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A branched-chain organic acid linked to multiple sclerosis: First identification by NMR spectroscopy of CSF

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Abstract

^{1}H NMR spectroscopy of cerebrospinal fluid (CSF) is currently being used to study metabolic profiles characteristic of distinct multiple sclerosis (MS) manifestations. For select MS patient groups, we have previously detected significantly increased concentrations of several identified metabolites and one unidentified compound. We now present, for the first time, the identification of the latter molecule, β-hydroxyisobutyrate (BHIB). A combination of dedicated 1D and 2D ^{1}H NMR experiments was employed for signal assignment. To our knowledge, BHIB has not previously been identified in ^{1}H NMR spectra of biofluids or biological tissues. Our assignment suggests new biochemical pathways involved in specific MS pathologies.

Keywords: Multiple sclerosis; Neurochemistry; Metabolomics; Cerebrospinal fluid (CSF); Inflammatory (active) plaques; ^{1}H NMR spectroscopy; 3-Hydroxyisobutyrate; Branched-chain amino acids; Gluconeogenesis

Multiple sclerosis (MS) is a remarkably heterogeneous disease of the central nervous system [1]. At present, numerous clinical and biomedical studies are being carried out to characterize MS in its various forms and stages. Among other methods, in vivo ^{1}H magnetic resonance spectroscopy of the brain and high-resolution ^{1}H NMR spectroscopy of cerebrospinal fluid (CSF) have been employed to detect differences in metabolic profiles between MS patient groups and controls. Thus, we have previously reported moderately increased CSF levels of lactate, creatinine, and fructose in MS vs. control patients [2]. In addition, two unidentified ^{1}H NMR signals, resonating at 1.06 and 1.08 ppm, were also elevated in MS patients (peaks referred to as 'XS1.06' and 'XS1.08' [2]). In a more recent study, the same unidentified signals were found to be significantly increased in CSF of clinically isolated syndrome (CIS) patients with vs. without inflammatory plaques (here referred to as 'U', unassigned doublet [3]). Up until now, biochemical and biological interpretation of the accumulation of the 'U' compound was hampered by the lack of assignment of these ^{1}H NMR signals to a specific molecule.

We have previously presented an approximate assignment of the peaks occurring at 1.06 and 1.08 ppm [4]. A series of pH-dependent one-dimensional ^{1}H NMR spectra had yielded, for this doublet, a titration curve that qualitatively resembled those of lactate and β-hydroxyisovalerate methyl resonances, showing comparable pK_a values (ca. 4.0–4.5) [4]. This result indicated that the two peaks to be assigned most likely represent a methyl doublet of a hydroxylated short-chain carboxylic acid. Moreover, with the aid of our data base comprising ^{1}H NMR spectra of

Abbreviations: AOIV, α-oxoisovalerate; BHIB, β-hydroxyisobutyrate; CIS, clinically isolated syndrome; CSF, cerebrospinal fluid; MS, multiple sclerosis; TSP-d_4, (sodium) 3-(trimethylsilyl)-2,2′,3,3′-tetradeuteropropionate.

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over 150 original substances, we have been able to exclude a number of commercially available candidate compounds for 'U'. We now present the full identification of the molecule in question. Our assignments are based on further one- and two-dimensional ^{1}H NMR spectra of CSF, and on comparison with a commercially unavailable compound synthesized in our laboratory (β-hydroxyisobutyrate, BHIB).

Materials and methods

CSF collection and sample preparation. CSF was obtained by lumbar puncture as described previously [5]. Samples were primarily used for routine diagnostic purposes; lumbar puncture for metabolic NMR analysis alone is not permissible for ethical reasons. In general, 1–3 ml CSF were available from each patient [5]. Samples were stored at −80 °C following lumbar puncture. For MRS signal assignment, CSF from 3–5 patients (max. 8 ml total volume) was combined and concentrated by lyophilization as published elsewhere [5]. A total of three concentrated samples were generated. The lyophilizate was redissolved in 620 μl D_2O, and 60 μl of a 15 mM solution of (sodium) 3-(trimethylsilyl)-2,2′,3,3′-tetradeuteropropionate (TSP-d_4) in D_2O was added for chemical-shift referencing. DCl and NaOD solutions were used to adjust pH to neutrality. All chemicals were purchased from Sigma–Aldrich (Saint Quentin, Fallavier, France).

BHIB synthesis. BHIB was synthesized by hydrolyzing 1 ml of the ester, methyl β-hydroxyisobutyrate, in 10 ml of a 10 M NaOH solution for ca. 2 h at room temperature. The resulting solution was neutralized with HCl and lyophilized. The lyophilizate was redissolved in 1 ml D_2O and centrifuged. The completeness of the ester hydrolysis was verified by ^{1}H NMR of the supernatant.

NMR spectroscopy. 1D ^{1}H spectra were acquired on 400 and 500 MHz FT-NMR spectrometers (Bruker, Wissembourg, France). Samples were examined using 5-mm Wilmad 528-PP tubes (Carlo Erba-SDS, Val de Reuil, France), spinning at a rate of 20 Hz. Standard Bruker Eurotherm variable temperature units were employed to maintain a sample temperature of 28 °C. ^{1}H NMR spectra were acquired using a repetition time of 15 s and a pulse width (90°) of 12.1 μs, preceded by water proton presaturation for 6 s (power setting 0.001 W). The acquisition time was 6.56 s (64 k) for each transient; up to 64 transients per spectrum were collected. NMR signals were apodized (exponential multiplication using 0.2 Hz line broadening), Fourier transformed, and phase and baseline corrected. Spectra were referenced to TSP-d_4. 2D ^{1}H NMR experiments were performed without sample spinning, by using standard pulse sequences for J-resolved and COSY spectroscopy. The number of t_1 increments varied between 128 and 512 for COSY experiments. Further acquisition and processing parameters have been detailed previously [4].

NMR assignments of signals other than BHIB were based on chemical-shift values previously obtained for ^{1}H NMR spectra of CSF samples [2,4], and on spiking with commercially available original compounds.

Results

1D ^{1}H NMR spectra were obtained for CSF, BHIB, and CSF upon BHIB addition. Fig. 1a shows a complete CSF spectrum. The peaks to be assigned were relatively weak, but could be easily analyzed after 32–64 transient accumulations. The chemical-shift region between 0.8 and 1.5 ppm contains several methyl signals, for the most part previously assigned doublets and triplets from aliphatic short-chain carboxylic acid and amino acid protons [4]. Fig. 1b shows the spectrum of a solution of BHIB obtained by hydrolysis of BHIB methyl ester. The absence of significant impurities demonstrates the completeness of the hydrolytic reaction. The integral ratios immediately yielded the assignments to the methyl, methine and methylene moieties, corresponding to 3:1:2 protons (from high to low field, as annotated in Fig. 1c). The methine signal revealed a complex multiplet, due to coupling with the methyl and methylene protons. The methylene signal consists of two groups of resonances, each consisting of four individual peaks. Each group is due to one methylene proton characterized by strong coupling with the geminal proton (apparent coupling constant 10.8 Hz), and weak coupling with the vicinal proton (coupling constants 7.8 or 5.8 Hz). Note the roof effect due to the relatively small chemical-shift difference between the two methylene protons (0.066 ppm, corresponding to 27 Hz).

BHIB spiking of a concentrated CSF sample resulted in exact superposition on the methyl peaks to be assigned (Fig. 2). Subsequently, 1D ^{1}H NMR spectra of the spiked sample were acquired for nine pH values ranging from 2.0 to 8.0 in order to obtain a titration curve for the chemical shift of the BHIB methyl signal (Fig. 3). Over the entire pH range, the methyl doublet due to the added BHIB remained exactly superimposed on the CSF doublet to be assigned.

Fig. 4 presents the high-field region of a J-resolved 2D ^{1}H NMR spectrum of a concentrated unspiked CSF sample, which we acquired to confirm the doublet structure of the methyl signals in question (A). A 1D skyline projection of the J-resolved spectrum is plotted on top of the 2D spectrum, to permit direct comparison of the J-resolved spectrum with a 1D spectrum not complicated by coupling patterns. The existence of spin-spin coupling between the doublet at 1.07 ppm and a resonance at 2.5 ppm in CSF was examined with a ^{1}H COSY experiment on a concentrated unspiked CSF sample (B). The corresponding cross peak was easily detected (Fig. 4).

Discussion

BHIB assignment

We identified β-hydroxyisobutyrate for the first time in ^{1}H NMR spectra of CSF. To our knowledge, ^{1}H NMR signals of this compound have not previously been assigned in any biofluid or biological tissue (intact or after extraction). A combination of dedicated 1D and 2D ^{1}H NMR experiments was employed for signal assignment. The addition of chemically synthesized BHIB allowed the identification, in 1D spectra, of the methyl doublet, which was confirmed by pH titration. The titration curve fell precisely on a previously published curve for the unassigned doublet 'U' in unspiked CSF [4]. A J-resolved 2D ^{1}H NMR spectrum of unspiked CSF provided the definite proof of the doublet structure of the pair of peaks at 1.06 and 1.08 ppm.

While this methyl doublet assignment to BHIB was rather straight forward with the original compound made available, unambiguous assignments of BHIB methylene and methine signals were not achievable based on spiking.

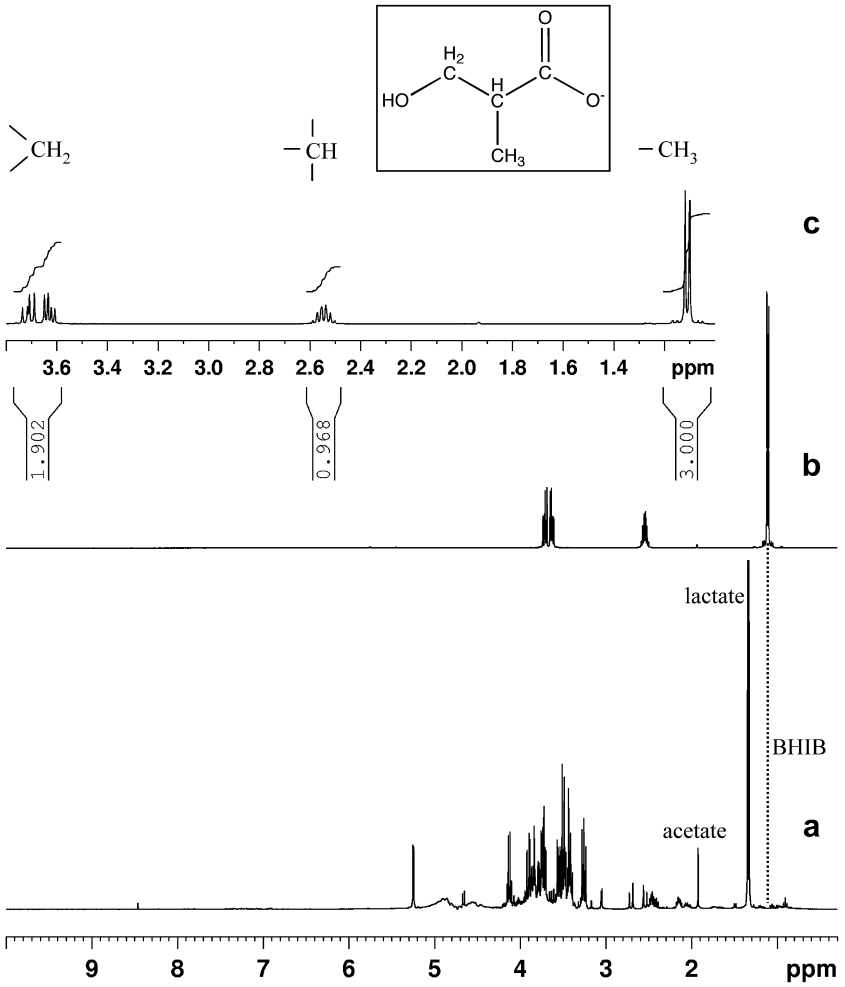

Fig. 1. (a) 1D ^{1}H NMR spectrum of lyophilized CSF redissolved in D_2O at pH 7.0 (sample from patient with active MS plaques). An expanded view of the relevant spectral region is displayed in Fig. 2. (b) 1D ^{1}H NMR spectrum of a BHIB solution in D_2O at pH 7.0. (c) BHIB multiplets in an expanded spectral region of (b), together with the molecular structure of BHIB.

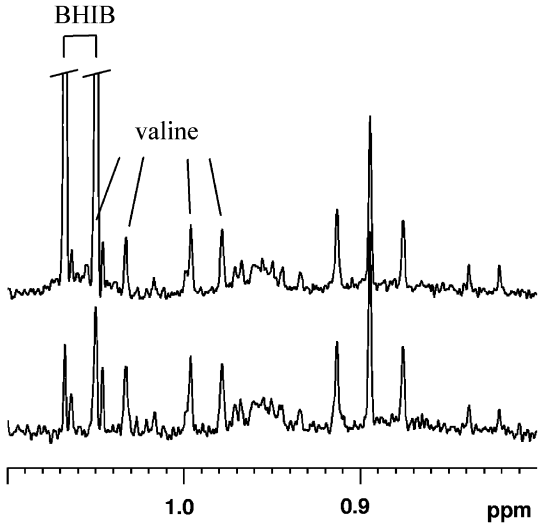

Fig. 2. Expanded spectral region of a CSF sample before (bottom) and after (top) addition of BHIB. For better orientation, peaks adjacent to the BHIB methyl doublet are also annotated (valine).

This is due to the relatively low intensities of these signals in unspiked CSF, in combination with relatively high intensities of overlapping peaks. However, the cross peak at 1.07/2.5 ppm in COSY spectra of unspiked CSF, in conjunction with the 1D ^{1}H NMR spectrum of the BHIB solution, clearly demonstrated the existence, at the expected chemical shift, of a methine group coupled with the methyl group assigned as described above. The remaining cross peak expected in a homonuclear COSY spectrum of BHIB is the methylene/methine peak at 2.5/3.6 ppm (values based on the 1D ^{1}H NMR spectrum of the BHIB solution). However, the low signal-to-noise ratio of this cross peak, in combination with 2D noise from strong sugar signals around 3.6 ppm, did not allow unambiguous detection of this cross peak in unspiked CSF.

The BHIB concentration range determined by 1D ^{1}H NMR spectroscopy of control CSF (before concentrating the samples), was of the same order of magnitude (14–22 μM [3]) as values previously determined by gas chromatography–mass spectrometry (GC–MS) (ca. 2–34 μM [6]).

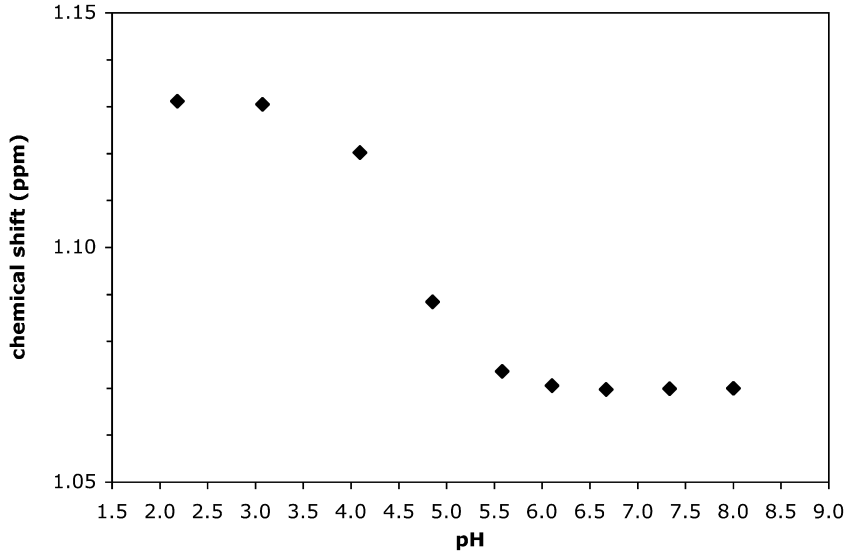

Fig. 3. Titration curve for the BHIB methyl doublet following BHIB addition to a concentrated CSF sample (pH range 2–8). This curve falls precisely on a titration curve previously determined for the unassigned doublet 'U' in a pure CSF sample [4].

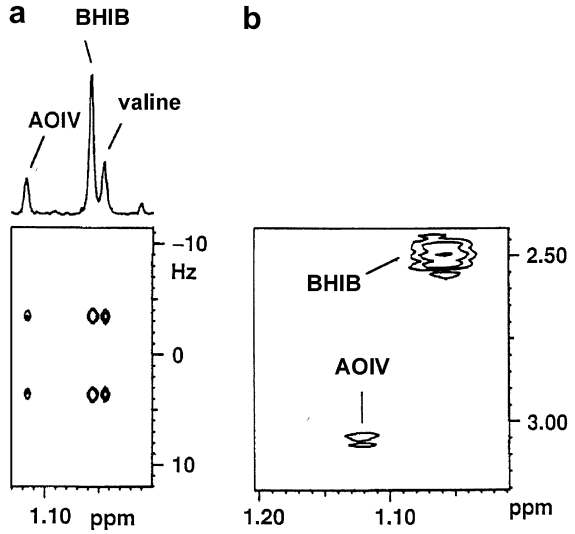

Fig. 4. (a) High-field region of a J-resolved 2D spectrum of concentrated CSF at pH 7.0, including its 1D projection. The BHIB doublet at 1.07 ppm is readily recognized. (b) Relevant region of a COSY spectrum of concentrated CSF at pH 7.0. The correlation peak at 1.07/2.5 ppm is consistent with a previously reported cross peak for 'U' [4], and with the CH_3–CH correlation expected for BHIB based on Fig. 1c. For better orientation, signals adjacent to the BHIB methyl doublet are also annotated: α-oxoisovalerate (AOIV) and valine.

As a matter of fact, the commercial availability of BHIB methyl ester may explain why BHIB in biofluids has long been identified by GC–MS where carboxylic acids are often derivatized (esterified) before analysis. In contrast, metabolic ^{1}H NMR spectroscopy of CSF samples does not require derivatization; therefore, unesterified BHIB (commercially unavailable) needs to be added to samples for NMR spiking experiments.

BHIB metabolism

BHIB is present not only in CSF, but also in other biofluids. For instance, BHIB has been detected by GC–MS in urine, plasma and amniotic fluid [6,7]. The BHIB concentration ranges determined for our CSF control samples were somewhat lower than those generally found in plasma (17–58 μM [7], 6–51 μM [6]) and serum (10–45 μM [8]). In contrast, BHIB ranges reported for urine (4.1–19 μM [7]) are somewhat lower than our CSF values, while BHIB levels are considerably higher in amniotic fluid (42–117 μM [7]). BHIB is a typical partial-degradation product of branched-chain amino acids released from muscle for hepatic and renal gluconeogenesis [9]. For valine, BHIB is quantitatively the most important intermediate. Because of its presence in blood it has been postulated to be an inter-organ metabolite [8,9]. Thus, a BHIB increase in CSF may be due to an increased uptake from blood since BHIB tends to be more concentrated in serum than in CSF.

Little is known about the role of BHIB in brain metabolism. However, it has been suggested that in the metabolic disease, 3-hydroxyisobutyric aciduria, BHIB accumulation in urine [10–13] or amniotic fluid [10] may be caused by tissue with inhibited NAD^+-dependent 3-hydroxyisobutyrate dehydrogenase due to an increased $NADH/NAD^+$ ratio [14–16]. Consequently, increased BHIB concentrations in urine were ascribed to respiratory-chain deficiency leading to impaired oxidation of NADH and secondary inhibition of 3-hydroxyisobutyrate dehydrogenase, presumably for the most part in liver and muscle [14,16]. However, it should be noted that the BHIB increases measured in CSF of MS patient groups [2,3] were by far less pronounced than the BHIB accumulation observed in 3-hydroxyisobutyric aciduria patients. The mechanism of BHIB enhancement in MS patient groups remains to be elucidated.

Conclusion

BHIB was identified, for the first time, in NMR spectra of cerebrospinal fluid. A combination of dedicated 1D and 2D ^{1}H NMR experiments was employed for complete BHIB identification. BHIB, whose concentration is significantly increased in some MS patients, is a typical partial-degradation product of branched-chain amino acids. The origin of the BHIB increase awaits further clarification.

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