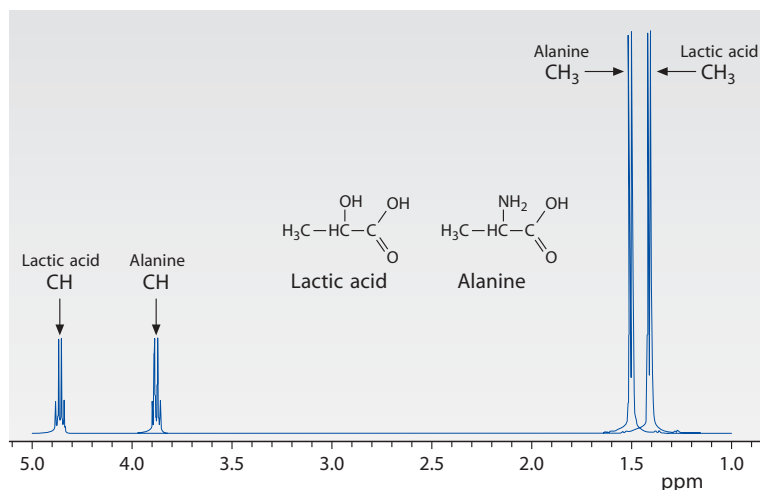


## F.1 Introduction

The laboratory diagnosis of inherited metabolic diseases cannot always be achieved by analysis of amino acids and organic acids alone. Often, however, 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 [1–3]. Proton NMR spectroscopy of body fluids shows the majority of proton-containing compounds. Therefore it provides an overall view on metabolism. In the diagnostics of hereditary metabolic diseases this is a great advantage compared to other techniques. NMR spectroscopy on body fluids may be considered as an alternative analytical approach for diagnosing known but also as yet unknown inborn errors of metabolism.

## F.2 Background of Body Fluid Proton NMR Spectroscopy

Protons of a compound leave a characteristic fingerprint behind in the  $^1\text{H}$ -NMR spectrum. Signals derive from all proton-containing small molecules. Several spectral parameters are useful for body fluid analysis. These are the peak area, the resonance position and the splitting of resonances. The peak area is important for quantification. The technique provides reliable quantitative data about proton-containing metabolites in the sample. Sensitivity is in the low micromolar range. The resonance position and the splitting pattern help identifying the compound and give structural information on the molecule. The signal from a particular proton or group of equivalent protons may consist of one or more peaks (=splitting) under the influence of its chemical environment. Singlet, doublet, triplet, quartet or multiplet resonances may occur. Figure F.1 gives a schematic presentation of the NMR spectra of lactate and alanine to illustrate that molecules with a high degree of similarity can be observed as separate resonances in the spectrum. The methyl groups of lactate and alanine show as doublet resonances. The proton from their methylene groups gives a quartet in the spectrum. The



**Fig. F.1.**  $^1\text{H}$ -NMR spectrum of lactic acid and alanine illustrating the “fingerprint” of both molecules in the spectrum

multiplicity is determined by the number of protons attached to neighboring carbon-atoms. The doublets originating from the methyl protons of lactic acid and alanine have a slightly dissimilar resonance position (0.10 ppm) caused by differences in the chemical environment of the methyl group in the two molecules ( $\text{NH}_2$  group versus OH group). This explains how the two substances, in spite of their partial structural similarity, can be distinguished in the spectrum. Peak areas in the NMR spectrum are proportional to the concentration of a metabolite. The areas of the doublet and the quartet resonances are in the proportion of three to one, because three protons contribute to the signal of the methyl group against one proton of the methylene group.

### F.3 Potential of the Method

Proton NMR spectroscopy of body fluids is a powerful new tool in diagnosing known and novel inborn errors of metabolism. Potentially it may replace some of the conventional diagnostic techniques in metabolic screening laboratories in future. At the moment the technique should be used mainly diagnostically in those cases where the metabolic specialist is convinced that a patient suffers from a metabolic disease but cannot find the diagnosis with conventional techniques. An important advantage of NMR spectroscopy over conventional techniques that are being used in basic screening of hereditary diseases is the non-selective character of the technique.

**Table F.1.** Body fluid NMR spectroscopy

Indications
<p><b>Clinical</b></p> <p>There is a strong clinical suspicion that this patient has an as yet undiagnosed inborn error of metabolism:</p> <ul style="list-style-type: none"> <li>– Two or more children in the same family with unexplained similar clinical signs and symptoms.</li> <li>– Unusual body odour.</li> <li>– Unknown metabolite observed by in vivo NMR spectroscopy (or other technique).</li> </ul> <p><b>Biochemical</b></p> <p>There is a strong biochemical suspicion that this patient has an as yet undiagnosed inborn error of metabolism:</p> <ul style="list-style-type: none"> <li>– An abnormal unknown metabolite (<math>\mu\text{molar}</math> range or higher) observed repeatedly in body fluids with other technique. Medication as origin of this metabolite has already been excluded. As the NMR spectrum also contains structural information on metabolites it may be possible to derive the structure of the compound directly from the NMR spectrum.</li> <li>– Confirmation of a special diagnosis with an independent technique.</li> <li>– Reliable quantification of a metabolite that otherwise is difficult to quantify.</li> </ul>

#### F.4 Indications for NMR Spectroscopy

Optimal results from NMR analysis of body fluids requires close cooperation between the referring clinician or chemist and the NMR spectroscopy group. Detailed information on the patient and the medication of the patient should be provided to result in an optimal interpretation of the NMR data. As the available measurement time on the NMR machines is limited samples can only be accepted on specific clinical or biochemical indications (Table F.1).

#### F.5 Sample Choice, Sample Preparation and Measurement

Traditionally urine is the body fluid of choice to find the way towards the diagnosis in inborn errors of metabolism. Often urine is 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 heparinised plasma). It may be necessary to use CSF in diseases affecting the central nervous system. Minimal sample volumes require 1 ml for all body fluids. For a proper interpretation of the spectrum, the request to do NMR spectroscopy should include information on the medication.

As the technique requires no derivatisation or extraction there is no loss of metabolites in sample pretreatment. Sample preparation is limited to the

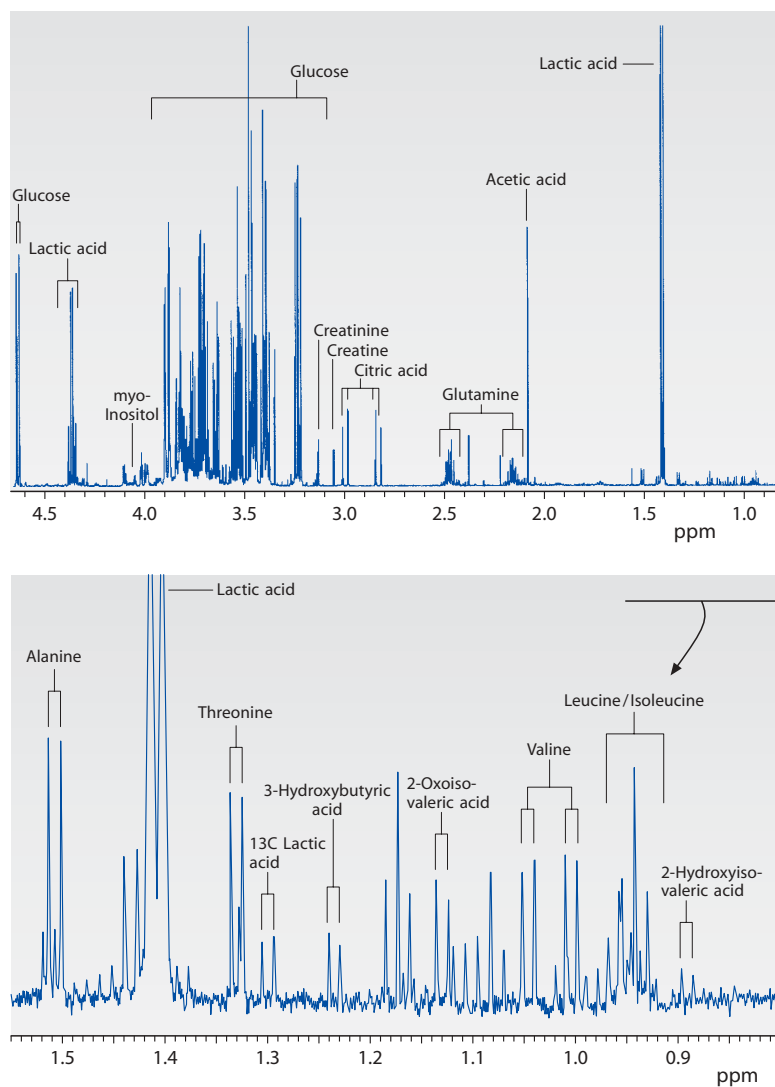
removal of proteins from serum and CSF samples on a 10 kD filter, pH standardisation of samples and addition of an internal standard.

The measurement itself requires adjustment per sample of the magnetic field in order to achieve good homogeneity (“shimming”). Automatic sample changers and automatic shimming programs take care of efficient measurement of larger series of samples. The actual measurement takes up about 30 minutes. The signal is recorded by the computer and can be further analyzed later on by means of software programs that have been especially designed for this purpose. The software required to interpret a spectrum of a body fluid and to quantify it has not yet been developed to the level of complete automation.

## F.6 The Resulting Spectrum and its Interpretation

In complex matrices like body fluids many resonances will be present in the NMR spectrum. The spectrum provides an overall view on metabolism. Basically, all proton containing small molecules ( $MW < 500$ ) can be observed with proton NMR spectroscopy. Under the conditions used, however,  $-NH_2$  and  $-COOH$  protons may actually be NMR-invisible as they exchange rapidly. Protons from proteins and from molecules that are protein bound are also largely invisible. It is impossible to give a full list of metabolites that can be observed in body fluid NMR spectra. For various body fluids such lists are available in literature [4–7]. Typically spectra from urine samples contain well over 300 resonances while the number of resonances in a CSF sample is limited to approximately 100. At least 50 compounds were identified in CSF samples. From NMR spectra it could be concluded that 3-hydroxyisovaleric acid turned out to be a normal constituent of CSF samples. Up till now this has been unknown [6]. In this way  $^1H$ -NMR spectroscopy provides new insight. The spectrum (Fig. F.2) is an example of a normal CSF. The lower panel magnifies part of the spectrum showing more details of that specific part.

Table F.2 summarizes the metabolites that are observed with NMR in more than 50% of the samples in a body fluid *and* remain undetected when routine metabolic screening techniques are applied. It is assumed that routine screening comprises measurement of 1. Urine: organic acids, amino acids, purines and pyrimidines, monosaccharides and polyols, mucopolysaccharides, oligosaccharides; 2. Plasma: amino acids, carnitine (esters), glucose, lactate, pyruvate; 3. CSF: amino acids and glucose.



**Fig. F.2.** A normal proton NMR spectrum (600 MHz) of cerebrospinal fluid. *Upper panel:* overall view; *lower panel:* expanded view of a part of the spectrum

**Table F.2.** Metabolites observed with NMR in more than 50% of the samples in a body fluid which may be undetectable with routine metabolic screening techniques

Urine	Plasma	Cerebrospinal fluid
2-Oxoisovaleric acid	2-Hydroxyisovaleric acid	2-Hydroxyisovaleric acid
Dimethylamine	2-Oxoisovaleric acid	2-Oxoisovaleric acid
Dimethylglycine	3-Hydroxyisovaleric acid	3-Hydroxybutyric acid
Creatine	Acetoacetic acid	3-Hydroxyisovaleric acid
Creatinine	Citric acid	Acetoacetic acid
Choline	Creatine	Citric acid
Carnitine (esters)	Creatinine	Creatine
Betaine		Creatinine
Trimethylamine N-oxide		Choline
1-Methylnicotinamide		Myoinositol
Allantoin		N-acetylneuraminic acid (tentative)
Urocanic acid		Glycolic acid
Indoxyl sulphate		Ascorbic acid
		Lactate
		Pyruvate

## F.7 Quantification, Sensitivity and Reproducibility

Quantification of a metabolite can be performed by adding an internal standard to the sample (plasma and CSF). In practically all studies trimethylsilyl-2,2,3,3-tetradeuteriopropionic acid (=TSP), which provides a singlet resonance from 9 equivalent methyl-protons, is used as such. Metabolites in urine can be expressed per creatinine without using an internal standard. For various compounds good correlations have been obtained with conventional techniques. The sensitivity obtained with  $^1\text{H}$ -NMR spectroscopy depends on the strength of the magnetic field that is available. It depends on the number of protons contributing to the resonance(s) and on the multiplicity of the resonance. Furthermore the region of the spectrum where the compound resonates and the measuring time play a part. Examples of the sensitivity level that can be reached are (600 MHz apparatus and 30 min measuring time per sample): 2  $\mu\text{mol/l}$  for betaine (singlet, 9 protons), 10  $\mu\text{mol/l}$  for lactic acid (doublet, 3 protons) and 30  $\mu\text{mol/l}$  for glucose (doublet, one proton). For molecules where the detection relies on a multiplet resonance the sensitivity may be significantly lower. A representative coefficient of variation has a value of 2.5% (1.2 mmol alanine/l).

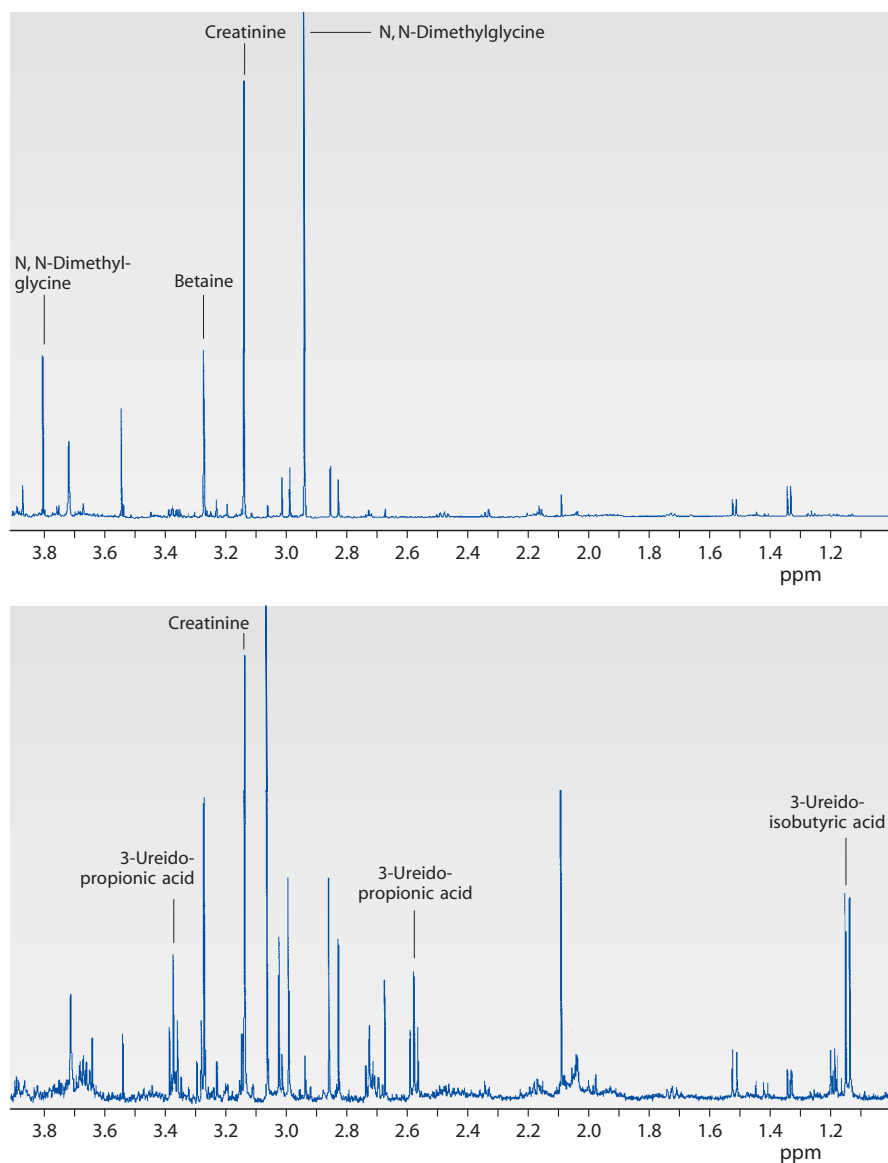
## F.8 NMR Spectroscopy in Inborn Errors of Metabolism

NMR spectroscopy on body fluids can be successfully applied to diagnose more than 60 of the known inborn errors of metabolism including most organoacidurias, aminoacidurias and diseases in purine- and pyrimidine metabolism. An overview of diseases that can be diagnosed is available [8].

NMR spectroscopy can be used to find inborn errors of metabolism characterised by the presence of metabolites that cannot be detected with conventional screening methods. Trimethylaminuria or “fish odour syndrome” may serve as an example [9]. It is a hereditary disease based on an enzyme deficiency in the liver. Trimethylamine (=TMA) derives from bacteria that convert it from dietary choline. Normally there is an enzyme in the liver that oxidizes TMA efficiently. TMA and trimethylamine N-oxide (=TMAO) are both secreted in the urine. TMA has the smell of rotten fish causing a social problem for the patient. Until recently no technique could measure TMA and TMAO simultaneously to prove this deficiency at the metabolic level. This is very well possible with NMR spectroscopy.

Until now in vitro NMR spectroscopy was used in the detection of three novel inborn errors of metabolism. These are dimethylglycine dehydrogenase deficiency [10], ureidopropionase deficiency [11] and a defect in polyol metabolism (ribose-5-phosphate isomerase deficiency) where increased arabitol and ribitol characterise this disease at the metabolite level [12, 13]. The characteristic metabolites in these diseases cannot be picked up with conventional screening techniques (dimethylglycine, ureidopropionic acid, ureidoisobutyric acid) or can only be detected with techniques that are not commonly used in metabolic screening laboratories (the detection of arabitol and ribitol requires GC of monosaccharides and polyols). The urine NMR spectrum in Fig. F.3 shows the characteristic resonances of dimethylglycine in dimethylglycine dehydrogenase deficiency (Fig. F.3: upper panel) and of both ureido-compounds in ureidopropionase deficiency (Fig. F.3: lower panel). The examples of these novel diseases clearly illustrate the advantage of the non-selective character of NMR spectroscopy and the power of the overall view on metabolism that it provides. Conventional techniques used in the screening for inborn errors of metabolism provide information on roughly 15–20% of metabolites known to play a role in human metabolism. Considering that most of the other metabolites actually contain protons that make them visible with NMR, it is to be expected that NMR spectroscopy will be able to open new perspectives for the field of inborn errors of metabolism.

In case a high concentration of an unknown metabolite is found with conventional screening techniques proton NMR spectroscopy may help to identify the compound. The  $^1\text{H}$ -NMR spectrum contains structural information on the molecules in the sample. Of course medication will first have to be excluded as a source. In such cases the 1-dimensional NMR spectrum



**Fig. F.3.** NMR spectra of urine from patients with dimethylglycine dehydrogenase deficiency (*upper panel*) and ureidopropionase deficiency (*lower panel*)

already may help to identify the unknown. Additional two-dimensional NMR techniques are available and may be used for further structure analysis.



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