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600 MHz ^{1}H-NMR spectroscopy of human cerebrospinal fluid: effects of sample manipulation and assignment of resonances

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Abstract: Extensive assignments of resonances in the 600 MHz ^{1}H-NMR spectra of cerebrospinal fluid are reported. These have been achieved by the measurement of a combination of two-dimensional experiments comprising homonuclear J-resolved, COSY45, and double-quantum filtered COSY (DQCOSY) spectra. By these means the previous total of 18 endogenous metabolites, of which in general only selected resonances have been assigned, has been augmented to 46 molecules including all of the resonances of both α- and β-anomers of glucose. With only a few exceptions all resonances have been assigned for all of the metabolites. In addition, the effect of freeze-drying on the 600 MHz ^{1}H-NMR spectrum of human cerebrospinal fluid (CSF) is presented using both lyophilization with reconstitution into either H_2O or D_2O. Freeze-drying and reconstitution into H_2O causes a significant sharpening of many small molecule resonances, including notably those of glutamate and glutamine as well as other amino acids and in addition causes the loss of volatile components, principally acetone. Further exchange of the H_2O solvent by D_2O causes no additional changes in the spectra.

Keywords: *Cerebrospinal fluid; ^{1}H-NMR; endogenous metabolites; COSY; J-resolved.*

Introduction

Analysis of the high resolution NMR spectra of body fluids in terms of quantification of endogenous metabolites has found widespread application for the understanding of intermediary biochemistry in animals and humans [1]. Measurement of such known small molecule endogenous metabolites has led to an improved understanding of the toxic effects of xenobiotics in animal studies [2] and the diagnosis of various pathological states [3]. Although most analysis has been performed on urine or blood plasma, studies have also been reported on a wide variety of other fluids. In order to marshall the large amount of data implicit in such spectra, the coupling of NMR data sets with computer-based multivariate statistical methods has been investigated and has led to the more efficient understanding of such biochemical changes [4, 5].

Assignment of resonances is not necessary for such pattern recognition approaches which discriminate samples into different classes and indeed an automatic method of spectral data reduction may be preferred for consistency, as recently exemplified [6]. However, detailed assignment of the resonances is necessary if mechanistic hypotheses are to be formulated based on the altered biochemistry.

Cerebrospinal fluid (CSF) surrounds the brain and fills the ventricles as well as the subarachnoid space. It is the secretion product of various central nervous system structures and it obtains its ultimate composition by exchange with the blood and adjacent brain tissues. It is usually collected by lumbar puncture from the lumbar subarachnoid space but can also be taken suboccipitally, for example during myelography. Because of the close proximity of the CSF to the brain, it may be that malfunction or diseases in this area may be manifested in the biochemical composition of the CSF, particularly as the ratio of blood serum to CSF concentrations of endogenous biochemicals can be markedly different from

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unity. CSF is usually a clear, colourless, mobile liquid but coloured or turbid samples can result from pathological conditions.

The biochemical compositions of CSF has been well characterized by standard techniques and a number of low molecular weight metabolites have been quantified in a range of pathological states [7]. A comprehensive NMR study of CSF to investigate changes in biochemical content as a consequence of disease processes is being undertaken and these findings will be reported elsewhere in due course. As a necessary prerequisite, this paper describes the detailed assignment of many of the small molecule metabolites found in CSF and has led to the assignment of most of the resonances for 46 such metabolites.

There are a number of ^{1}H-NMR studies of CSF in the literature. The earliest report was by Bales *et al.* [8] and later Petroff *et al.* [9] and Bell *et al.* [10] have reported the assignment of a number of resonances, 17 in all, excluding water. Koschorek *et al.* [11] have measured ^{1}H-NMR spectra of CSF for 84 patients at 360 MHz and quantified the levels of 19 signals some of which were assigned to specific metabolites (lipids, valine, 3-hydroxybutyrate, phospholipids, lactate, alanine, acetate, acetone, citrate, creatinine, creatine and glucose) and others which remained unassigned. For 42 patients with lumbar disk herniations, no significant differences from controls were found. However, they observed differences between controls and one patient with a medulloblastoma which showed a decreased glucose level and new signals in the δ0.8–1.0 and δ1.28–1.42 regions which they assigned to valine and alanine. More recently, the same group [12] have reported results on 66 patients with tumours, arteriovenous malformations, aneurysms, infarctions and lumbar back pain, including a group of 19 controls, with classification based on discriminant analysis. Commodari *et al.* [13] have detected changes in the ^{1}H-NMR spectra of CSF in nine patients suffering a variety of conditions including the detection of methylmalonate in a patient with Vitamin B_{12} deficiency. One result of all of these studies has been the specific assignment of some or all of the resonances for 18 endogenous metabolites.

The use of 600 MHz ^{1}H-NMR spectroscopy as a technique for extracting maximum information from body fluids including CSF has been proposed recently along with the use of two-dimensional methods for biofluids including J-resolved spectroscopy [14]. It is now shown that through the use of 600 MHz ^{1}H-NMR with both J-resolved and COSY methods it is possible to markedly augment the number of assigned metabolites in CSF.

A large number of *ex vivo* lumbar puncture samples from a variety of disease states have been examined together with a number of control samples, as well as a number of *post mortem* samples from Alzheimer's patients and related controls [15]. Results of these studies are being published separately together with the use of pattern recognition methods for their classification.

Experimental

^{1}H-NMR spectra were measured on samples as received, and after freeze-drying with reconstitution in an equal volume of either H_2O or D_2O. Sodium trimethylsilyl-[2,2,3,3-$^2H_4$]-propionate (TSP) was added as a chemical shift reference (δ0.00) and internal quantitation standard. All spectra were acquired at a frequency of 600 MHz using Varian VXR-600S and Bruker AMX-600 spectrometers at ambient temperature. Suppression of the water peak was achieved using a one-dimensional NOESY pulse sequence or by continuous secondary irradiation during the recycle delay.

For one-dimensional spectroscopy, typically 256 transients were accumulated in quadrature mode into 32 K data points with a spectral width of 8000 Hz. A 2.7 s relaxation delay was included to allow T_1 relaxation. A 0.2–1.0 Hz line-broadening was applied before Fourier transformation. Some spectra were measured using an automatic sample changer and in this case a spectral width of 18518 Hz, with 131072 time domain data points and a total recycle time of 6.0 s (including 3.0 s for water saturation) was used. Two-dimensional J-resolved spectra [16], (JRES), were measured using a spectral width of 6500 Hz, 2 s relaxation delay, 8192 data points, an acquisition time of 0.63 s, 64 increments with a 30 Hz spectral width in F1 and eight scans per FID. A sine-bell weighting function shifted by 31.4% was applied and zero-filling to 16384 points were carried out prior to Fourier transformation. Two-dimensional COSY spectra were obtained using the COSY45 [17] variant to lessen the diagonal peaks. Typical parameters were: acquisition time, 0.32 s; 4096 data points; 2 s relaxation

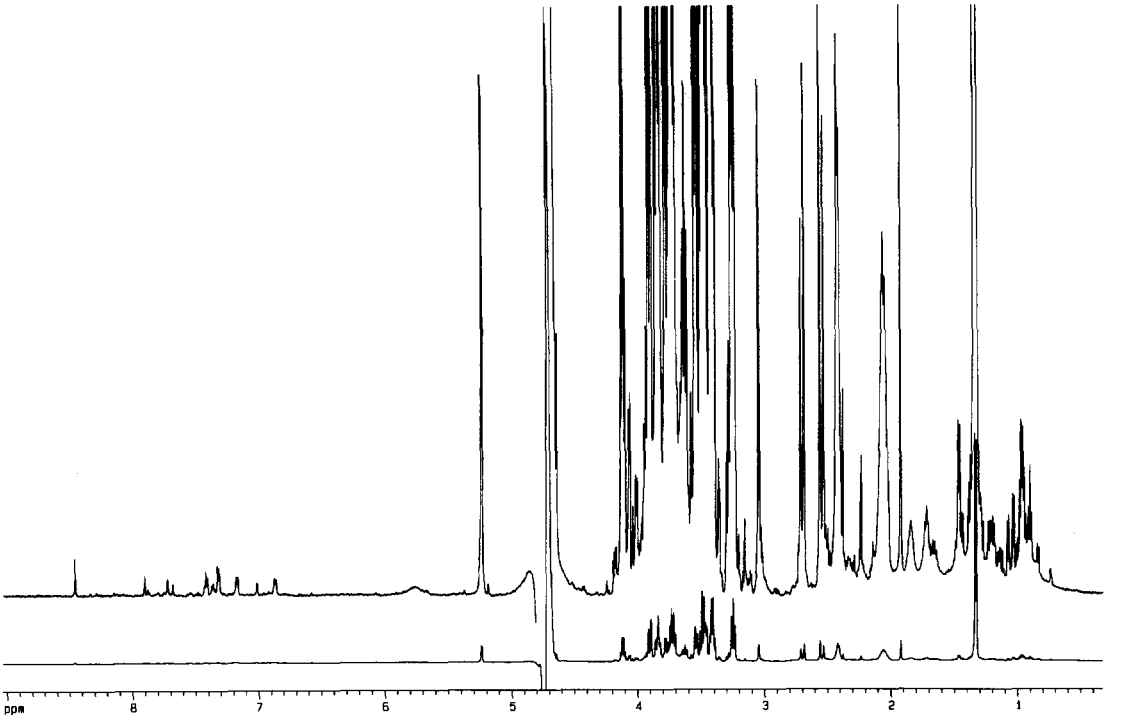


Figure 1
600 MHz ^{1}H-NMR spectrum of *ex vivo* control human CSF. The upper trace is a 32× vertical expansion.

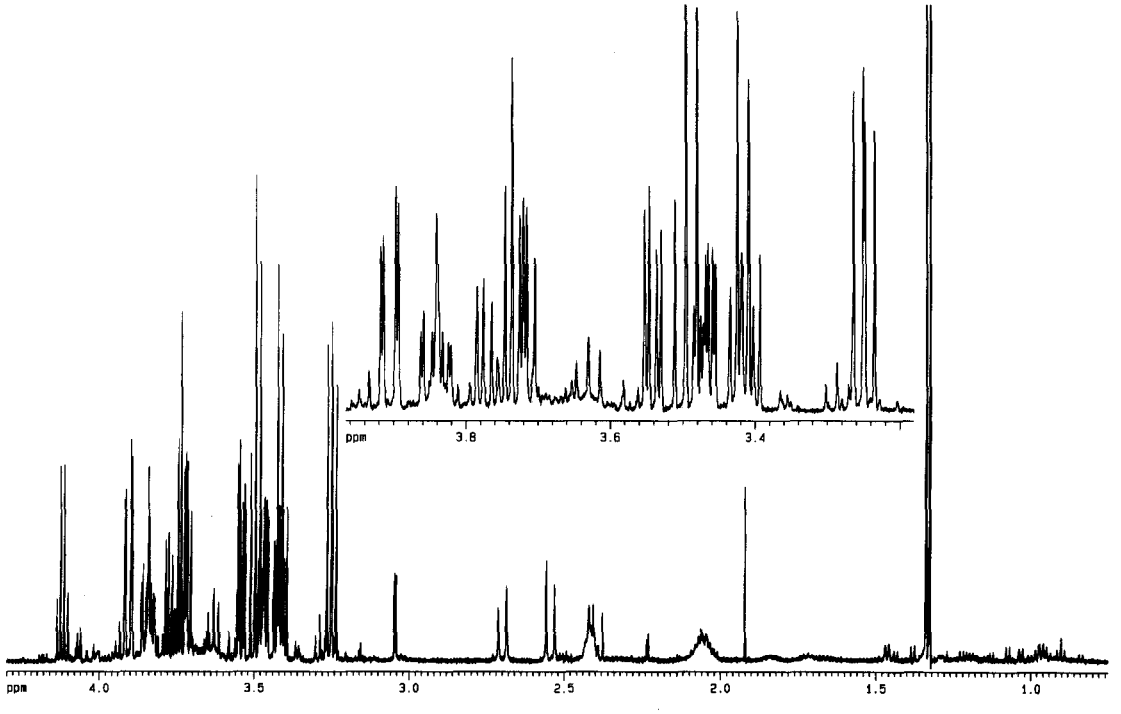


Figure 2
Expansion of the aliphatic region of the spectrum shown in Fig. 1 (δ0.7–4.3) with Lorentzian–Gaussian resolution enhancement (LB = −1.0, GB = 0.42), showing the high level of spectral complexity. The insert is an expansion of the region δ3.18–3.97.

delay; 40 transients per increment; 512 increments; spectral width 6500 Hz; sine-bell weighting, shifted by 15.7% and zero-filled to 4096 points before Fourier transformation; spectral width 6500 Hz. In addition, double-quantum filtered COSY spectra [18], (DQCOSY), were also measured using similar parameters.

Results

A typical one-dimensional 600 MHz ^{1}H-NMR spectrum of CSF indicating the extreme complexity of overlapped lines is shown in Fig. 1. Figure 2 shows an expanded spectrum both horizontally and vertically such that the high level of complexity can be observed. The assignments of the resonances can be distinguished more easily on the later figures which are of the two-dimensional spectra. Thus, these assignments have been made principally through the use of two-dimensional JRES spectra [16] and COSY45 spectra [17]. Figure 3 shows the 600MHz ^{1}H-JRES spectrum in which the multiplicities due to coupling constants on each resonance are rotated orthogonal to the chemical shift axis. This provides added resolution enabling peak multiplicity patterns to be more clearly resolved. As a consequence, the projection of the two-dimensional spectrum on to the F2 (chemical shift) axis provides a ^{1}H-NMR spectrum containing no ^{1}H–^{1}H spin-couplings. One disadvantage of this experiment is that the results have to be presented in absolute value mode, making accurate measurements of coupling constants difficult. Also, because the experiment is a spin-echo pulse sequence, intensity of peaks and projections are unreliable measures of true concentrations.

Figures 4–6 show a 600 MHz ^{1}H-COSY45 contour plot. In this representation, the one-dimensional spectrum appears along the diagonal with off-diagonal peaks at coordinates of the chemical shifts of coupled protons. Figure 4(a) shows the region about the diagonal from δ0.72 to 2.98 and Fig. 4(b) shows the higher frequency region from δ2.98 to 5.22. Figure 5 is an expansion of the most crowded region of the spectrum and Fig. 6 is an

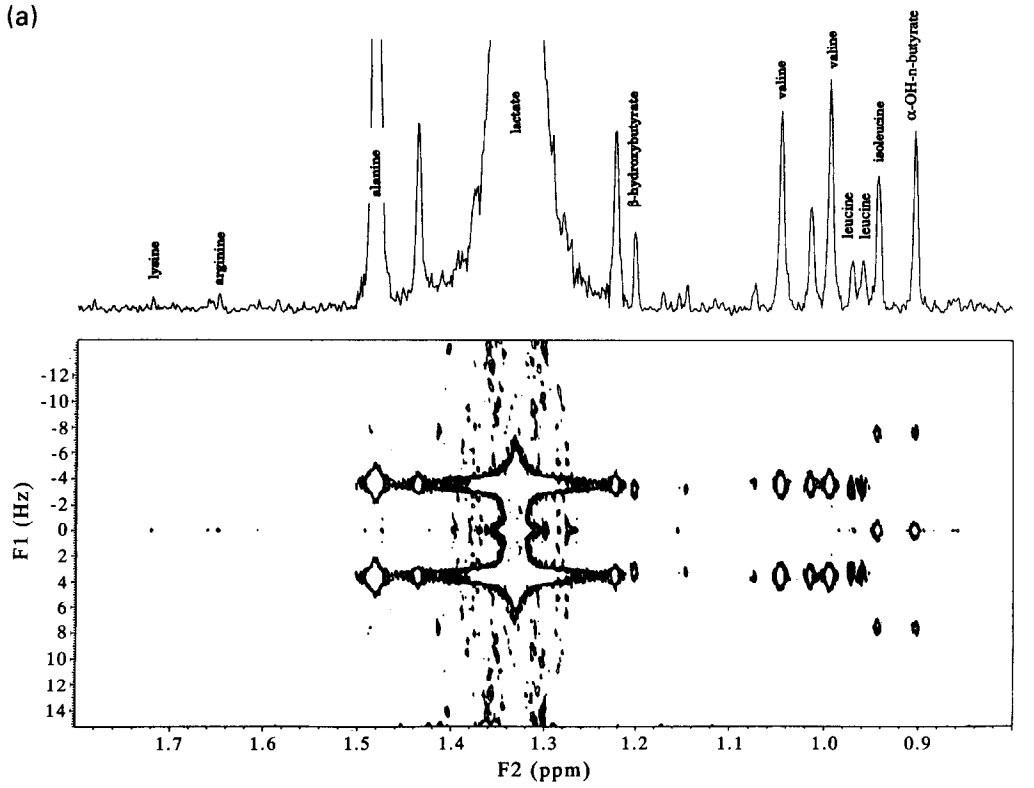
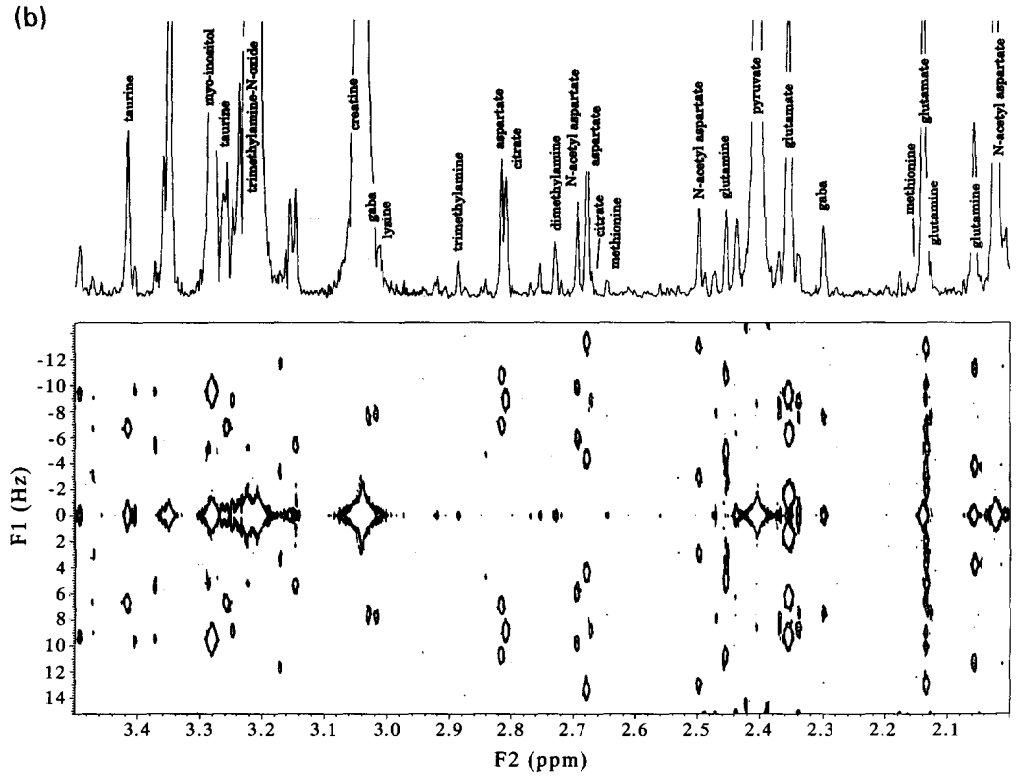


Figure 3
600 MHz ^{1}H-JRES NMR spectrum of lyophilized CSF reconstituted into D_2O with skyline projections and assignments (a) δ0.8–1.8, (b) δ2.0–3.5, (c) δ3.5–4.3.


(b)
taurine
myo-inositol
taurine
trimethylamine-N-oxide
creatine
gaba
lysine
trimethylamine
aspartate
citrate
dimethylamine
N-acetyl aspartate
aspartate
citrate
methionine
N-acetyl aspartate
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pyruvate
glutamate
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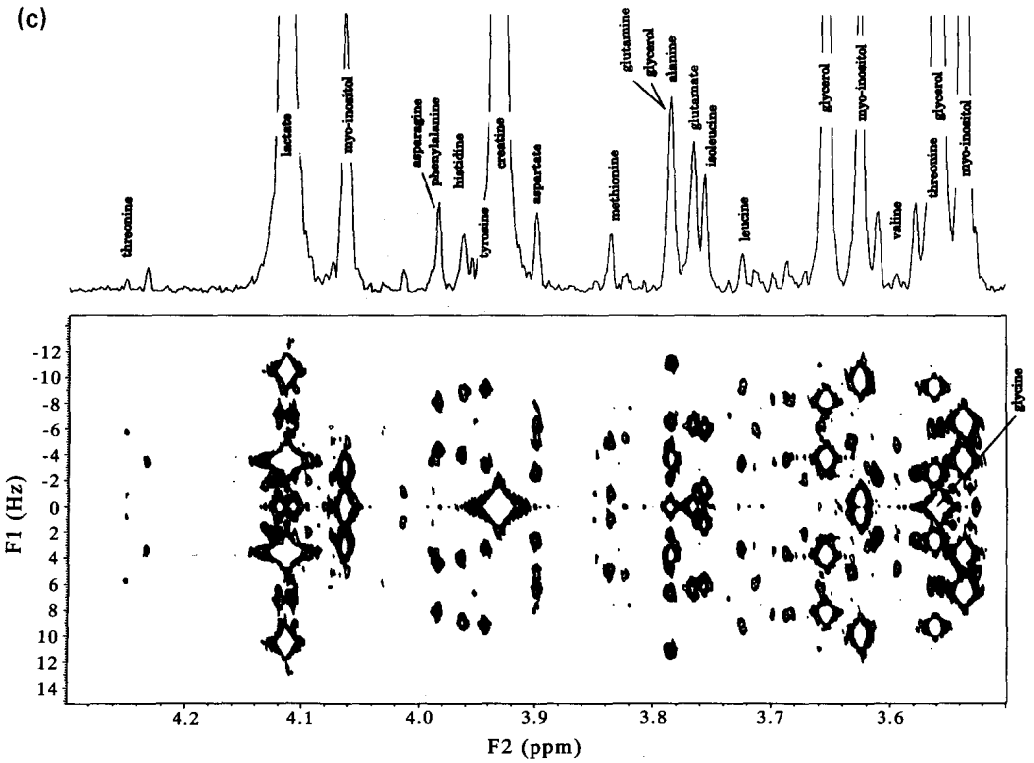
N-acetyl aspartate
F1 (Hz)
F2 (ppm)


(c)
threonine
lactate
myo-inositol
asparagine
phenylalanine
histidine
tyrosine
creatine
aspartate
methionine
glutamine
glycerol
alanine
glutamate
isoleucine
leucine
glycerol
myo-inositol
valine
threonine
glycerol
myo-inositol
glycine
F1 (Hz)
F2 (ppm)

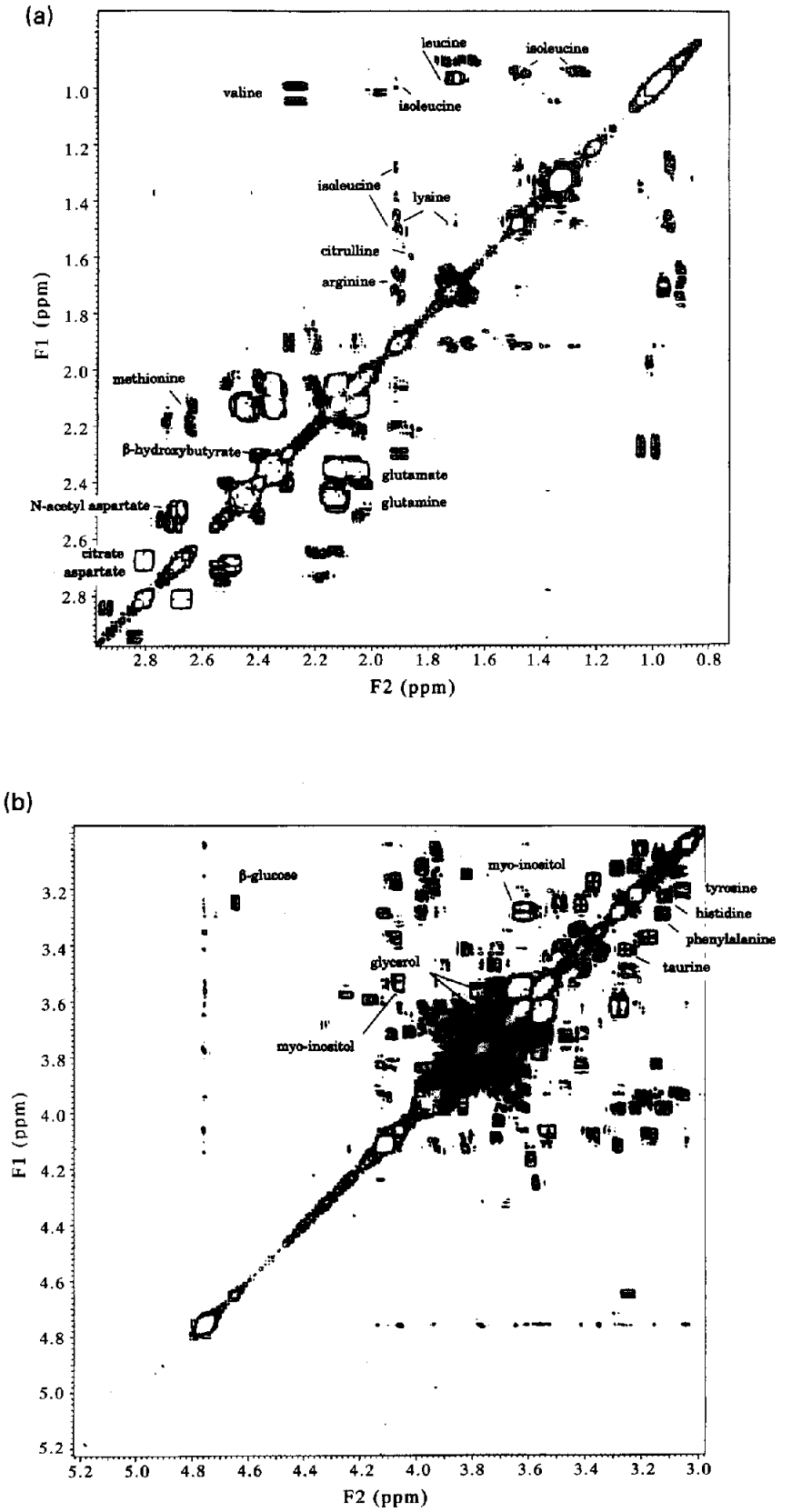


Figure 4
600 MHz ^{1}H-COSY45 NMR spectrum of CSF. Aliphatic region only. (a) δ2.98–5.22, (b) δ0.72–2.98.

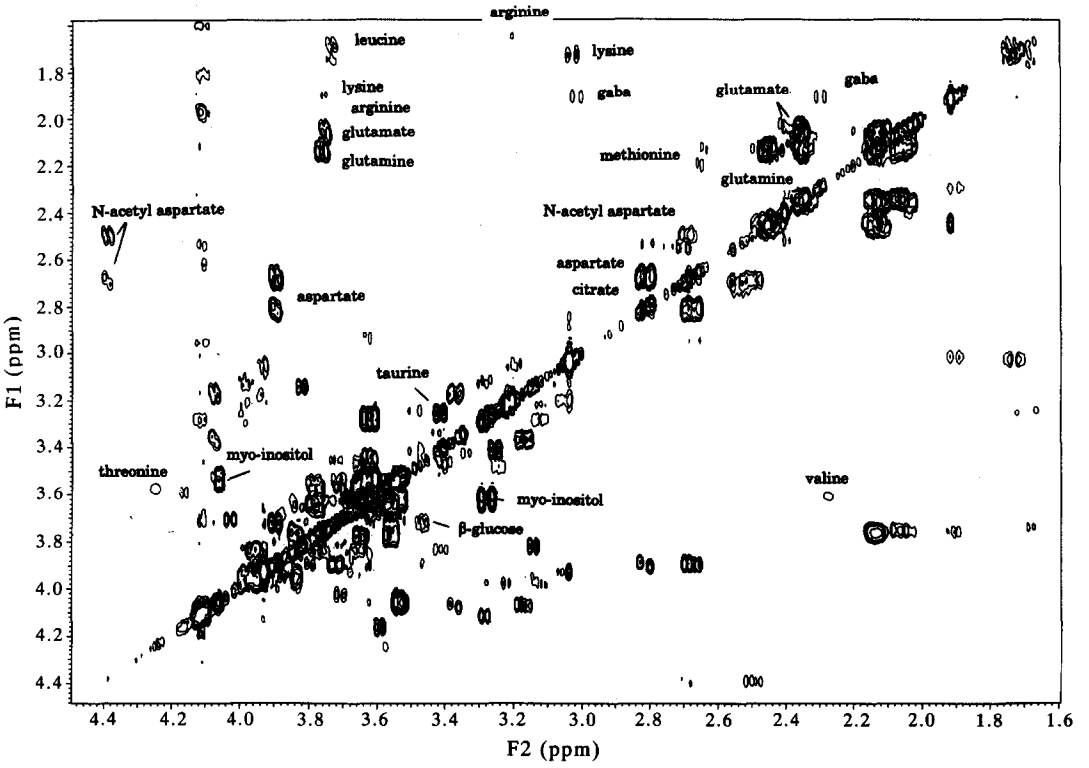


Figure 5
600 MHz ^{1}H-COSY45 NMR spectrum of CSF, expanded region.

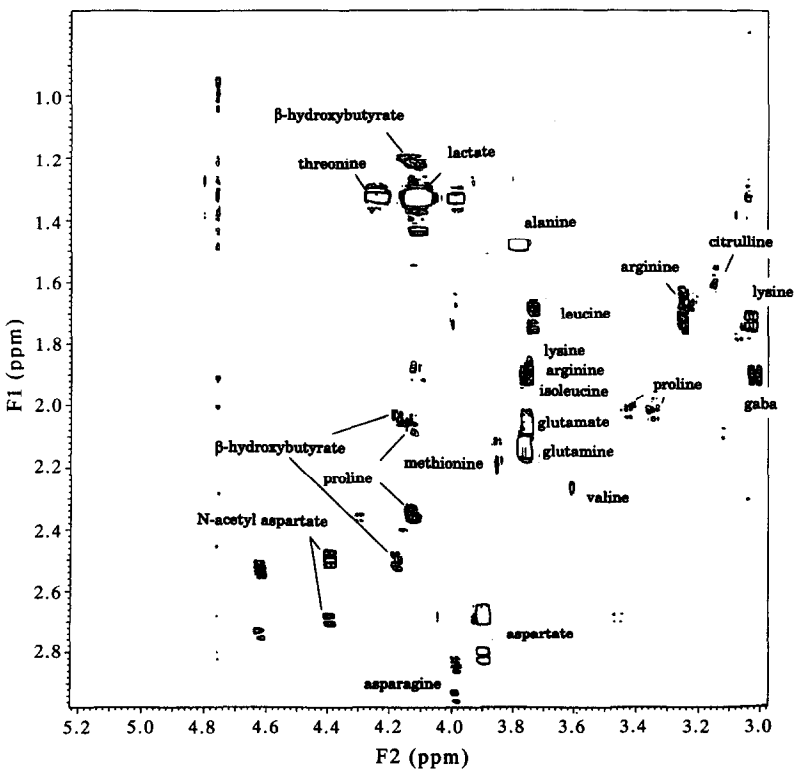


Figure 6
600 MHz ^{1}H-COSY45 NMR spectrum of CSF, off diagonal region.

expansion of the off-diagonal region which, amongst other resonances, connects those of α-CH protons of amino acids with side-chain proton chemical shifts.

Approximately 250 model compounds have also been measured and a database of structures and ^{1}H-NMR parameters generated (S. Garland, D. Paulls, B.C. Sweatman and J.C. Lindon, unpublished work). Standard additions of known compounds to aliquots of CSF also confirmed assignments in cases of ambiguity.

Examination of *ex vivo* control samples shows a remarkable consistency in the aliphatic region of the one-dimensional proton NMR spectra. Many assignments can be made by inspection [1]. Some general observations can be made, some of which have been noted before [10]. Thus glutamate and glutamine (for assignments see later) have distinctly broadened resonances. The effect of freeze-drying the samples with reconstitution into H_2O causes small but consistent low frequency shifts to a number of resonances. These shifts of about δ0.02–0.04 in magnitude can be observed for resonances from valine, alanine, lysine, arginine, threonine and for the aromatic resonances of histidine, tyrosine and phenylalanine. This phenomenon is not seen on all resonances and appears to be limited to amino acids only, possibly reflecting a change in binding to CSF proteins after the freeze-drying step which may have denatured the proteins. In addition, the effect on the glutamate and glutamine resonances is more marked. The β-CH_2 signals from both of these molecules are seen as an overlapped multiplet at about δ2.00–2.05 in the NMR spectrum of untreated CSF, but after freeze-drying these are sharpened, shifted to low frequency and separately resolved. Similarly, the γ-CH_2 resonances of both compounds are manifested as a broad multiplet at about δ2.5 and after freeze-drying, these are also much sharper and separately resolved at chemical shifts of δ2.46 and δ2.36 for glutamine and glutamate, respectively. The proportions of the two species (glutamine:glutamate) can now be determined easily by integration to be about 1:3 to 1:4. This contrasts with previous amino acid analysis of these compounds in CSF which gave a ratio of glutamine:glutamate of at least 20:1 [19]. However in that study, it was pointed out that enzymes in CSF can cause the hydrolysis of asparagine and glutamine to aspartate and glutamate and as the present samples were not deproteinized, this may explain the elevated amounts of these latter species seen in the present study.

Any proteins or other macromolecules present in the samples generally would give rise to broad resonances and would be of low intensity. Although resonances from macromolecules can be significant features in other biofluids such as blood plasma, they do not interfere with the small molecule resonances in CSF described here. It may be that some of the small molecules detected in this study are partially bound to CSF proteins in a fast exchange regime on the NMR timescale, which could then affect their relaxation times or chemical shifts. The reliability of the database of chemical shifts of model compounds and the lack of chemical shift changes on standard compound addition indicates that, apart from the observations above, any protein binding effects are minor.

The singlet peak at δ2.25 is lost completely after freeze-drying and has been assigned to acetone on the basis of its volatility and chemical shift.

No exchangeable protons from OH or NH groups are observed because the water resonance has been suppressed using a one-dimensional NOESY sequence which because of transfer of saturation will also suppress other peaks in chemical exchange with the water. To observe these types of proton, it would be necessary to remove the water resonance by non-excitation employing for example binomial or selective WEFT pulse sequences. Some information may be obtainable through the use of freeze-dried samples reconstituted into DMSO-d_6.

The only effects of refreeze-drying and reconstitution into D_2O is to cause the loss of a small unassigned triplet at δ1.19, the slight sharpening of a multiplet at δ1.54 and to remove the intensity anomalies seen in the H_2O solution due to partial suppression effects close to the water resonance. There are no further effects on any other resonances including those of glutamate and glutamine.

The major components in CSF detectable by ^{1}H-NMR spectroscopy are in the millimolar concentration range [7] and have been assigned previously [8–13]. In this study, through the application of two-dimensional NMR methods, it has proved possible to assign resonances

from compounds which have CSF concentrations down to about 100 micromolar.

Assignments

Amino acids

Alanine was readily identifiable in the one-dimensional spectrum giving a doublet at δ1.48 and a corresponding quartet visible in the JRES spectrum at δ3.79, with the connectivity established through a COSY cross-peak. Valine has also been identified previously and in this case the two non-equivalent methyl groups give doublets at δ0.99 and δ1.04 with the coupled β-CH at δ2.28 and in turn the α-CH appearing only in the COSY and JRES spectra at δ3.59. Glycine gives rise to a singlet confirmed by addition of authentic material at δ3.56 and observable in the JRES spectrum. The assignments for arginine were obtained from the COSY spectrum. The δ-CH_2 is clearly seen at δ3.24 with a connectivity to the γ-CH_2 at δ1.66 visible in the one-dimensional spectrum which in turn is coupled to the β-CH_2 at δ1.91 and which in turn is coupled to the α-CH at δ3.75. The cross-peak between δ3.24 and δ1.66 represents the best marker for arginine. The assignment for lysine follows a similar argument with the ε-CH_2 resonating at δ3.02 being coupled to the δ-CH_2 at δ1.71. This in turn is coupled to the γ-CH_2 at δ1.48 shown by a weak connectivity in the COSY spectrum which is then coupled to the β-CH_2 at δ1.91 with the connectivity to the α-CH observable giving the chemical shift of the latter at δ3.75. The best marker for lysine in the COSY spectrum is the clear cross-peak between δ3.02 and δ1.71, and in the one-dimensional spectra the resonances of the δ-CH_2 are clearly observable at δ1.71. The resonances for methionine are relatively easy to assign. The *S*-methyl gives rise to a singlet in the one-dimensional and JRES spectra at δ2.14. The γ-CH_2 group gives rise to a triplet also observable in the one-dimensional and JRES spectra at δ2.64. This in turn is coupled to the β-CH_2 at δ2.16 visible only in the COSY spectrum and which is connected to the α-CH at δ3.84 (COSY and JRES). Threonine also gives characteristic resonances. The side chain methyl group gives a doublet at δ1.33 with a COSY connectivity to the attached CH at δ4.24, the latter also being visible in the one-dimensional and JRES spectra. The β-CH is also coupled to the α-CH which appears in the COSY spectrum at δ3.56. Glutamate and glutamine give rise to prominent resonances in the NMR spectra of CSF especially after lyophilization. The assignments were confirmed by the addition of authentic materials. Thus the γ-CH_2 of glutamine appears as a multiplet at δ2.46 coupled as evidenced from the COSY spectrum to the β-CH_2 at δ2.14. The α-CH of glutamine is then characterized in the COSY spectrum by a cross-peak at δ3.79. Similarly the γ-CH_2 of glutamate appears in the one-dimensional spectrum at δ2.36 which from the COSY spectrum is coupled to the non-equivalent protons of the β-CH_2 group at δ2.06 and δ2.14. These in turn are coupled to the α-CH at δ3.76 visible in the JRES spectrum. Addition of glutamate to freeze-dried CSF causes the existing resonances of this compound to be shifted. The β-CH_2 chemical shift of glutamate moves to higher frequency whilst the γ-CH_2 shifts to low frequency. Similarly, addition of glutamine causes a high frequency shift of the γ-CH_2 resonance. These changes presumably reflect a modification in the equilibrium position of free and metal bound glutamate and glutamine complexes. Further evidence for such complexation in CSF arises from the observation of different chemical shifts for glutamate and glutamine in phosphate-buffered saline where the γ-CH_2 chemical shifts for glutamate and glutamine are reversed with respect to those in CSF and the β-CH_2 signals are also less non-equivalent. The other acidic amino acid, aspartate is easily assigned from the JRES and COSY spectra with the non-equivalent β-CH_2 protons and the α-CH appearing as an ABX spin system at δ2.68, δ2.81 (CH_2) and δ3.90 (CH). Isoleucine and leucine give complex spectra which can only be assigned through examination of the COSY spectrum. Thus the terminal CH_3 of isoleucine appears as a triplet at δ0.94 in the one-dimensional and JRES spectra. From the COSY connectivity this is coupled to a non-equivalent CH_2 group at δ1.28 and δ1.47. These in turn are coupled to the β-CH at δ1.91 which is also coupled to a methyl signal at δ0.98 from the COSY spectrum. The β-CH at δ1.91 is also coupled to the α-CH at δ3.75. The best marker for isoleucine is the triplet signal clearly visible in the one-dimensional spectrum at δ0.94. Leucine has a second order spectrum even at 600 MHz. The β-CH_2 and γ-CH give a complex multiplet centred at about δ1.72 with a connectivity to two methyl doublet signals at δ0.96

and δ0.97. This methyl geminal pair appears as a triplet in the one-dimensional ^{1}H spectrum of leucine in phosphate-buffered saline at 600 MHz but was shown to be two overlapping doublets by measurement of a JRES spectrum (not shown) in agreement with the result seen in the CSF spectrum. The connectivity between the signal at δ1.72 and the α-CH at δ3.73 is clearly seen in the COSY spectrum with the α-CH resonance visible in the JRES spectrum.

The aromatic amino acids are easily distinguished in the one-dimensional spectrum with histidine giving two characteristic singlets at δ7.04 (H4) and δ7.76 (H2). The aromatic resonances of tyrosine comprise the expected pattern for an AA'BB' spin system with shifts at δ6.90 (H3, H5) and δ7.20 (H2, H6) confirmed by the COSY connectivity. Similarly the phenyl resonances from phenylalanine appear at δ7.33 (H2, H6), δ7.38 (H4) and δ7.43 (H3, H5) all confirmed in the COSY spectrum. From studies on model compounds the β-CH_2 signals of tyrosine are expected to be the most shielded and so the shifts are assigned to the peaks at δ3.06 and δ3.20. Also the corresponding resonances from the α-CH for tyrosine are expected to be at the highest field and indeed the connection to the resonance at δ3.94 confirms this. Distinction between the phenylalanine and histidine $CHCH_2$ moieties is very difficult even at 600 MHz. They are distinguished on the basis of the relative amounts in the CSF, phenylalanine being the major of the two by about 50%. Therefore examination of the COSY and the JRES spectra (allowing for difficulties of quantitation) indicates that the α-CH of phenylalanine resonates at δ3.98 with the β-CH_2 protons at δ3.28 and δ3.12 and with the α-CH of histidine at δ3.96 with the corresponding CH_2 protons at δ3.22 and δ3.12.

The assignment of the remaining amino acids is more speculative. An ABX spin system is observed with the CH at δ3.98 with the corresponding CH_2 at δ2.84 and δ2.97. These resonances are close to those expected for asparagine but standard additions would not be very successful given the difficulty of observing the resonances in the one-dimensional spectrum. However the cross-peaks in the COSY provide good markers. Cysteine cannot be observed in the spectra and would have resonances very close to the aromatic amino acids. Similarly tryptophan is not detected at all in the aliphatic region as the cross-peak between the CH and CH_2 would be obscured by a *myo*-inositol resonance (see later). However minor peaks are observed in the aromatic region consistent with the aromatic resonances, only H2 being obscured by the phenylalanine (H2, H6) resonance. The observed shifts are δ7.73 (d, H4), δ7.20 (H5), δ7.28 (H6), δ7.54 (d, H7), all of these resonances showing appropriate connectivities in the COSY spectrum. Alternatively, these observed chemical shifts are also consistent with those of indoxyl sulphate with again that of H2 being obscured, this latter assignment being preferred from the known relative amounts of tryptophan and indoxyl sulphate in CSF [7]. Serine is very difficult to observe giving a tightly coupled multiplet for the $CHCH_2$ moiety close to the diagonal in the COSY plot and in the very crowded region between δ3.85 and 4.00, and so it was not detected. The remaining natural amino acid, proline, gives a complex spectrum and there are cross-peaks in the COSY spectrum which correspond to the expected chemical shifts. Thus a connectivity is seen between two resonances at δ2.06 and δ2.36 and a resonance at δ4.14. This matches the α-CH to β-CH_2 resonance in proline. In addition a resonance at δ2.03 (assigned to the γ-CH_2) shows a connectivity to two resonances at δ3.36 and δ3.45 and these match the expected shifts for the γ and δ methylenes, respectively, of proline.

Modified and other amino acids

A number of related molecules have already been assigned in the ^{1}H-NMR spectra of body fluids [1]. These include taurine which gives rise to two triplet resonances. These are evident in the one-dimensional, JRES and COSY spectra at δ3.41 for the CH_2SO_3 group and δ3.25 for the CH_2N group. GABA, γ-aminobutyric acid, contains three CH_2 groups. The one nearest the carboxyl terminus resonates at δ2.30 appearing as a triplet in the one-dimensional and JRES spectra showing a COSY connection to a resonance at δ1.89 being the β-CH_2 group which is in turn coupled as shown by the COSY spectrum to a resonance at δ3.03 appearing in the one-dimensional and JRES spectra and arising from the CH_2N group. In the CSF of Alzheimer's patients we have previously observed resonances of *N*-acetylaspartate, confirmed by addition of authentic compound [15]. This gives the familiar $CHCH_2$ ABX pattern visible in the

one-dimensional, JRES and COSY spectra, with the CH_2 protons resonating at δ2.50 and δ2.70 and the CH appearing at a distinctive shift of δ4.40. The *N*-acetyl resonance is visible at δ2.03. Small peaks are observed in the COSY and JRES spectra which are consistent with the presence of the amino acid citrulline, which is part of the urea cycle. The δ-CH_2 of citrulline has a shift of δ3.15 and this should be coupled to the γ-CH_2 with a shift of δ1.57. There is indeed a cross-peak in the COSY spectrum at these chemical shifts. Additional support for this structure arises from the cross-peak between this γ-CH_2 resonance and that for the β-CH_2 at δ1.85. This latter resonance should also be coupled to an α-CH resonance at about δ3.76 but this cannot be observed in the crowded region of the spectrum even in the COSY plot. There is no evidence for the presence of ornithine in any of the CSF samples examined. Also no β-alanine, which would give rise to triplets at δ3.19 and δ2.56, has been detected.

Organic acids

Many of these simple molecules have been assigned previously [1]. In this study the singlet of acetate at δ1.92, the singlet of pyruvate at δ2.41, and the AB pattern of citrate at δ2.67 and δ2.80 are observed as well as a singlet at δ6.53, assigned to fumarate. In addition, the signals of β-D-hydroxybutyrate are visible. The γ-CH_3 group gives a doublet in the one-dimensional and JRES spectra at δ1.20 and from the COSY plot the connection to the β-CH at δ4.18 can be made. This in turn is coupled in the COSY spectrum to the non-equivalent α-CH_2 protons at δ2.05 and δ2.51. Lactate gives rise to prominent well characterized resonances at δ1.33 (doublet, CH_3) and δ4.11 (quartet, CH). Formate gives rise to a singlet at δ8.46. Acidification of a sample from pH 7 to 2 resulted in the predictable high frequency shift of lactate with the methyl chemical shift moving to δ1.45 and the CH shift at δ4.43. Also under these circumstances the citrate and acetate resonances shift to high frequency. Acetoacetate is visible in the NMR spectra after lyophilization giving rise to a singlet for the methyl groups at δ2.29. The resonance of the methylene group, expected at δ3.45, is hidden in the complex overlapping resonances in the glucose region of the spectrum. Aromatic resonances in the one-dimensional spectrum and confirmed by cross-peaks in the COSY spectrum are consistent with those of indoxyl sulphate or tryptophan, the former assignment being preferred on the grounds of the known relative CSF concentrations [7]. Thus the doublet at δ7.73 (H4) is connected in the COSY spectrum to the triplet at δ7.20 (H5) which in turn is coupled to the triplet at δ7.28 (H6) which then shows a COSY connectivity to the doublet at δ7.54 (H7).

Oxaloacetate would be expected to give rise to a singlet at δ2.38 but this has not been observed as it would be obscured by the large complex multiplet from glutamine. Similarly, the presence of α-oxoglutarate cannot be confirmed unambiguously since although a triplet is observed in the JRES spectrum at δ2.47 consistent with one of the CH_2 groups, the other CH_2 group which is expected at δ3.01 cannot be detected in either the one-dimensional or JRES spectrum as it is obscured, if present, by other resonances. A singlet is detected in the JRES spectrum at δ2.44 consistent with the presence of succinate.

Figure 3(a) shows a clear triplet at δ0.90 which is correlated with a complex set of resonances at δ1.70 in the COSY spectrum. This in turn shows a possible weak correlation to a peak at δ3.99. These chemical shifts are consistent with the presence of α-hydroxy-*n*-butyrate. The shifts are not consistent with those of the related amino acid, α-amino-*n*-butyrate. Similarly, the triplet of α-hydroxy-*n*-valerate expected at δ0.92 has not been seen so far. In addition, the singlet for α-hydroxyisobutyrate expected at δ0.84 has also not been observed. On the other hand, a doublet can often be seen at δ0.84 which is consistent with the chemical shift of α-hydroxyisovalerate.

Organic bases and related compounds

Many of the compounds give rise to singlets and have been assigned previously [1]. Thus the resonances of dimethylamine (DMA) at δ2.72, trimethylamine (TMA) at δ2.88 and trimethylamine-*N*-oxide (TMAO) at δ3.23 can be observed. Distinction is possible between creatine and creatinine. Figure 3(b) and (c) shows singlets at δ3.04 and δ3.93 consistent with the chemical shifts of creatine. The ^{1}H-NMR spectra are not sensitive to whether the species is creatine or phosphocreatine. However, in other samples singlets have also been observed at δ3.05 and δ4.06 which are consistent with the presence of creatinine. The detection of choline is supported by obser-

vation of a singlet at δ3.21 and additional evidence is provided by the observation of a triplet from the NCH_2 group at δ3.49. The resonance of the CH_2O group would be hidden by the large lactate peaks.

A pair of doublets is observed in the aromatic region of the spectrum at chemical shifts of δ7.53 and δ5.80 with a coupling constant of about 7 Hz and with a COSY connectivity. These two groups of resonances are consistent with the pyrimidine nucleoside base uracil. A second set of similar peaks is detected at δ7.85 and δ5.90 and these remain unassigned at present.

Carbohydrates and polyols

In previous studies the presence of glucose has been inferred from observation of only the anomeric proton resonances for the α and β forms. Using authentic glucose and through the use of JRES and COSY spectra it has been possible to assign all of the resonances of both the α- and β-anomers. As well as the diagnostic H1 shifts of δ5.23 (α) and δ4.64 (β) the remainder of the shifts are listed in Table 1. Thus from the JRES spectra and COSY connectivity plots, H2 of β-glucose appears as a double doublet at δ3.24 which in turn is coupled to a triplet at δ3.49 (H3) which identifies H4 as another triplet as δ3.40. H5 of β-glucose appears as a characteristic eight line multiplet at δ3.47 showing strong coupling to H4 and to the two anisochronous protons of the CH_2(C6) group at δ3.90 and δ3.72 with the appropriate coupling patterns. The resonances from α-glucose are lower in intensity and can be mapped out starting with the H2 proton at δ3.53 (double doublet) coupled to H3 which appears as a triplet at δ3.71. This is in turn coupled to H4 also a triplet at δ3.41. H5 of α-glucose resonates at δ3.84 and appears as an eight line multiplet with 3 J couplings. The two protons of the CH_2 group appear at δ3.84 and δ3.74. The closeness of the chemical shifts of one of the C6-CH_2 protons and H5 ensures that the multiplicity seen in the JRES and one-dimensional spectra is not first order.

A low intensity doublet is seen only in the one-dimensional spectrum of CSF at δ4.52 with a coupling constant of the same magnitude as that of the H1 proton of β-glucose. This resonance is at the correct chemical shift for H1 of β-galactose and is therefore provisionally assigned as such. All other resonances of galactose (both α and β anomers) are hidden under other resonances.

Myo-inositol gives rise to some prominent features in the ^{1}H-NMR spectra of CSF. The signals from this molecule have been assigned previously [10] and the assignments have been confirmed through the use of COSY spectroscopy. Thus H2 resonates at δ4.06 (triplet) and is coupled to H1, H3 at δ3.53 (doublet of doublets), which is in turn coupled to H4, H6 at δ3.63 (doublet of doublets) and which is finally coupled to H5 at δ3.28 (triplet). In addition prominent resonances of glycerol can also be seen in the spectra consisting of an ABX-like spin system with the CH_2 group appearing at δ3.56 and δ3.65 coupled to the CH group at δ3.79.

A doublet of doublets at δ3.90 observed in the JRES spectrum, partially obscured by the aspartate α-CH resonance, has a COSY connectivity to another doublet of doublets at δ3.70, these chemical shifts being consistent with those of the CH_2 group of mannitol. Two CH resonances in mannitol are expected at about δ3.83 (C3H) and δ3.78 (C2H), the former coupled to the C2H peak and the latter also coupled to the resonances at δ3.83 and δ3.90. The δ3.78 peak cannot be detected because of severe overlap but a doublet is observed in the JRES spectrum at δ3.82 as expected although at a low intensity. Arabitol, if present, would give resonances visible in the JRES spectrum between δ3.58 and 3.94 and these are not observed.

Other species

The singlet at δ2.20, which is lost on freeze-drying, has been assigned to acetone on the basis of its chemical shift and volatility. A number of resonances remain unassigned particularly in the aromatic proton region of the spectrum and in the complex overlapped region between δ3.1 and 4.2 where carbohydrates usually resonate.

Conclusions

The use of high field ^{1}H-NMR spectroscopy coupled with the concerted application of homonuclear J-resolved and COSY techniques has enabled the identification of many new assignments in the NMR spectra of human CSF. This opens up further possibilities for investigating changes in the levels of these

Table 1
Assignments of resonances in the 600 MHz ^{1}H-NMR spectra of human CSF

Shift (δ)	Multiplicity	Molecule	Assignment
0.90	t	α-OH-*n*-butyrate	CH_3
0.94	t	isoleucine	δ-CH_3
0.96	d	leucine	δ-CH_3
0.97	d	leucine	δ-CH_3
0.98	d	isoleucine	β-CH_3
0.99	d	valine	CH_3
1.04	d	valine	CH_3
1.20	d	β-OH-butyrate	γ-CH_3
1.28	m	isoleucine	half γ-CH_2
1.33	d	lactate	CH_3
1.33	d	threonine	γ-CH_3
1.47	m	isoleucine	half γ-CH_2
1.48	m	lysine	γ-CH_2
1.48	d	alanine	CH_3
1.57	m	citrulline	γ-CH_2
~1.66	m	leucine	β-CH_2, γ-CH
1.66	m	arginine	γ-CH_2
1.70	m	α-OH-*n*-butyrate	CH_2
1.71	m	lysine	δ-CH_2
1.85	m	citrulline	β-CH_2
1.89	m	GABA	β-CH_2
1.91	m	lysine	β-CH_2
1.91	m	arginine	β-CH_2
1.91	m	isoleucine	β-CH
1.92	s	acetate	CH_3
2.03	m	proline	γ-CH_2
2.03	s	NAA	$COCH_3$
2.05	m	β-OH-butyrate	half α-CH_2
2.06	m	proline	half β-CH_2
2.06	dd	glutamate	half β-CH_2
2.14	m	glutamate	half β-CH_2
2.14	s	methionine	CH_3
2.14	m	glutamine	β-CH_2
2.16	m	methionine	β-CH_2
2.25	s	acetone	CH_3
2.28	septet	valine	β-CH_2
2.29	s	acetoacetate	CH_3
2.30	t	GABA	CH_2COO
2.36	m	proline	half β-CH_2
2.36	m	glutamate	γ-CH_2
2.41	s	pyruvate	CH_3
2.44	s	succinate	CH_2
2.46	m	glutamine	γ-CH_2
2.47	t	α-oxoglutarate	γ-CH_2
2.50	dd	NAA	half CH_2
2.51	m	β-OH-butyrate	half α-CH_2
2.64	t	methionine	γ-CH_2
2.67	d	citrate	half CH_2
2.68	dd	aspartate	half CH_2
2.70	dd	NAA	half CH_2
2.72	s	DMA	CH_3
2.80	d	citrate	half CH_2
2.81	dd	aspartate	half CH_2
2.84	dd	asparagine	half CH_2
2.88	s	TMA	CH_3
2.97	dd	asparagine	half CH_2
3.02	t	lysine	ε-CH_2
3.03	t	GABA	CH_2NH
3.04	s	creatine	CH_3
3.05	s	creatinine	CH_3
3.06	dd	tyrosine	half β-CH_2
3.12	dd	histidine	half β-CH_2
3.12	dd	phenylalanine	half β-CH_2
3.15	t	citrulline	δ-CH_2
3.20	dd	tyrosine	half β-CH_2
3.21	s	choline	$N(CH_3)_3$
3.22	dd	histidine	half β-CH_2
3.23	s	TMAO	CH_3
3.24	t	arginine	δ-CH_2
3.24	dd	β-glucose	H2
3.25	t	taurine	CH_2NH
3.28	dd	phenylalanine	half β-CH_2
3.28	t	inositol	H5
3.36	m	proline	half δ-CH_2
3.40	t	β-glucose	H4
3.41	t	α-glucose	H4
3.41	t	taurine	CH_2SO_3
3.45	m	proline	half δ-CH_2
3.47	ddd	β-glucose	H5
3.49	t	β-glucose	H3
3.49	t	choline	NCH_2
3.53	dd	α-glucose	H2
3.53	dd	inositol	H1, H3
3.56	s	glycine	CH_2
3.56	dd	glycerol	half CH_2
3.56	dd	threonine	α-CH
3.59	d	valine	α-CH
3.63	dd	inositol	H4, H6
3.65	dd	glycerol	half CH_2
3.70	dd	mannitol	half-CH_2
3.71	t	α-glucose	H3
3.72	dd	β-glucose	half CH_2–C_6
3.73	dd	leucine	α-CH
3.74	m	α-glucose	half CH_2–C_6
3.75	dd	isoleucine	α-CH
3.75	dd	lysine	α-CH
3.75	dd	arginine	α-CH
3.76	t	glutamate	α-CH
3.79	q	alanine	α-CH
3.79	ddd	glycerol	C_2-H
3.79	dd	glutamine	α-CH
3.82	d	mannitol	C_3H
3.84	m	α-glucose	half-CH_2-C_6
3.84	ddd	α-glucose	H5
3.84	dd	methionine	α-CH
3.90	dd	mannitol	half-CH_2
3.90	dd	aspartate	α-CH
3.90	dd	β-glucose	half CH_2-C_6
3.93	s	creatine	CH_2
3.94	dd	tyrosine	α-CH
3.96	dd	histidine	α-CH
3.98		asparagine	α-CH
3.98	dd	phenylalanine	α-CH
3.99	m	α-OH-*n*-butyrate	CH
4.06	t	inositol	H2
4.11	q	lactate	CH
4.14	m	proline	α-CH
4.18	m	β-OH-butyrate	β-CH
4.24	m	threonine	β-CH
4.40	dd	NAA	α-CH
4.52	d	β-galactose	H1
4.64	d	β-glucose	H1
5.23	d	α-glucose	H1
5.80	d	uracil	H5
6.53	s	fumarate	CH=CH
6.90	m	tyrosine	H3, H5
7.04	s	histidine	H4
7.20	m	indoxyl sulphate	H5
7.20	m	tyrosine	H2, H6
7.28	m	indoxyl sulphate	H6
7.33	m	phenylalanine	H2, H6
7.38	m	phenylalanine	H4
7.43	m	phenylalanine	H3, H5
7.53	d	uracil	H6
7.54	d	indoxyl sulphate	H7
7.73	d	indoxyl sulphate	H4
7.76	s	histidine	H2
8.46	s	formate	CH

Abbreviations: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; dd, doublet of doublets; ddd, doublet of doublets of doublets.

endogenous biochemicals as a result of pathological conditions.

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