3A

Fig. 1 Comparison of three different patterns of tissue configuration in samples extracted from tumours diagnosed as GBM and the corresponding stained tissue slice (left), ^{1}H (centre) and ^{31}P (right) spectra. Upper, sample 1 (4% Tum, 96.0% Nec, 0.0% NAT): extensive necrosis dominates the sample, low signals of metabolites and prominent signals of FA can be appreciated in the ^{1}H and ^{31}P. Centre, sample 7 (94.2% Tum, 5.8% Nec, 0.0% NAT) dense tumoral cell growth (higher amounts of metabolites and lower of FA are present). Down, sample 10 (46.1% Tum, 1.0% Nec, 52.8% NAT): border tissue with dense tumoral growth is clearly delimited in this case; the signals of metabolites area also prominent, but present very different proportions. All the quantified signals are assigned except the signals of FA8 and Adenosine which are out of the region showed in the spectra

Fig. 2 ^{1}H-^{31}P HSQC (up) and TOCSY (down) spectra with the correspondent 1D ^{1}H and ^{31}P spectra for the sample 10. The displayed region in the TOCSY show the ^{1}H-^{1}H X**P**-O-**CH**$_2$-**CH**$_2$-N**X** crosspeaks in the TOCSY for GPC, PC, GPE and PE. The ^{1}H-^{31}P HSQC region in the figure shows with higher intensity the ^{1}H-^{31}P crosspeaks of X**P**-O-**CH**$_2$-CH$_2$-NX of GPC, PC, GPE and PE, and CH$_2$OH-CHOH-**CH$_2$-P**O4-CH2-… of GPC and GPE. Less intense but also appear the crosspeaks of X**P**-O-CH$_2$-**CH**$_2$-NX for GPE and GPC. The path of homo-heteronuclear assignments are drawn in dashed line in blue for GPC, in red for GPE, in green for PC and in yellow for PE. The signals can be observed in the correspondent 1D ^{31}P spectrum on the right margin of HSQC spectrum, but not in the 1D ^{1}H crowded spectra on top of HSQC spectrum

Fig. 3 The evaluation of the signals was performed correcting for the overlapping nearby peaks as illustrated here for the case of creatine CH$_2$ protons (upper left). The posterior quantification of the creatine concentrations showed a good correlation between the evaluations using the CH$_2$ and the CH$_3$ signals (>0.99 with p<0.001; upper right). The comparison of concentrations calculated using ^{1}H and ^{31}P spectra for PC (0.94, p<0.001; lower left) and GPC (0.92, p<0.001; lower right) showed also an internal consistency of the quantification method

Fig. 4 Linear fit for pH versus the chemical shift difference between Cho and PC N(CH$_3$)$_3$ protons evaluated in ^{1}H water presaturation spectra (correlation coefficient 0.86, p< 0.001). The dotted lines represent the 95% prediction bounds and show that only a broad estimation of the pH (approximately $\pm$ 0.45 pH units) is possible from the measured chemical shift difference

REFERENCES

[1] Cheng LL, Chang IW, Louis DN, Gonzalez RG (1998) Cancer Res 58:1825-1832

[2] Opstad KS, Bell BA, Griffiths JR, Howe FA (2008) Magn Reson Med 60:1237-1242

[3] Sjobakk TE, Johansen R, Bathen TF, Sonnewald U, Juul R, Torp SH, Lundgren S, Gribbestad IS (2008) NMR Biomed 21:175-185

[4] Martinez-Bisbal MC, Marti-Bonmati L, Piquer J, Revert A, Ferrer P, Llacer JL, Piotto M, Assemat O, Celda B (2004) NMR Biomed 17:191-205

[5] Erb G, Elbayed K, Piotto M, Raya J, Neuville A, Mohr M, Maitrot D, Kehrli P, Namer IJ (2008) Magn Reson Med 59:959-965

[6] Wilson M, Davies NP, Brundler MA, McConville C, Grundy RG, Peet AC (2009) Mol Cancer 8:6

[7] Martinez-Bisbal MC, Monleon D, Assemat O, Piotto M, Piquer J, Llacer JL, Celda B (2009) NMR Biomed 22:199-206

[8] Martínez-Granados B, Monleón D, Martínez-Bisbal MC, Rodrigo JM, del Olmo J, Lluch P, Ferrández A, Martí-Bonmatí L, Celda B (2006) NMR Biomed 19:90-100

[9] Hubesch B, Sappey-Marinier D, Roth K, Meyerhoff DJ, Matson GB, Weiner MW (1990) Radiology 174:401-409

[10] Albers MJ, Krieger MD, Gonzalez-Gomez I, Gilles FH, McComb JG, Nelson MD,Jr, Bluml S (2005) Magn Reson Med 53:22-29

[11] Wijnen JP, Scheenen TW, Klomp DW, Heerschap A (2010) NMR Biomed 23:968-976

[12] Podo F (1999) NMR Biomed 12:413-439

[13] Griffiths JR, Cady E, Edwards RH, McCready VR, Wilkie DR, Wiltshaw E (1983) Lancet 1:1435-1436

[14] Robitaille PL, Robitaille PA, Gordon Brown G, Brown GG (1991) Journal of Magnetic Resonance (1969) 92:73-84

[15] Griffiths JR (1991) Br J Cancer 64:425-427

[16] Payne GS, Troy H, Vaidya SJ, Griffiths JR, Leach MO, Chung YL (2006) NMR Biomed 19:593-598

[17] De Silva SS, Payne GS, Thomas V, Carter PG, Ind TE, deSouza NM (2009) NMR Biomed 22:191-198

[18] Wang Y, Cloarec O, Tang H, Lindon JC, Holmes E, Kochhar S, Nicholson JK (2008) Anal Chem 80:1058-1066

[19] Lehnhardt FG, Rohn G, Ernestus RI, Grune M, Hoehn M (2001) NMR Biomed 14:307-317

[20] Srivastava NK, Pradhan S, Gowda GA, Kumar R (2010) NMR Biomed 23:113-122

[21] Akoka S, Barantin L, Trierweiler M (1999) Anal Chem 71:2554-2557

[22] Albers MJ, Butler TN, Rahwa I, Bao N, Keshari KR, Swanson MG, Kurhanewicz J (2009) Magnetic Resonance in Medicine 61:525-532

[23] Ben Sellem D, Elbayed K, Neuville A, Moussallieh FM, Lang-Averous G, Piotto M, Bellocq JP, Namer IJ (2011) J Oncol 2011:174019

[24] Bourne R, Dzendrowskyj T, Mountford C (2003) NMR Biomed 16:96-101

[25] Martinez-Bisbal MC, Esteve V, Martinez-Granados B, Celda B (2011) J Biomed Biotechnol 2011:763684. Epub 2010 Sep 5

[26] Celda B, Montelione GT (1993) Journal of Magnetic Resonance, Series B 101:189-193

[27] Esteve V, Celda B (2008) Magn Reson Mater Phy MAGMA 21:484-484

[28] Collins TJ (2007) BioTechniques 43:25-30

[29] Govindaraju V, Young K, Maudsley AA (2000) NMR Biomed 13:129-153

[30] Fan TW- (1996) Prog Nucl Magn Reson Spectrosc 28:161-219

[31] Ulrich EL, Akutsu H, Doreleijers JF, Harano Y, Ioannidis YE, Lin J, Livny M, Mading S, Maziuk D, Miller Z, Nakatani E, Schulte CF, Tolmie DE, Kent Wenger R, Yao H, Markley JL (2008) Nucleic Acids Res 36:D402-8

[32] Kriat M, Vion-Dury J, Confort-Gouny S, Favre R, Viout P, Sciaky M, Sari H, Cozzone PJ (1993) J Lipid Res 34:1009-1019

[33] Subramanian A, Shankar Joshi B, Roy AD, Roy R, Gupta V, Dang RS (2008) NMR Biomed 21:272-288

[34] Daykin CA, Corcoran O, Hansen SH, Bjornsdottir I, Cornett C, Connor SC, Lindon JC, Nicholson JK (2001) Anal Chem 73:1084-1090

[35] Griffin JL, Lehtimaki KK, Valonen PK, Grohn OH, Kettunen MI, Yla-Herttuala S, Pitkanen A, Nicholson JK, Kauppinen RA (2003) Cancer Res 63:3195-3201

[36] Petroff OAC, Prichard JW (1995) In: Kraicer J, Dixon SJ (eds) Methods in Neurosciences, Academic Press,

[37] Barton S, Howe F, Tomlins A, Cudlip S, Nicholson J, Anthony Bell B, Griffiths J (1999) Magnetic Resonance Materials in Physics, Biology and Medicine 8:121-128

[38] Sitter B, Sonnewald U, Spraul M, Fjosne HE, Gribbestad IS (2002) NMR Biomed 15:327-337

[39] Coen M, Hong YS, Cloarec O, Rhode CM, Reily MD, Robertson DG, Holmes E, Lindon JC, Nicholson JK (2007) Anal Chem 79:8956-8966

[40] Russell D, Rubinstein LJ (1998) Russel and Rubinstein's Pathology of Tumors of the Nervous System. Arnold, London

[41] Tynkkynen T, Tiainen M, Soininen P, Laatikainen R (2009) Anal Chim Acta 648:105-112

[42] Kjaergaard M, Brander S, Poulsen F (2011) J Biomol NMR 49:139-149

[43] Robert O, Sabatier J, Desoubzdanne D, Lalande J, Balayssac S, Gilard V, Martino R, Malet-Martino M (2011) Anal Bioanal Chem 399:987-999

[44] Chadzynski GL, Bender B, Groeger A, Erb M, Klose U (2011) J Magn Reson 212:55-63

[45] Weljie AM, Jirik FR (2011) Int J Biochem Cell Biol 43:981-989

[46] Barba I, Cabanas ME, Arus C (1999) Cancer Res 59:1861-1868

[47] Liimatainen T, Hakumaki JM, Kauppinen RA, Ala-Korpela M (2009) NMR Biomed 22:272-279

[48] Opstad KS, Bell BA, Griffiths JR, Howe FA (2008) NMR Biomed 21:677-685

[49] Schmitz JE, Kettunen MI, Hu D, Brindle KM (2005) Magnetic Resonance in Medicine 54:43-50

[50] Glunde K, Artemov D, Penet MF, Jacobs MA, Bhujwalla ZM (2010) Chem Rev 110:3043-3059

[51] Hertz L (2008) Neuropharmacology 55:289-309

[52] Takahashi T, Otsuguro K, Ohta T, Ito S (2010) Br J Pharmacol 161:1806-1816

Labels on spectra (top to bottom, left column):

FA6,7 · FA5 · FA4 · FA3 · FA2 · FA1

Lac

Cr

GPC

PC

Cho

NAA

GABA

Ala

Tau

mI

Gly

GSH

Gln · Glu

Pi

GPE

GPC

PE

PC

4.0   3.5   3.0   2.5   2.0   1.5   1.0   ppm

5   4   3   2   1   0   −1   −2   ppm