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Characterisation of the ^{1}H and ^{13}C NMR spectra of *N*-acetylaspartylglutamate and its detection in urine from patients with Canavan disease

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Abstract

^{1}H and ^{13}C NMR spectra of *N*-acetylaspartylglutamate (NAAG) have been recorded and interpreted. The values of the ^{1}H chemical shifts and $^{1}H-^{1}H$ coupling constants at different pH were obtained by iterative computer fitting of 1-D ^{1}H NMR spectra. This provided information on the solution conformation of the investigated molecule. Proton-decoupled high resolution ^{13}C NMR spectra of NAAG have been measured in a series of dilute water solution of various acidity. These data have provided a basis for unequivocal determination of the presence of NAAG in the urine sample of a patient suffering from Canavan disease. NMR spectroscopy provides a possibility of detecting NAAG in body fluids.

Keywords: ^{1}H, ^{13}C NMR; *N*-Acetylaspartylglutamate; Canavan disease

1. Introduction

N-acetylaspartylglutamate (NAAG) is the neuropeptide localised to neurons with a high affinity for metabotropic glutamate receptors, mGluR3 [1]. It is an antagonist at NMDA receptors. It is catabolised by carboxypeptidase-II, which is expressed on astrocyte membranes, to *N*-acetylaspartate (NAA) and glutamate. Recent reports show that there is an increase of NAAG in the brain of people during an epileptic attack and immediately after it (the ictal and early postictal states) [2,3]. Conversely its quantity is lowered in the average and advanced phases of multiple sclerosis [4].

Canavan disease [5] is autosomal recessive neurodegenerative disorder affecting cerebral white matter. The disease is caused by an aspartoacylase deficiency and is characterised by an increased level of NAA in urine and brain. It was also shown that, the level of NAAG increases in urine of patients suffering from Canavan disease [6,7].

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The complete literature assignment of the NMR spectrum at different pH is not available with only partial data [8–12]. Generally there is lack of ^{13}C NMR data. We decided to perform suitable measurements on NAAG and provide a full assignment.

The application of different NMR techniques to the analysis of physiological fluids has become very popular during the recent decade [13–23]. The NMR methods provide information not only on the presence of a particular substance in a sample, but also on its quantity and its molecular structure. The complementary usage of different NMR techniques is a very useful approach in biomedical research. 1-D ^{1}H NMR, ^{13}C NMR and 2-D correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), heteronuclear single quantum coherence (HSQC) and heteronuclear multi-bond correlation (HMBC) methods were used in our investigation. The NMR spectra of NAAG were recorded at different pH and chemical shift data were collected. Finally, the results were used to confirm the presence of NAAG in urine of children suffering from Canavan disease.

2. Materials and methods

Measurements were performed on samples of unprocessed urine or 0.15 M water solution of NAAG (Sigma Chemical Co.), or on a mixture of NAA (Sigma Chemical Co.) and NAAG in proportion 4: 1. The 0.5 ml of urine or the NAAG solution was transferred to a 5-mm o.d. high precision NMR tube. Then 50 μl D_2O was added containing 3-trimethylsilyl-2,2,3,3-tetradeuteropropionic acid (TSP; sodium salt; Dr. Glaser AG Basel, concentration 0.2 μl/ml) as the spectrometer field lock and chemical shift reference ($\delta(^{1}H,\ ^{13}C)$, 0.0 ppm). The sample pH was controlled directly in the NMR tube using a pH electrode and a pH-meter (Cole-Parmer Instrument Co). This measuring system was standardised using pH 1.68, 4, 7, 10 (Cole-Parmer Instrument Co) buffers. The pH of the NAAG solution or of the NAAG and NAA mixture solution was adjusted by adding small amounts of 1 M HCl or 0.55 M $NaOH \cdot H_2O$ solutions.

The proton-decoupled ^{13}C NMR spectra were recorded using a Varian UNITY plus spectrometer operating at 11.7 T magnetic field. The standard measurement parameter set was: pulse width, 7 μs (the 90° pulse width was 12.5 μs), acquisition time 1 s, spectral width 200 ppm, WALTZ 16 ^{1}H decoupling. Four thousand to eight thousand scans were accumulated and after zero-filling to 64 K, the FID signals was subjected to Fourier transformation after application of a 1 Hz line broadening. Similar measurement and processing conditions were applied when using MERCURY Vx and GEMINI 2000 spectrometer operating at 9.4 and 4.7 T, respectively, for some measurement. The only essential difference was in the accumulation time.

^{1}H NMR spectra with presaturation of water were recorded using standard VARIAN software. The values of ^{1}H chemical shifts and ^{1}H–^{1}H coupling constants reported in Table 1 were obtained by iterative fitting the line positions in the 1-D ^{1}H NMR spectra using the program SYMUL written by Adam Gryff-Keller [24].

The ^{1}H–^{1}H COSY, TOCSY, and the ^{1}H–^{13}C HSQC and HMBC 2-D spectra were also recorded using the standard VARIAN software.

The experimental parameters were as follows:

^{1}H–^{13}C HSQC with a selective pulse (for first 180° carbon pulse, offset 35 ppm, bandwidth 80 ppm and shape SINC) for mixture of NAA and NAAG were recorded using 1024 datapoints, and spectral width of 6000 Hz at 500 MHz, and eight scans per increment. In F_1, 26 increments were used. The delay between scans was 1.7 s and the spectra were zero-filled to 512 datapoints in F_1.

HSQC with a selective pulse (for first 180° carbon pulse, offset 35 ppm, bandwidth 80 ppm and shape SINC) for a sample of urine from a patient suffering from Canavan disease previously diagnosed by a GC–MS method were recorded using 4096 datapoints, and spectral width of 6000 Hz at 500 MHz, and 64 scans per increment. In F_1, 256 increments were used. The delay between scans was 1.7 s and the spectra were zero-filled to 2048 datapoints in F_1.

The measurement temperature was 30 °C.

Table 1
Scalar coupling constants (Hz) between protons J_{ij} and proton chemical shifts (ppm) of NAAG at different pH values

J_{ij}	pH		
	2.45	3.95	6.75
2–3	4.92 (±0.01)	4.62 (±0.01)	4.61 (±0.02)
2–3′	9.35 (±0.02)	8.47 (±0.02)	8.42 (±0.04)
2–6	7.23 (±0.04)	7.55 (±0.04)	7.46 (±0.01)
3–3′	−14.22 (±0.04)	−13.98 (±0.03)	−14.28 (±0.03)
3–4	7.35 (±0.04)	8.13 (±0.03)	10.56 (±0.03)
3–4′	7.85 (±0.03)	7.44 (±0.02)	6.09 (±0.02)
3′–4	7.50 (±0.02)	5.73 (±0.02)	4.90 (±0.01)
3′–4′	6.20 (±0.03)	9.19 (±0.03)	11.11 (±0.03)
4–4′[a]	−9.75	−10.84	−15.28
8–9	7.93 (±0.01)	8.89 (±0.04)	9.62 (±0.02)
8–9′	5.36 (±0.01)	4.62 (±0.02)	4.38 (±0.01)
8–11	7.33 (±0.04)	7.51 (±0.04)	7.32 (±0.03)
9–9′	−16.91 (±0.02)	−16.43 (±0.03)	−15.97 (±0.01)
δ			
2	4.43 (±0.02)	4.22 (±0.01)	4.14 (±0.02)
3	2.22 (±0.04)	2.13 (±0.02)	2.06 (±0.03)
3′	2.01 (±0.03)	1.94 (±0.02)	1.90 (±0.02)
4	2.47 (±0.01)	2.37 (±0.01)	2.20 (±0.01)
4′	2.48 (±0.01)	2.37 (±0.02)	2.21 (±0.01)
6	8.36 (±0.04)	8.04 (±0.04)	7.95 (±0.04)
8	≈4.68[b]	≈4.75[b]	4.63 (±0.04)
9	2.82 (±0.02)	2.66 (±0.03)	2.54 (±0.02)
9′	2.91 (±0.02)	2.81 (±0.03)	2.74 (±0.02)
11	8.38 (±0.04)	8.36 (±0.04)	8.26 (±0.03)

[a] The determination precision of $J_{44'}$ is poor, which is the result of a very small $\delta 4 - \delta 4'$, chemical shift difference between the interacting protons.
[b] The signal is found partially in water resonance.

3. Results and discussion

The chemical shift assignments in the proton spectrum of the investigated compound were the starting point for further analysis of the ^{1}H and ^{13}C NMR spectra. The proton ^{1}H NMR of NAAG in the water solution at pH 2.45 is shown in Fig. 1a. The signals in proton spectrum lying in 2–5 ppm range were from protons of aspartyl and glutamate residue. Other signals (8–9 ppm) were from amide protons. Those assignments were done by the use of correlation spectroscopy (^{1}H–^{1}H COSY) and TOCSY techniques [25]. In the next step the computer analysis of the 1-D ^{1}H NMR spectrum at pH 2.45, 3.95 and 6.75 was performed (Table 1). The obtained values of the coupling constants were determined for the first time and provide information on the solution conformation of the investigated molecule. Scalar couplings are widely used in the analysis of NMR spectra as indicators of the molecular framework. Together with NOE-derived ^{1}H–^{1}H distances, they can be used to obtain molecular structures in solutions [26,27]. Scalar couplings between vicinal (three-bond) protons and torsion angles (the Karplus relation [28,29]) are very important for obtaining conformations of small organic molecules and for large molecules such as proteins [30].

The results collected in Table 1 give information about configuration of NAAG in solution. Considering values of four vicinal coupling constants between protons of the glutamate CH_2CH_2 fragment of NAAG (protons 3, 3′, 4 and 4′) it can be shown that at the highest pH used they are very different. Two of them ($^3J_{34}$ and $^3J_{3'4'}$) are high, and close to the expected value for a *trans* arrangement of the interacting protons, whereas the two remaining ($^3J_{34'}$ and $^3J_{3'4'}$) are much smaller and close to the value expected for a *gauche* arrangement. These results suggest the strong preference of conformation **A** (Fig. 2a) at high pH. When the pH of the solution is decreased, the differences between values of these coupling constants disappear and eventually all four values fall into the narrow (6.2–7.8 Hz) range. This observation points out that in acidic solutions all three *staggered* conformations are similarly populated.

The remaining vicinal coupling constants including those involving the amide protons exhibit a lower sensitivity toward pH changes. Also for the glutamyl residue the small value of $^3J_{23}$ at about 4.7 (4.61–4.92) Hz indicates that the involved protons are in a *gauche* arrangement. At the same time, the value of $^3J_{23'}$ (8.42–9.35 Hz), the second coupling constant which depends mainly on the dihedral angle about the C-2–C-3 bond, could arise only from averaging of the *trans* and *gauche* conformation couplings. Thus, NAAG assumes the conformation **D** of Fig. 2b and only one of two other possible conformations. An increase of $^3J_{23'}$ with decreasing pH may reflect an increase in the population of the conformer **D**.

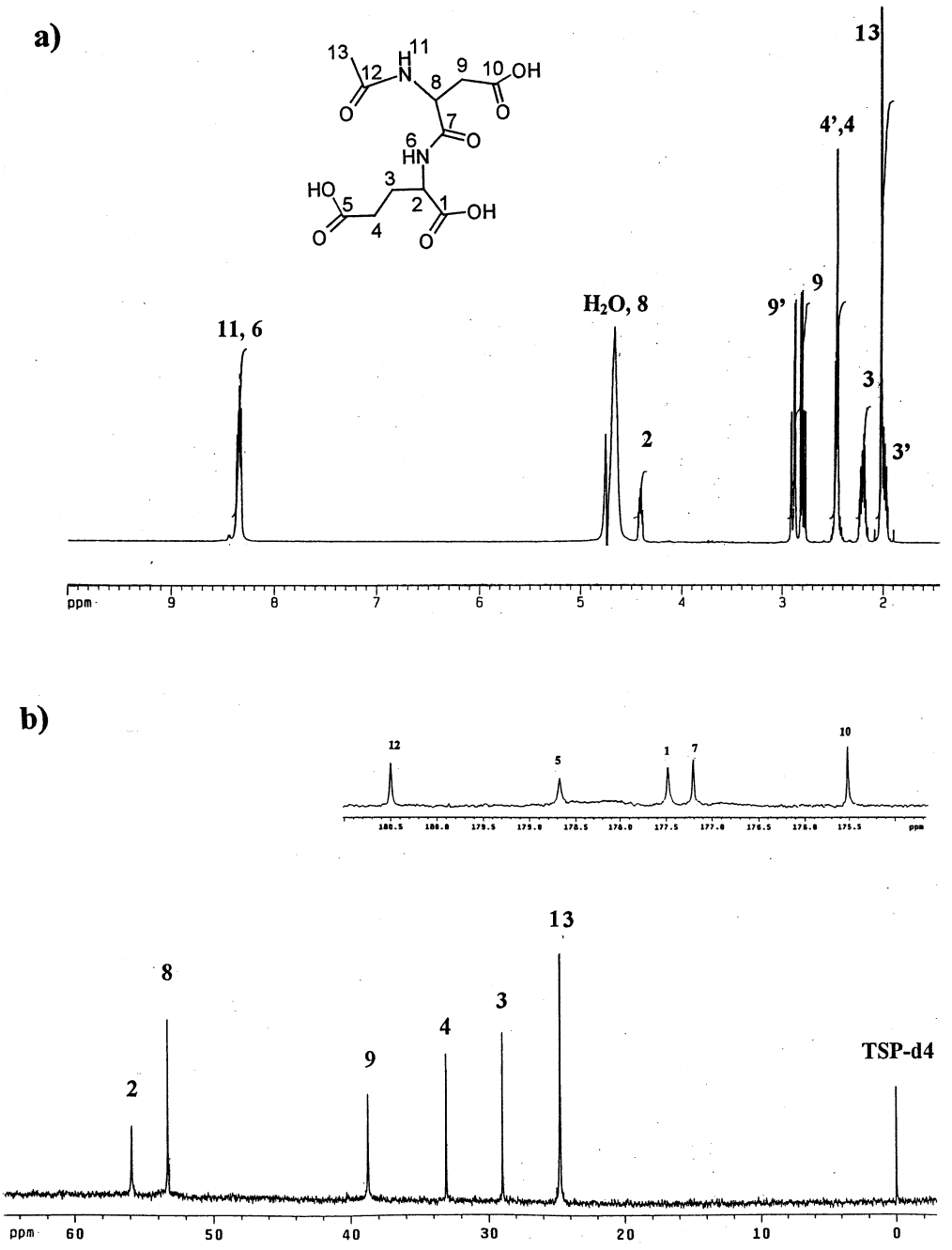

Fig. 1. NMR spectra of NAAG in water solution, pH 2.45; (a) ^{1}H spectrum; (b) ^{13}C spectrum.

The same situation is observed for $^{3}J_{89}$ and $^{3}J_{89'}$ coupling constants which are connected with the conformation of the aspartyl part of NAAG. In this case, however, the pH effect is somewhat stronger than for $^{3}J_{23}$ and $^{3}J_{23'}$ couplings and $^{3}J_{89}$ increases at high pH and $^{3}J_{89'}$ increases at low pH.

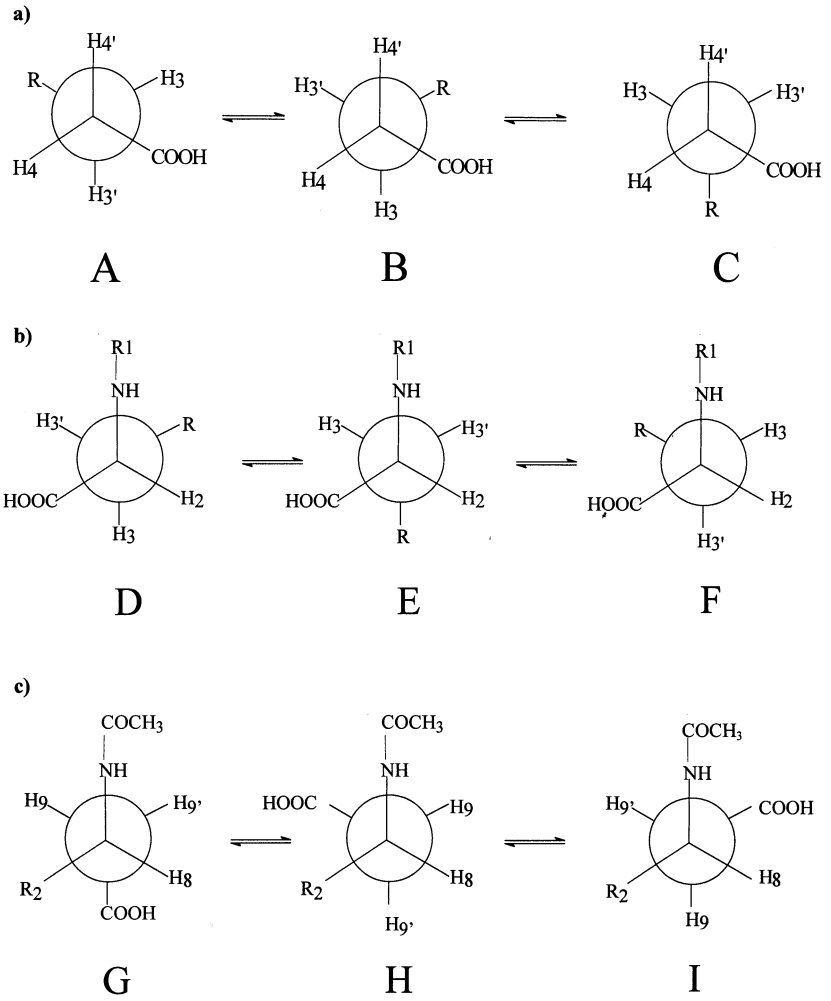

Fig. 2. *Staggered* conformations of NAAG; (a) the glutamic CH_2CH_2 fragment (protons 3, 3′, 4 and 4′, R-molecule residue); (b) the glutamic fragment (protons 2, 3, and 3′, R-glutamate residue, R1-aspartyl residue); (c) the aspartic fragment (protons 8, 9, 9′, R-glutamate residue).

These results suggest the strong preference of conformation **G** (Fig. 2c) at high pH. When the pH of the solution is decreased, the differences between values of these coupling constants disappear. This observation points out that in acidic solutions all three *staggered* conformations are similarly populated. The results are in agreement with data obtained for NAA using transition metal ions [31,32].

When comparing the values of the geminal coupling constants it is seen that the largest pH effect is manifested for $^2J_{44'}$. Unfortunately, the determination precision of just this parameter is poor, which is the result of a very small $\delta 4 - \delta 4'$ chemical shift difference between the interacting protons. It is interesting that the other geminal coupling constant between protons which are next to a carboxylic group ($^2J_{99'}$) is much less pH-sensitive. This type of coupling is known to be influenced among other factors by the electronegativity of substituents, and by the relative orientation of a neighbouring carbonyl group, i.e. by

conformation [33,34]. The former factor can be, indeed, pH-sensitive, as the electronegativity of anionic ($-COO^-$) and neutral (–COOH) forms of the carboxylic group are quite different, and the dissociation degree changes with pH.

The coupling constants between the amide protons and the adjacent α-CH protons do not change considerably with change of pH. Thus the average these parts of the spatial configuration of atoms in amide molecule do not change in conformation in all ranges of the measured pH.

With regard to diagnostic applications, ^{13}C spectra of the dipeptide NAAG at ten different pH values from 1.8 to 9.4 were also measured. The proton decoupled ^{13}C NMR spectrum of NAAG in the water solution at pH 2.45 is shown in Fig. 1b. The signal at 0 ppm originated from TSP-d_4 being the chemical shift reference. The signals lying downfield (20–60 ppm) were from methyl, methylene and methine carbons of NAAG, while those lying (170–185 ppm) were carbonyl carbons. The latter signals were of the characteristic low intensities because of the long relaxation times and reduced nuclear Overhauser enhancement factors. The detailed assignment of all the ^{13}C NMR signals of NAAG in a water solution was established on the basis, HSQC and HMBC [25].

The sensitivity of particular carbons in NAAG to pH variation was represented by the chemical shift difference ($\Delta\delta$) between the solutions of pH 1.8 and 9.4. These ranges are given in the last row of Table 2. The observed sensitivity order was as follows:

$$\Delta\delta_{C5} > \Delta\delta_{C4} > \Delta\delta_{C10} > \Delta\delta_{C9} > \Delta\delta_{C1} > \Delta\delta_{C3} > \Delta\delta_{C2} > \Delta\delta_{C8} \gg \Delta\delta_{C12} > \Delta\delta_{C7} > \Delta\delta_{C13}$$

It can be noted that values of the chemical shift for the carboxylic carbons C-5, C-10, C-1 are large and for the carbonyl carbons C-7, C-12 are small. It is obvious that this enhanced sensitivity was related to the association–dissociation phenomena of their carboxylic protons. This is well known [14] and has been observed earlier for argininosuccinic acid [13].

Protonated carbons, which are close to carboxylic carbons, also show large pH sensitivity:

$$\Delta\delta_{C4} > \Delta\delta_{C9} > \Delta\delta_{C3} > \Delta\delta_{C2} > \Delta\delta_{C8}$$

and one can suppose, that association–dissociation phenomena carboxylic protons has influence on sensitivity to those carbons. The position of the methyl group signal does not change on the whole range of pH value ($\Delta\delta_{C\text{-}13}$ 0.02).

The results above show, that the pH environment is very important in NMR spectroscopic analysis of body fluids. In Canavan disease, both NAA as well as the dipeptide NAAG accumulate in urine of patients suffering from this disease [6]. ^{13}C spectra of NAA were measured at different pH (Table 3). Next NAA and NAAG were mixed in a

Table 2
pH Dependence of ^{13}C chemical shifts δ (ppm) of NAAG in water solution

pH	^{13}C chemical shifts δ (ppm) of carbon number										
	1	2	3	4	5	7	8	9	10	12	13
1.80	178.58	55.81	28.93	33.06	180.43	175.51	53.29	38.70	177.20	177.41	24.76
2.45	178.67	55.92	29.00	33.10	180.50	175.51	53.33	38.76	177.20	177.47	24.76
4.85	179.57	57.93	30.94	35.81	183.90	175.80	54.65	41.29	177.20	177.15	24.76
5.15	181.16	58.02	31.11	36.18	184.37	175.81	54.69	41.42	180.57	177.12	24.78
6.20	181.20	58.13	31.31	36.61	184.95	175.84	54.75	41.54	180.60	177.11	24.76
6.75	181.19	58.16	31.42	36.73	184.96	175.80	54.78	41.61	180.68	177.07	24.76
7.20	181.21	58.17	31.36	36.71	185.00	175.86	54.76	41.56	180.70	177.12	24.76
8.20	181.32	58.16	31.34	36.71	185.06	175.86	54.75	41.56	180.75	177.11	24.76
9.20	181.27	58.16	31.39	36.72	185.03	175.83	54.76	41.58	180.73	177.10	24.76
9.40	181.30	58.17	31.36	36.72	185.06	175.86	54.75	41.58	180.77	177.11	24.76
$\Delta\delta$	2.74	2.36	2.49	3.66	4.65	0.35	1.49	2.91	3.57	0.40	0.02

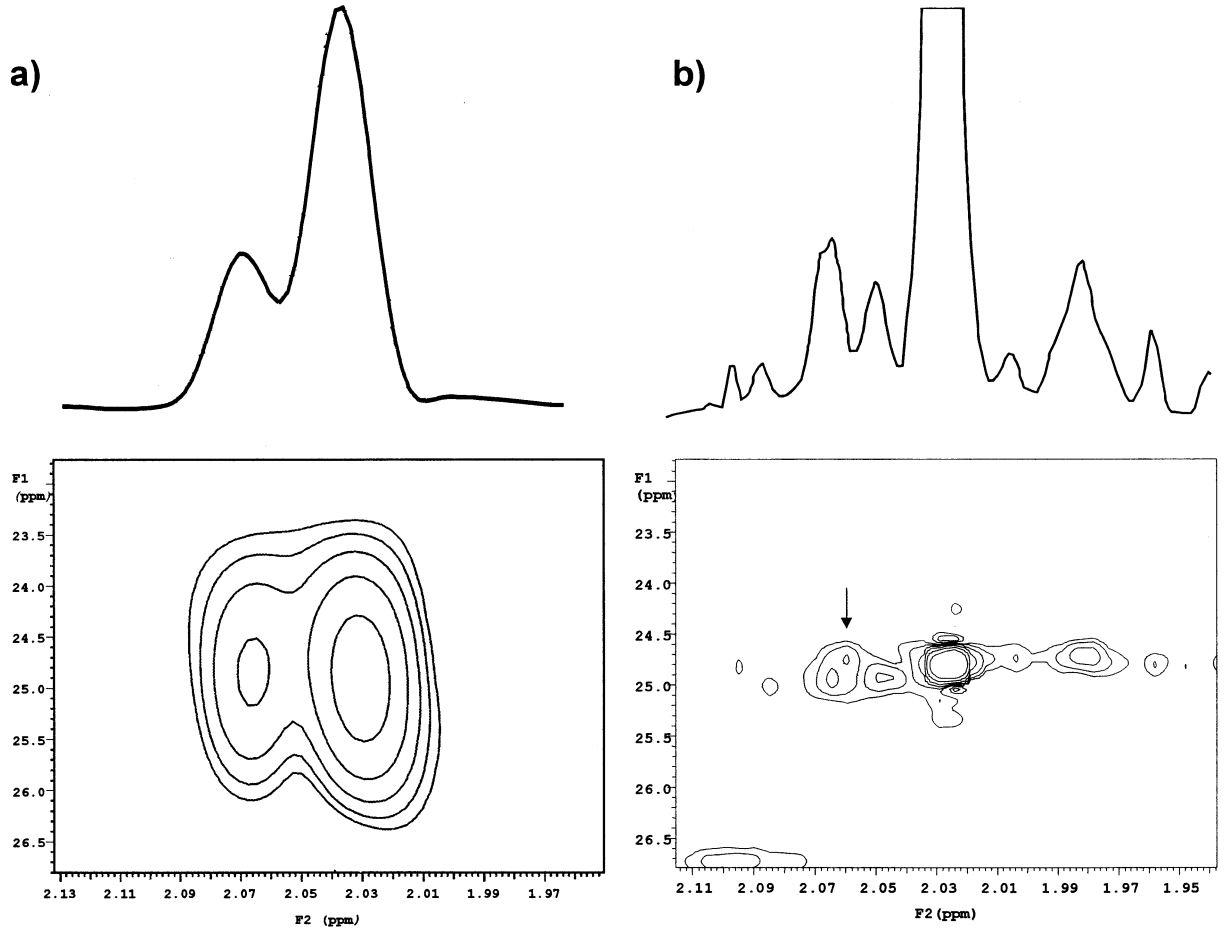

Fig. 3. $^{1}H-^{13}C$ HSQC spectrum using a selective pulse for the methyl range; (a) of mixture NAA and NAAG (4:1) at pH 6.20; (b) of urine sample of patient suffering from Canavan disease at pH 6.44.

proportion of 4:1 and ^{13}C NMR spectra were measured. Taking into account the small concentration of NAAG in relation to NAA and the wide variety of different metabolites in urine, chemical shifts were measured for the methyl signals of protons and carbons of both compounds. One can conclude from Tables 2 and 3, that only above pH 5.15 are the signals of the methyl carbons of both compounds are sufficiently resolved. The ^{1}H NMR chemical shifts of the methyl protons for NAAG (δ 2.06 ppm) and NAA (δ 2.03 ppm) are constant on the whole range of pH. Unfortunately in straightforward ^{1}H and in ^{13}C NMR spectra of urine of patient suffering from Canavan disease were a lot of metabolites and signals overlap in methyl range. It was not possible unmistakably identify NAAG in sample of urine [35]. Therefore, we decided on usage 2-D NMR correlation

Table 3
pH Dependence ^{13}C chemical shifts δ (ppm) of NAA in water solution

pH	C_{Me}	C_{α}	C_{β}	CO_{NH}	COO_{β}	COO_{α}
1.80	24.76	52.91	39.22	176.83	177.99	178.32
2.45	24.76	53.02	39.30	176.81	178.07	178.42
5.15	24.88	55.87	42.20	176.41	181.41	181.41
6.20	24.86	56.08	42.41	176.41	181.60	181.72
7.50	24.87	56.11	42.44	176.41	181.64	181.77
8.20	24.86	56.11	42.43	176.41	181.65	181.77
9.40	24.87	56.12	42.44	176.41	181.65	181.77

techniques. The HSQC experiment (proton detected $^{1}H-^{13}C$ correlation spectrum in this case) was measured for the methyl range of a mixture of NAA and NAAG (4:1). The chemical shift of the carbon at 24.88 ppm correlates with the ^{1}H chemical shift at 2.03 ppm (NAA) and the chemical shift of the carbon at 24.76 ppm correlates with the ^{1}H chemical shift at 2.06 ppm (NAAG) (Fig. 3a). This allowed the use of NMR spectroscopy to identify NAAG in the urine of a child suffering from Canavan disease. The $^{1}H-^{13}C$ HSQC spectrum of urine of this patient (pH 6.44) was measured for the methyl range and was found to be very similar to the mixture of standards (Fig. 3b). In order to identify NAAG in urine it may not be possible using 1-D spectra of ^{1}H and ^{13}C NMR because many metabolites with different methyl signals appear in such spectra. The use of the HSQC spectrum reduces possibility of misassignment.

NMR spectroscopy gives possibility to analyse of NAAG in body fluids and can be complementary and useful diagnostic tool.

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References

[1] J.T. Coyle, Neurobiol. Dis. 4 (1997) 231–238.
[2] Z. Pfund, D.C. Chugani, C. Juhasz, O. Muzik, H.T. Chugani, I.B. Wilds, N. Seraji-Bozorgzad, G.J. Moore, J. Cereb. Blood Flow Metab. 20 (2000) 871–878.
[3] I.M. Najm, Y. Wang, S.C. Hong, et al., Epilepsia 38 (1997) 87–94.
[4] C.A. Davie, G.J. Barker, A.J. Thompson, P.S. Tofts, W.I. McDonald, D.H. Miller, J. Neurol. Neurosurg. Psychiatry 63 (1997) 736–742.
[5] C.R. Scriver, A.L. Beaudet, W.S. Sly, D. Valle (Eds.), The Metabolic and Molecular Basis of Inherited Disease, eighth ed, McGraw-Hill, New York, 2001.
[6] A.P. Burlina, V. Ferrari, P. Divry, W. Gradowska, C. Jacobs, M.J. Bennett, A.C. Sewell, C. Dionisi-Vici, A.B. Burlina, Eur. J. Pediatr. 158 (1999) 406–409.
[7] A.P. Burlina, A. Corazza, V. Ferrari, P. Erhard, B. Kunnecke, J. Seelig, A.B. Burlina, Eur. J. Pediatr. 153 (1994) 538–539.
[8] L.B. Piotrovsky, M.A. Dumpis, L.N. Poznyakova, L.N. Alexandrova, N.F. Sepetov, Zh. Org. Chim. 24 (1988) 111–116.
[9] R.A. Komoroski, A.H. Fowler, H.J. Lubansky, C.D. Rice, W.M. Jay, Spectrosc. Lett. 24 (1991) 1123–1144.
[10] D. Holowenko, J. Peeling, G. Sutherland, NMR Biomed. 5 (1992) 43–47.
[11] M. Martin, J. Labouesse, P. Canioni, M. Merle, Magn. Reson. Med. 29 (1993) 692–694.
[12] V. Govindaraju, K. Young, A.A. Maudsley, NMR Biomed. 13 (2000) 129–153.
[13] H. Krawczyk, A. Gryff-Keller, W. Gradowska, M. Duran and, E. Pronicka, J. Pharm. Biomed. Anal. 26 (2001) 401–408.
[14] T.W.M. Fan, Prog. Nucl. Magn. Reson. Spectrosc. 28 (1996) 161–219.
[15] J.C. Lindon, J.K. Nicholson, Trends Anal. Chem. 16 (1997) 190–200.
[16] J.C. Lindon, J.K. Nicholson, J.R. Everett, in: G.A. Webb (Ed.), Annual Reports on NMR Spectroscopy, vol. 38, Academic Press, London, 1999, pp. 1–88.
[17] J.C. Lindon, J.K. Nicholson, S. Holmes, J.R. Everett, Concepts Magn. Reson. 12 (2000) 289–232.
[18] F.J. Bamforth, V. Dorian, H. Vallance, D.S. Wishart, J. Inherit. Metab. Dis. 22 (3) (1999) 297–301.
[19] J. Lombardi, C.R. Graham, Jr, J. Nutr. Biochem. 6 (1995) 516–521.
[20] N. Beckmann, Carbon-13 NMR Spectroscopy of Biological Systems, Academic Press, San Diego, CA, 1995, pp. 269–322.
[21] H. Krawczyk, A. Gryff-Keller, Bull. Pol. Acad. Chem. 47 (1999) 21–24.
[22] S.L. Wehrli, G.T. Berry, M. Palmieri, A. Mazur, L. Elsas, III, S. Segal, Pediatr. Res. 42 (1997) 855–861.
[23] S.H. Moolenaar, J. Poggi-Bach, U.F.H. Engelke, et al., Clin. Chem. 45 (1999) 459–464.
[24] S. Szymański, M. Witanowski, A. Gryff-Keller, Annu. Rep. NMR Spectrosc. 8 (1978) 227–289.
[25] S. Braun, H.-O. Kalinowski, S. Berger, One Hundred and Fifty and More Basic NMR Experiments, Wiley-VCH, New York, 1998.
[26] H. Friebolin, Basic One and Two-Dimensional NMR Spectroscopy, third ed, VCH, Weinheim, 1998.
[27] J.K.M. Sanders, B.K. Hunter, Modern NMR Spectroscopy. A guide for Chemists, second ed, Oxford University Press, Oxford, 1993.
[28] M. Karplus, J. Chem. Phys. 30 (1959) 11–15.
[29] M. Karplus, J. Am. Chem. Soc. 85 (1963) 2870–2871.
[30] V.F. Bystrov, Prog. NMR Spectrosc. 10 (1976) 41–81.
[31] D. Baron, N. Lumbroso-Bader, Can. J. Chem. 64 (1986) 1317–1327.
[32] Y. Rubin, G.P. Connelly, R.E. Lenkinski, J. Inorg. Biochem. 60 (1995) 31–43.

[33] H. Günther, NMR Spectroscopy, second ed, Wiley, Chichester, 1995, pp. 108–112.
[34] M. Barfield, D.M. Grant, J. Am. Chem. Soc. 85 (1963) 1899–1904.
[35] S.H. Moolenaar, U.F.H. Engelke, S.M.G.C. Hoenderop, A.C. Sewell, L. Wagner, R.A. Wevers, Handbook of ^{1}H NMR Spectroscopy in Inborn Errors of Metabolism, SPS Verlagsgesellschaft mbH, Heilbronn, 2002.