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The magician from Transsylvania
On the use of proton NMR spectroscopy in the clinical chemistry laboratory

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Nikola Tesla (1856-1943) was born in the village of Smiljan in the province of Lika in Croatia, then part of the Austro-Hungarian Empire. He is referred to as the magician from transsylvania. Tesla was the genius who ushered in the age of electrical power. In 1891 Tesla became a United States citizen. He then was at the peak of his creative powers. He developed in rapid succession the induction motor, new types of generators and transformers, a system of alternating-current power transmission, fluorescent lights, and a new type of steam turbine. He also became intrigued with wireless transmission of power. Nowadays we find his name back as a measure of magnetic field strength. NMR spectroscopy makes use of high magnetic field strength. In the setting of the clinical chemistry laboratory proton NMR spectroscopy has found an application in diagnosing inherited metabolic diseases. This paper explains the structural information deriving from NMR spectra and shows examples from body fluid H-NMR spectra. It explains how the technique can contribute to finding diagnoses of known and as yet unknown metabolic diseases.

Introduction to body fluid NMR spectroscopy

The laboratory diagnosis of inherited metabolic diseases cannot always be achieved by analysis of amino acids and organic acids alone. Often additional investigations also do not lead to the diagnosis while there is a strong suspicion of a metabolic disease. In such cases NMR spectroscopy of body fluids can be a complementary technique to be used as a last resort to find the diagnosis (1-7). ^{1}H-NMR spectroscopy of body fluids shows the majority of proton-containing compounds and therefore provides an overall view of metabolism. For the diagnosis of inherited metabolic diseases, this is a great advantage compared with other techniques. The spectral parameters chemical shift, spin-spin coupling, and signal intensity are important for body fluid analysis. The properties of these parameters will be described briefly here.

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NMR spectroscopy of body fluids may be considered as an alternative analytical approach for diagnosing known, but also as yet unknown, inborn errors of metabolism. In this way novel inborn errors of metabolism have been delineated. Examples are: 1. dimethylglycinuria (8), 2. a novel polyol disease with ribose 5-phosphate isomerase deficiency (9), 3. ureidopropionase deficiency (10). The technique can also be applied to cerebrospinal fluid. For patients with a clinical suspicion of a neurometabolic disease CSF investigations may lead to a diagnosis that cannot be found easily or not at all in other body fluids. The paper of Wolf et al (11) describes two patients with an increased concentration of N-acetylaspartylglutamate in CSF found with NMR spectroscopy. The paper describes this dipeptide as a biochemical hallmark for a novel neurometabolic disease with severe hypomyelination.

Chemical shift

The chemical shift (resonance position) can be used to discriminate the ^{1}H-NMR spectra of molecules, even when their chemical structure is only slightly different. For example, two molecules that have a quite similar chemical structure are lactic acid and alanine. The only difference is that lactic acid has a hydroxyl group whereas alanine has an amino group.

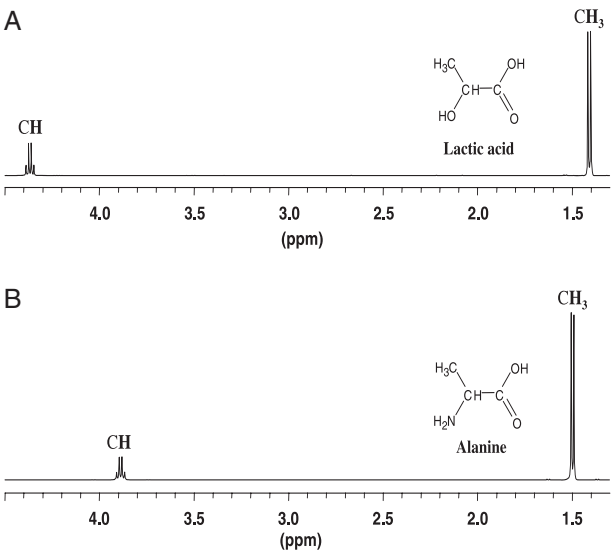

Figure 1. ^{1}H-NMR spectra of lactic acid (A) and alanine (B) dissolved in D_2O measured at 500 MHz; pD = 2.5

As shown in figure 1, the CH_3-protons of lactic acid resonate at 1.41 ppm and the CH-proton resonates at 4.37 ppm under the conditions used. Due to the small difference in chemical structure, the resonance positions of the CH_3- and CH-protons of alanine are slightly different, i.e. 1.50 ppm for the CH_3-protons and 3.89 ppm for the CH-proton.

Spin-spin coupling

In ^{1}H-NMR spectra, signals arising from one or more equivalent protons are often split into two or more components. This is also illustrated in Figure 1A, showing the ^{1}H-NMR spectrum of lactic acid. The splitting of the resonances is caused by an interaction between neighboring protons. Some rules governing this splitting are: 1) no splitting is caused between equivalent protons, e.g. the CH_3-group protons of lactic acid; 2) a proton that is coupled to *n* equivalent protons gives rise to (*n* + 1) lines. The relative intensities of these lines are given by the binomial distribution. In figure 1, the equivalent CH_3-group protons coupled to the CH-group proton give rise to two lines (a doublet) with relative intensities of 1:1. The CH-group proton coupled to the equivalent CH_3-group protons gives rise to four lines (a quartet) with relative intensities of 1:3:3:1. Since the hydroxyl proton in lactic acid exchanges rapidly with water protons under the conditions used, it does not couple to any of the non-exchangeable protons in this molecule.

Signal intensity

The peak area or signal intensity of a resonance in a ^{1}H-NMR spectrum is proportional to the number of protons contributing to the signal when appropriate experimental conditions are used. For example in figure 1, the doublet is assigned to the CH_3-group (3 protons contributing) and the quartet is assigned to the CH-group (1 proton contributing). Therefore, the peak area of the doublet is three times as large as the peak area of the quartet. Since the peak area is proportional to the number of protons contributing to the signal, it is also proportional to the concentration of the molecule concerned. Therefore, it is possible to use NMR spectroscopy for metabolite quantification. The sensitivity of the technique is in the low micromolar range for most metabolites.

Body fluid ^{1}H-NMR spectroscopy

Using the spectral parameters chemical shift, multiplicity, and signal intensity, body fluid ^{1}H-NMR spectra can be used for identification and quantification of proton-containing metabolites. These are useful properties for body fluid analysis for diagnosing inborn errors of metabolism. ^{1}H-NMR spectra of all relevant body fluids can be recorded, i.e. urine, serum or heparinized plasma, and cerebrospinal fluid (CSF). In a review paper on NMR spectroscopy of biofluids by Lindon *et al*., more than 100 resonances were assigned in serum and CSF spectra, and even more than 200 in urine spectra (6). An advantage of ^{1}H-NMR spectroscopy compared to conventional metabolic screening techniques is the fact that NMR spectroscopy is not selective. No derivatization or

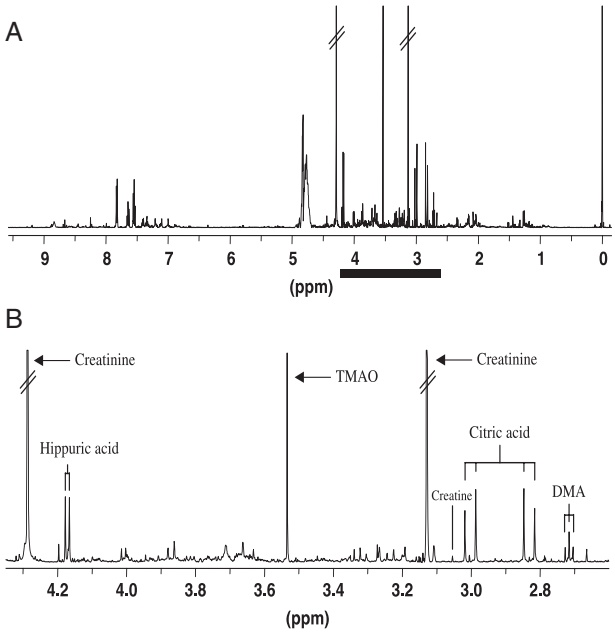

Figure 2. ^{1}H-NMR spectrum of urine from a healthy volunteer (A) and an expansion of a part of this spectrum (B) recorded at 500 MHz. DMA = dimethylamine; TMAO = trimethylamine N-oxide.

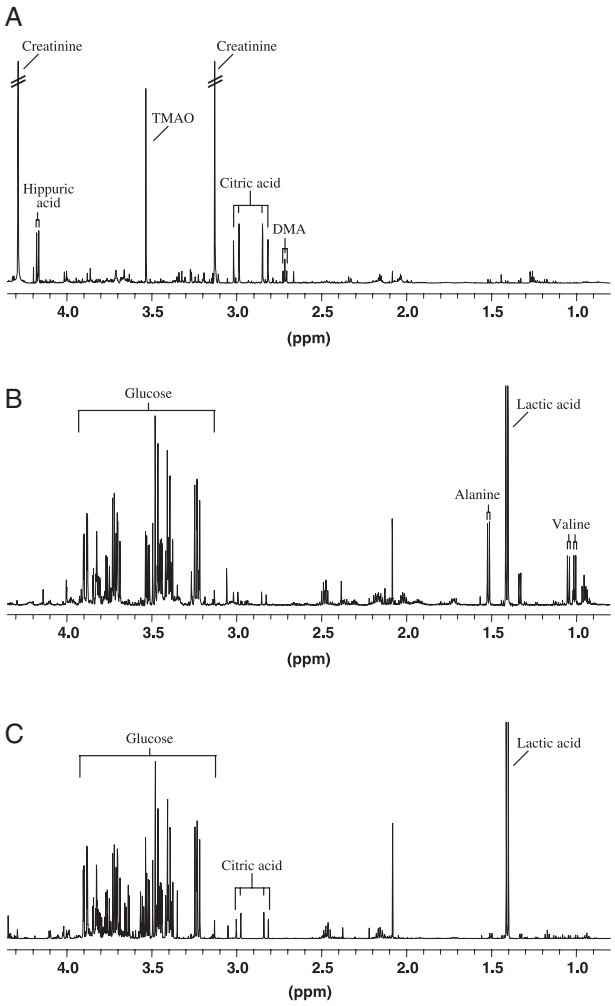

Figure 3. Normal ^{1}H-NMR spectra of urine (A), serum (B), and CSF (C) recorded at 500 MHz. The scales of the spectra are adapted to the size of the figure. TMAO = trimethylamine N-oxide, DMA = dimethylamine.

extraction steps are needed. A body fluid ^{1}H-NMR spectrum therefore provides an overview of almost all proton-containing metabolites in a concentration range above the detection limit of the NMR method used. The detection limit depends on a number of factors such as field strength of the NMR spectrometer, the multiplicity, the number of protons contributing to a resonance, and the region of the spectrum where the resonance is observed (crowded or less crowded with resonances). In general, the detection limit is in the low micromolar range in the less crowded regions of the spectrum. An example of a urine spectrum from a healthy volunteer and an expansion of a part of this spectrum are shown in figure 2.

Examples of a normal urine, serum, and CSF ^{1}H-NMR spectrum are shown in Figure 3. The minimal sample volume required measuring undiluted urine, CSF, and serum or heparinized plasma is 1 ml for a standard NMR system. Only limited sample preparation is required:

1. Serum, plasma, and CSF samples contain substantial amounts of protein. The broad resonances of these proteins may disturb the detection and quantification of the smaller metabolites. Therefore, serum, plasma, and CSF samples are deproteinized before measurement using a 10 kD filter.
2. The internal reference TSP (trimethylsilyl-2,2,3,3-tetradeuteropropionic acid) is added to the samples. It is used to calibrate the spectra. Since it is added in a known concentration, it can also be used for metabolite quantification. This procedure is used in body fluids in which metabolite concentrations are expressed per liter, e.g. serum, plasma, and CSF.

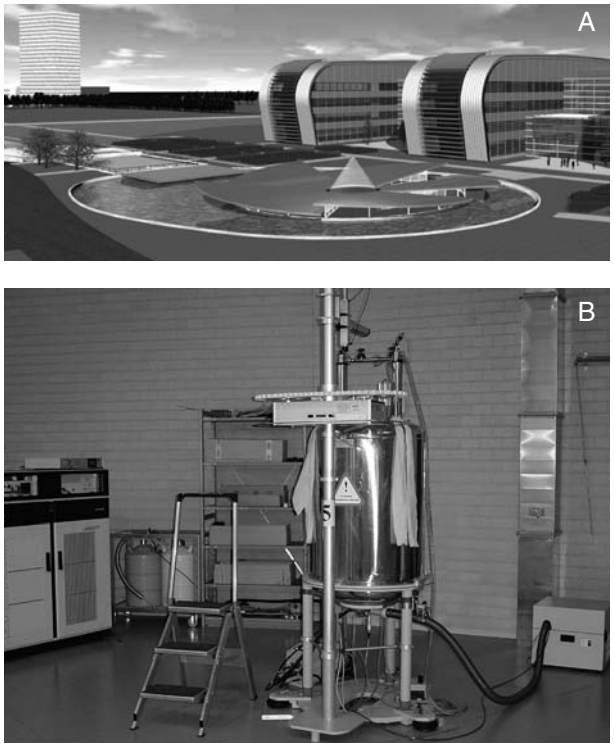

Figure 4. (A) The new NMR facility building in Nijmegen houses various field strength spectrometers. (B) 500 and 600 MHz machines (14.5 Tesla) are used for body fluid NMR measurements.

3. The proton chemical shifts of most metabolites are pH-dependent. Therefore, for accurate assignment of resonances and for optimal comparison between spectra, the pH of the samples needs to be adjusted. We always use pH 2.5.

Case selection

NMR spectroscopy of body fluids may be considered when the patient is suspected to suffer from a so far undiagnosed inborn error of metabolism or when further investigations that may lead to a diagnosis are required. Measurements are performed in the recently opened NMR facility building (a 'water-lily in the pond') on the Radboud Nijmegen University campus (figure 4). For the effective use of the NMR we have used strict indications to accept samples for measurement. These indications have formed the basis for the patient selection in all our studies.

Clinical indications are as follows.

1. Two or more children in the same family have unexplained similar clinical signs and symptoms.
2. The patient has an unusual body odour.
3. An unknown metabolite is observed using in vivo NMR spectroscopy of the brain or other organ.

Biochemical indications are as follows.

1. An abnormal unknown metabolite is repeatedly observed in body fluids with another technique. NMR spectroscopy may provide structural information about the metabolites involved.
2. Confirmation of a special diagnosis with an independent technique is needed.
3. Reliable quantification of a metabolite that is difficult to quantify otherwise is needed.
4. Consistent abnormal results are found using conventional screening techniques for one or more metabolites that did not lead to a classifying diagnosis. NMR spectroscopy may provide additional information.

Sample choice

Traditionally urine is the body fluid of choice to lead the way towards the diagnosis in inborn errors of metabolism and is often used as a first approach. Urine NMR spectra are very complex. Moreover, some metabolites are rapidly excreted but others remain preferentially in the blood. In relevant cases it is advised also to investigate the serum (or heparinized plasma). It may be necessary to use CSF in diseases affecting the central nervous system. For a proper interpretation of the spectrum, the request to do NMR spectroscopy apart from the clinical signs and symptoms of the patient should include information on the medication and on special dietary regimens or habits.

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