# High-Resolution $^1$H-NMR Spectroscopy of Blood Plasma for Metabolic Studies

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Although spin-echo techniques are often used to obtain $^1$H-NMR spectra of serum or plasma samples, they do not provide reliable quantitative analyses of metabolites. We present a standardized procedure, optimized for sensitivity, for using single-pulse $^1$H-NMR spectroscopy to analyze deproteinized plasma. The detection limit for various metabolites ranges between 2 and 40 μmol/L. The method allows quantitative analysis of many compounds of interest in studies of inborn errors of metabolism, including betaine and dimethylglycine, which cannot be measured easily with other techniques. For lactate, tyrosine, threonine, and alanine, we obtained results that correlated well with those obtained by established techniques. We also present a library containing resonance positions of 38 compounds occurring in plasma samples in health and disease, including 14 as-yet-unidentified resonances. As an example of the diagnostic power of the technique we show a spectrum of a plasma sample from a patient with 5-oxoprolinuria (pyroglutamic aciduria; McKusick 266130), an enzymatic defect in glutathione biosynthesis.

**Indexing Terms:** *betaine/heritable disorders/nuclear magnetic resonance/metabolic screening/5-oxoprolinuria/lactate/amino acids*

Proton nuclear magnetic resonance ($^1$H-NMR) spectroscopy has been used to measure metabolites, drugs, and toxic agents in body fluids (*1, 2*). The technique is potentially nondestructive to the samples and requires little or no sample pretreatment. Because it includes no derivatization or extraction step, the technique is characteristically nonselective. However, it provides an overview of the quantitatively most important H-containing substances in the sample (*3, 4*). From such studies, investigators have realized that the technique could play a role in the diagnosis and follow-up of patients with inborn errors of metabolism (*5, 6*).

$^1$H-NMR spectroscopy of small metabolites in plasma presents technical complications (*4, 7–9*). The presence of high concentrations of proteins and lipids results in a broad envelope of overlapping proton resonances that obscure the signals from low-molecular-mass metabolites of interest. Current methods to obtain plasma NMR spectra generally use a spin-echo sequence to filter out interfering broad resonances (*1, 3, 4, 8, 9*). This causes a loss of sensitivity through suppression of resonances in spin-echo spectra because of phase modulation and relaxation effects. Furthermore, lactate and some other physiologically important carboxylic acids and aromatic amino acids appear to be substantially invisible to NMR in plasma (*10, 11*). To circumvent these limitations to the clinical applications of $^1$H-NMR in blood samples, we have developed a simple, reliable, and sensitive assay in which the most important methodological features are as follows:

1. Removal of plasma proteins by filtration, which eliminates broad protein resonances and allows standard single-pulse $^1$H-NMR.
2. Concentration of the sample by a factor 1 to 4, depending on the available sample volume, thereby improving the sensitivity of the assay.
3. Removal of water by evaporation and replacement with $D_2O$ to reduce the water resonance.
4. Standardization of the pH of the sample to improve the intersample reproducibility of resonances.

Here we describe the features of our assay and summarize some findings on 49 plasma samples from patients who were thought, from clinical evidence, to have an inborn error of metabolism. As an example of the diagnostic power of the technique, we have included a case of 5-oxoprolinuria, an inborn error of metabolism in glutathione biosynthesis. To our knowledge, $^1$H-NMR spectra of plasma samples from patients with inborn errors of metabolism have not previously been published.

## Materials and Methods

### Samples and Sample Pretreatment

Our laboratory receives for metabolic screening samples from patients clinically thought to have (inherited) metabolic disease. Because EDTA-anticoagulated plasma shows additional EDTA resonance lines, we prefer either serum or lithium heparinate-treated plasma. After centrifugation for cell separation, samples are stored without delay at −80°C until analysis. Because no specific measures are taken to prevent glycolytic flux in the blood samples, several samples have artificially increased lactate and pyruvate and decreased glucose content.

We deproteinize 2 mL of the samples by centrifugation (3000*g*, 2 h) over a filter with a molecular-mass cutoff of 10 kDa (Microsep no. OD010C45, membrane type omega; Filtron, Northborough, MA). To avoid contamination of the ultrafiltrate with glycerol, we clean the filters before use by passing 6 mL of 0.05 mol/L NaOH and 6 mL of distilled water through them. We evaporate the ultrafiltrate to dryness in an automatic concentrator (AS290 Automatic Speedvac Concentrator; Savant Instruments, Farmingdale, NY), dissolve it in 0.5 mL of $H_2O$, adjust the pH carefully to 2.50 ± 0.10 (at room temperature), and evaporate the solvent again. Finally we dissolve the sample in 0.5 mL of freshly

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prepared $D_2O$ solution containing 0.812 mmol/L trimethyl-2,2,3,3-tetradeuteropropionic acid (TSP, sodium salt; Merck, Darmstadt, Germany) as the chemical shift reference standard. Routinely, we thus concentrate samples by fourfold. However, depending on the available sample volume, the concentration factor varies between 1 and 4 in this study. Because of the removal of protein from the sample, the quantitative data obtained are expressed as metabolite concentrations per liter of ultrafiltrate, not per liter of plasma.

### NMR Measurements

We analyzed 400 μL of the samples on a 600 MHz spectrometer (AMX-600; Bruker Analytische Messtechnik, Karlsruhe, Germany) at 25°C, using a 60° radio frequency pulse and a 15-s pulse repetition time (132 scans). Shimming of the sample was judged to be adequate when the $^{29}Si$-$^1H$ long-range coupling of 3 Hz in the TSP resonance could be resolved. At half-peak height, the TSP resonance was 0.79 Hz (range in 11 samples, 0.39–1.26). Shimming the plasma samples generally allowed the 1-Hz J-coupling of the $\beta C_2$-glucose resonance at 3.21 ppm to be observed. The residual resonance of $H_2O$ was suppressed by low-power continuous wave presaturation during the relaxation delay. To evaluate the NMR spectrum, we used NMR-1 software (version 1.1.1./; New Methods Research, East Syracuse, NY). Chemical shifts were calibrated with respect to the chemical shift position of the TSP resonance. The free induction decay was recorded in 16 K datapoints with a sweep width of 6605 Hz, then Fourier-transformed after zero filling to 32 K. No digital filtering was applied. The phase was manually corrected, and resonances in the spectra were semiautomatically fitted to a Lorentzian lineshape model function. The quantitative data for metabolite concentrations reported here were calculated by comparing the area of the metabolite's corresponding resonance(s) with the area of the TSP resonance.

### Amino Acid and Lactate Analysis

For the correlation study we determined amino acids by conventional ion-exchange chromatography with ninhydrin detection on an LKB/Pharmacia (Uppsala, Sweden) Mark II amino acid analyzer; lactate was measured enzymatically with lactate dehydrogenase (EC 1.1.1.27). To obtain samples having various concentrations of metabolites, we used blood samples taken during ischemic forearm testing (*16*). The blood was collected on ice into a tube containing a mixture of sodium iodoacetate (80 mg), sodium fluoride (80 mg), and potassium oxalate (16 mg) to inhibit glycolysis. Samples were centrifuged within 0.5 h of collection to remove cells. The plasma samples were measured enzymatically both with and without deproteinization with $HClO_4$. For $^1H$-NMR analysis these samples were pretreated essentially as described above (protein removal by ultrafiltration).

## Results

### Normal Plasma Spectrum

Figure 1 shows a spectrum of a fourfold-concentrated plasma sample from an apparently healthy man. Assignments for resonances in the spectrum were made after two-dimensional-correlated spectroscopy (results not shown) and measuring of various standard solutions (Table 1). Table 1 shows only those resonances that were actually observed in our plasma samples. Unidentified resonances are included if they occurred in >5 of the 49 patients or if the intensity of the resonance was >2% of the TSP resonance; for the singlet of the creatinine methyl group, this corresponds to a creatinine concentration of 45 μmol/L. An advantage of working at low pH is the good separation between creatine and creati-

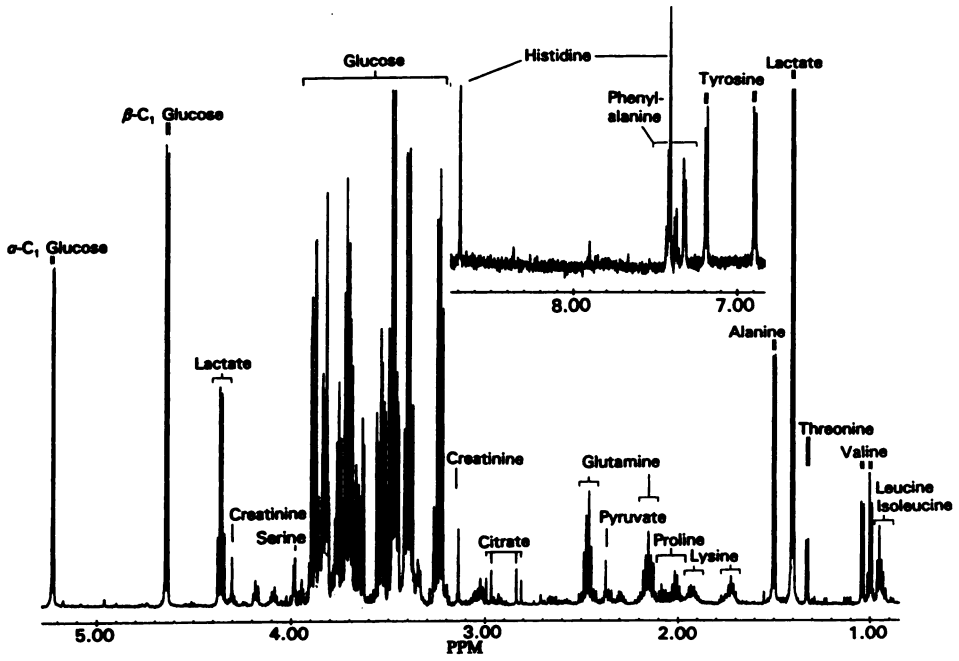


Fig. 1. 600 MHz $^1H$-NMR spectrum of plasma from a healthy volunteer (concentrated fourfold).

**Table 1. $^1$H-NMR resonances from metabolites observed in 49 plasma samples.[a]**

| Metabolite | Chemical shift and multiplicity [proton(s)] |
|---|---|
| TSP | 0.00 s [Si-$(CH_3)_3$] |
| U1 (n = 2)[b] | 0.85 s |
| α-Hydroxyisovaleriate[c] | 0.89 d [$CH_3$]; 0.98 d [$CH_3$]; 4.13 d [CH] |
| Isoleucine[c] | 0.94 t [$CH_3$]; 1.02 d [$CH_3$] |
| Leucine[c] | 0.95 d [$CH_3$]; 0.97 d [$CH_3$] |
| Valine[c] | 1.00 d [$CH_3$]; 1.04 d [$CH_3$] |
| U2 (n = 12) | 1.11 d |
| Propylene glycol[d] | 1.13 d [$CH_3$] (J-coupling = 6.4 Hz); 3.48 AB [$CH_2$] |
| 2-Oxoisovaleriate[c] | 1.13 d [$(CH_3)_2$] (J-coupling = 7.1 Hz) |
| U3 (n = 4) | 1.17 t |
| β-Hydroxybutyrate[c] | 1.23 d [$CH_3$]; 2.53 AB [$CH_2$] |
| U4 (n = 33) | 1.30 d |
| β-Hydroxyisovaleriate | 1.33 s [$(CH_3)_2$]; 2.55 s [CH] |
| Threonine[c] | 1.33 d [$CH_3$] |
| Lactate | 1.41 d [$CH_3$]; 4.36 q [CH] |
| Alanine[c] | 1.50 d [$CH_3$] |
| U5 (n = 14) | 1.53 d |
| U6 (n = 32) | 1.56 s |
| U7 (n = 4) | 1.58 s |
| U8 (n = 3) | 1.66 s |
| Lysine[c] | 1.72 m [$CH_2$]; 1.92 m [$CH_2$]; 3.01 t [$CH_2$] |
| Proline[c] | 2.02 m [$CH_2$]; 2.08 m [$CH_2$]; 2.39 m [$CH_2$] |
| Methionine[c] | 2.13 s [$CH_3$]; 2.67 t [CH] |
| Glutamine | 2.16 m [$CH_2$]; 2.47 m [$CH_2$]; 3.91 t [CH] |
| 5-Oxoproline | 2.20 m [$CH_2$]; 2.43 m [$CH_2$]; 2.55 m [$CH_2$]; 4.36 dd [CH] |
| Acetoacetate[c] | 2.31 s [$CH_3$] |
| Pyruvate | 2.37 s [$CH_3$] |
| Succinate | 2.67 s [$CH_2$-$CH_2$] |
| Sarcosine | 2.74 s [N-$CH_3$]; 3.65 s [$CH_2$] |
| Citrate | 2.91 AB [$CH_2$-C(OH)-$CH_2$] |
| *N,N*-Dimethylglycine[c] | 2.94 s [N-$(CH_3)_2$] |
| Creatine | 3.05 s [N-$CH_3$]; 4.06 s [N-$CH_2$] |
| U9 (n = 11) | 3.11 s |
| Creatinine | 3.13 s [N-$CH_3$]; 4.29 s [N-$CH_2$] |
| U10 (n = 21) | 3.14 s |
| Carnitine esters | 3.19 s [N-$(CH_3)_3$] |
| Carnitine | 3.21 s [N-$(CH_3)_3$] |
| Glucose[c] | 3.23 dd [CH: $\beta C_2$]; 3.3–3.9 (various); 4.64 d [CH: $\beta C_1$]; 5.22 d [CH: $\alpha C_1$] |
| Betaine | 3.26 s [N-$(CH_3)_3$]; 3.94 s [$CH_2$] |
| Triethylamine *N*-oxide | 3.54 s [N-$(CH_3)_3$] |
| EDTA | 3.59 s; 3.91 s |
| Mannitol | 3.6–3.8 (various) |
| Glycine | 3.72 s [$CH_2$] |
| Serine | 3.98 m [$CH_2$-CH] |
| U11 (n = 3) | 4.41 d |
| U12 (n = 6) | 4.51 d |
| OH-Phenylacetic acid | 6.85 d [arom. protons]; 7.16 d [arom. protons] |
| Tyrosine[c] | 6.89 d [arom. protons]; 7.19 d [arom. protons] |
| Phenylalanine[c] | 7.32 m [arom. protons] |
| U13 (n = 25) | 8.25 s |
| U14 (n = 8) | 8.46 s |
| Histidine[c] | 8.68 d [ring proton]; 7.41 d [ring proton]; 4.09 t [$CH_2$] |

[a] s = singlet, d = doublet, dd = doublet/doublet, t = triplet, q = quartet, m = multiplet, arom. = aromatic, and AB = AB-system.

[b] U1, U2, etc., resonances from unknown metabolites. Number of patients in whom the unknown resonance was detected is given in parentheses.

[c] Only the most important signals are shown.

[d] Medication-derived.

nine resonances (3.05 and 3.13 ppm, respectively). These metabolites practically overlap when spectra are recorded at neutral pH. Because of the high glucose content, identification of metabolites in the region between 3.30 and 3.95 ppm is difficult. The singlet resonance at 3.72 ppm of glycine, for instance, is hard to identify at physiological concentrations of glycine but can be recognized in a patient with nonketotic hyperglycinemia.

## Assay Validation

*Correlation study.* Correlation with conventional amino acid analysis (data not shown) was determined for NMR assays of alanine (methyl-group doublet at 1.50 ppm), threonine (methyl-group doublet at 1.33 ppm), and tyrosine (aromatic-proton doublet at 7.19 ppm). Passing and Bablok regression analysis (*12*) showed a good correlation for all three; e.g., for alanine, the regression line for 10 samples (range 270–702 μmol/L) had a slope of 1.04 (95% confidence interval 0.75–1.17) and an intercept of 9 (−50 to 128) μmol/L.

Lactate in plasma was measured three ways: enzymatically without deproteinization; enzymatically with $HClO_4$ for protein removal; and by $^1$H-NMR. The correlation between the three methods was excellent (Fig. 2). The results in deproteinized samples were somewhat higher (median 4.9%) than in the samples not deproteinized—a difference probably accounted for by the volume of the protein. The correlation (*12*) between the enzymatic assay (with deproteinization) and the NMR data for nine samples (range 0.9–9.2 mmol/L) is described by the regression line $y = 0.90x$ (95% confidence interval 0.85–1.09) + 0.27 (−0.6 to 0.5) mmol/L.

*Reproducibility.* Analytical reproducibility was studied by measuring the 2.67 ppm singlet resonance of succinate. At 64 scans per measurement, we obtained 128 ± 12 μmol/L (mean ± SD, CV 9.3%) in 10 separate measurements of this sample. Using 132 scans, we found 127 ± 7 μmol/L (n = 10, CV 5%). For 1.2 mmol/L alanine, the CV was 5.1% with 64 scans and 2.5% with 132 scans. Consequently, all further data were collected with 132 scans.

For correct interpretation of spectra the use of a library of relevant metabolites is required. Correct assignment of resonances depends heavily on the inter-sample reproducibility of the resonance positions. The pH of the sample in the spectrometer is critical and has therefore been closely standardized in our protocol. In 14 random plasma samples we found the following mean (± SD) values for the chemical shift (ppm): lactate 1.406 ± 0.002; alanine 1.501 ± 0.003; histidine 8.683 ± 0.003; and citrate 2.907 ± 0.005. Of the metabolites mentioned in Table 1, citrate has the most variable chemical shift.

*Detection limit.* The correlation study for alanine and threonine illustrates the sensitivity of the NMR technique, given the good correlation with amino acid analysis over a concentration range of 80–700 μmol/L. The detection limit depends on the number of equivalent protons that contribute to a resonance and on the multiplicity of the resonance. Instrumental and methodological

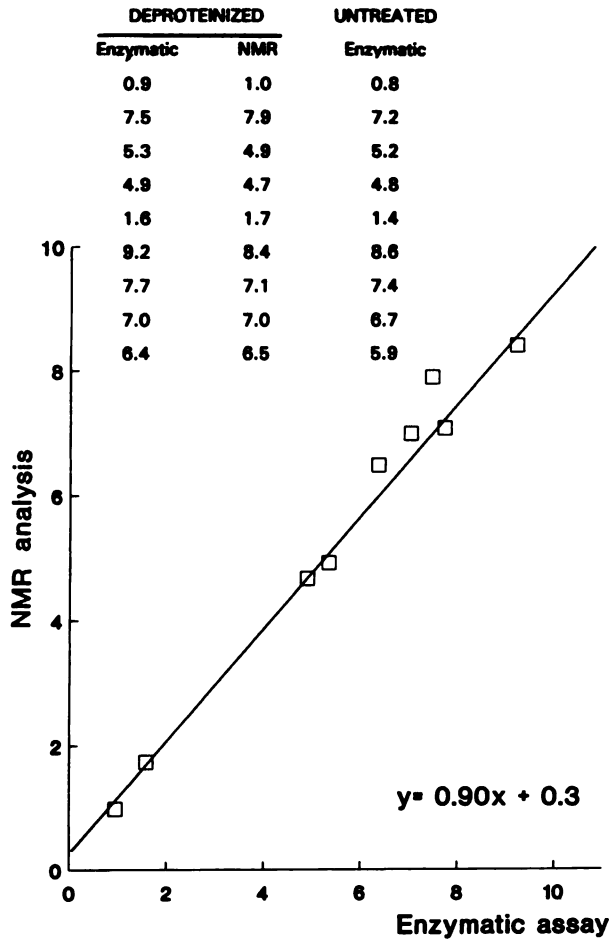


Fig. 2. Comparison of plasma lactate determination (mmol/L) by $^1$H-NMR and by an enzymatic method in samples deproteinized with $HClO_4$ (for enzymatic measurement) and with filtration (for NMR). *Inset:* Comparison with enzymatic measurement of lactate in nondeproteinized plasma samples.

characteristics, e.g., field strength, number of scans, and concentration factor, also determine the sensitivity. Assuming that a signal can be discriminated from the noise if the signal/noise ratio is ≥3, we determined the detection limit for the methyl-group doublet of threonine as 10 μmol/L. From this value, one can calculate the detection limit for other metabolites. Table 2 shows the estimated detection limits for other resonance types for unconcentrated samples under the conditions described in this study, and includes examples for relevant metabolites for the study of inborn errors of metabolism. The detection limits given are valid for all metabolites with a given resonance structure. The detection limit for lactate can therefore also be used for (e.g.) alanine.

### Interpretation of Spectra

For proper interpretation of the spectra, one must consider the influence of the patient's diet (*3*), the medication used, and the effects of age and sex. In very young children pre- or dysmaturity may influence the results (*14*). Data on the influence of these factors on many of the metabolites in Table 1 are available in the literature and are beyond the scope of this paper.

**Table 2. Estimated detection limit for relevant metabolites in unconcentrated plasma samples (600 MHz and 132 scans).**

| No. of contributing equivalent protons | Detection limit, μmol/L, for multiplicity | | | |
|---|---|---|---|---|
| | Singlet | Doublet | Triplet | Quartet |
| 1 | 15 | 30 | 30/60[a] | 40/120[a] |
| | | β $C_1$-Glucose | | |
| 2 | 8 | 15 | 15/30 | 20/60 |
| | Glycine | 2-OH-isovaleriate | 3-OH-propionate | |
| 3 | 5 | 10 | 10/20 | 13/40 |
| | Creatinine | Lactate | Isoleucine | |
| 4 | 4 | 8 | 8/16 | 10/30 |
| | Succinate | | | |
| 9 | 2 | | | |
| | Betaine | | | |

[a] Concentration at which only highest of the resonances of a proton or proton group is visible/concentration at which all resonances are observed.

Some of the unknown resonances in Table 1 may have been caused by medication, but it is not always the pharmacologically active substance of the medication or its metabolite(s) that show up in the spectrum. In three patients we observed a high-resonance doublet at 1.13 ppm, which at first was explained as 2-oxoisovaleriate, a metabolite normally present in low concentration in many plasma samples. By measuring the J-coupling of this doublet and noting also the presence of the additional AB-system at 3.48 ppm, we found that these resonances were from propylene glycol, a carrier vehicle in some medications.

Another difficult interpretation of the plasma spectrum involved two brothers with a similar clinical picture. They had similar plasma NMR spectra, which were governed by very high resonances from the mannitol being infused into both children.

### Inherited Metabolic Disease

As an example of the diagnostic power of the technique, Fig. 3 shows a case of 5-oxoprolinuria (also called pyroglutamic aciduria; McKusick 266130), a defect in glutathione biosynthesis involving either of two enzymes: glutathione synthase (EC 6.3.2.3) or 5-oxoprolinase (EC 3.5.2.9) (*15*). The diagnosis in this young child was confirmed by the characteristic urinary excretion of 5-oxoproline (1.9 mol/mol creatinine; data not shown) and its high plasma concentration (3.6 mmol/L; Fig. 3B); 5-oxoproline is normally not detectable in urine or plasma. Resonances from the 5-oxoproline standard (Fig. 3A) are obvious in this patient's plasma by age 8 days (Fig. 3B). We confirmed the identity of this compound by gas chromatography/mass spectrometry; by adding an extra amount of 5-oxoproline to the plasma of this patient and measuring the NMR spectrum; and by two-dimensional-correlated spectroscopy. The correlated spectroscopy NMR nicely illustrated the coupling of resonance peak complexes (data not shown).

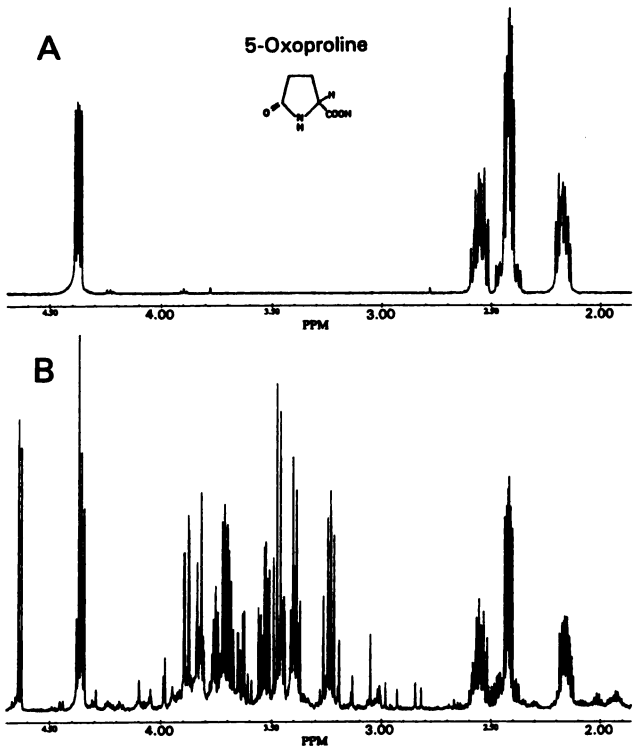


Fig. 3. Plasma $^1$H-NMR spectrum of a patient with 5-oxoprolinuria: (*A*) standard solution of 5-oxoproline, (*B*) patient's plasma.

## Discussion

The partial invisibility of several metabolites, e.g., lactate (*10*), some other carboxylic acids (*10*), and aromatic amino acids (*11*), in plasma subjected to NMR spin-echo techniques has severely inhibited clinical applications of $^1$H-NMR. We have shown that simple removal of protein by filtration provides an alternative that allows standard single-pulse $^1$H-NMR measurements of several metabolites (lactate, alanine, threonine, and tyrosine), with good correlation with conventional techniques. In all plasma samples, moreover, we could find resonances from phenylalanine, tyrosine, and histidine. Under the conditions we use, we have found no indications for NMR-invisible lactate or for a partial binding of lactate to serum protein. The correlation of the NMR-derived lactate concentration with enzymatic lactate values was very good, in agreement with the data of Bell et al. on ultrafiltrates (*10*). Thus we conclude that $^1$H-NMR can provide a multicomponent spectrum of metabolites in plasma, whereby reliable quantitative interpretation of data is possible.

The plasma NMR spectrum in the region between 3.30 and 3.95 ppm is dominated by many glucose resonances of high intensity, which obscure resonances of metabolites such as glycine, trimethylamine *N*-oxide, carnitine esters, and perhaps others; i.e., ~14% of the spectral information is lost. The water resonance potentially presents a similar problem. However, in this case, replacing the water in the sample with $D_2O$ provides good spectra with no water resonance line. Still, this manipulation may have other consequences. In some metabolites, protons may exchange with deuterium; e.g., the $\alpha$C-proton of methylmalonic acid exchanges, causing the 3.50 ppm quartet to disappear and the methyl-group doublet (1.37 ppm) to change into a singlet (1.37 ppm), which serves as the only fingerprint of methylmalonic acid in the spectrum. Furthermore, removal of $H_2O$ from the plasma ultrafiltrate before reuptake in $D_2O$ also removes more-volatile compounds (e.g., acetone or ethanol) from the sample. However, we are convinced that the quality of the single-pulse spectrum after removal of protein and $H_2O$ from the sample is better than that of the spin-echo spectra commonly used for plasma samples. The advantage of a better-quality spectrum that allows simultaneous quantitative measurements of many metabolites outweighs the disadvantage of the loss of spectral information from volatile compounds.

A $^1$H-NMR spectrum of human plasma shows many resonances, and its interpretation therefore requires expertise. A library indicating the positions at which various metabolites resonate is essential. A metabolite can be recognized only when there is not much intersample variability in the chemical shift of resonances. To minimize this variability, standardization of pH is absolutely essential. Lehnert and Hunkler (*5*) made a start, creating a library that contains many metabolites of interest for the study of inborn errors of metabolism. Because we used standardized conditions (especially pH), similar to theirs we could use these data as a basis (*5*). Table 1, however, provides many metabolites not included in their publication that may be of value for $^1$H-NMR study of plasma samples in health and disease.

Unlike other techniques used in screening for inborn errors of metabolism, $^1$H-NMR spectroscopy is essentially nonselective. Almost all H-containing metabolites will appear in the spectrum if their concentration is great enough. For most metabolites the detection limit in unconcentrated plasma samples by our method varies between 2 and 40 $\mu$mol/L, depending on the number of contributing protons and on the multiplicity of the resonance (Table 2). Of interest for the study of inborn errors of metabolism, the spectrum contains quantitative information on such important metabolites as creatine, dimethylglycine, and betaine, which are not measured by the conventional techniques used for screening for these diseases. The spectrum may also indicate abnormal concentrations of metabolites not known to be related to an inborn error of metabolism. However, the nonselective character of the technique demands thorough knowledge of the resonances that derive from medication. Knowledge on this aspect is still limited, and further studies are required.

To our knowledge, $^1$H-NMR data on serum or plasma samples from patients with inborn errors of metabolism have not been published—probably because of the technical limitations described above. The case of the child with 5-oxoprolinuria illustrates that $^1$H-NMR spectroscopy in plasma samples may be used diagnostically in children with inborn errors of metabolism. Although this particular diagnosis can easily be established by

analysis of urine with conventional techniques, the use of $^1$H-NMR spectroscopy in studies of metabolites in plasma from patients with inborn errors of metabolism seems promising. We have used plasma and not urine in this metabolic study because the many more resonances in the urinary NMR spectra make interpretation of results more difficult. By providing an overall view on all H-containing substances present in the sample in quantities surpassing the detection limit, the technique may bring new perspectives to the study of inherited metabolic disease.

$^1$H-NMR spectra were recorded at the Dutch hf-NMR facility (supervisor, S. S. Wijmenga) at the Department of Biophysical Chemistry (head, C. W. Hilbers). We thank J. Joordens and G. Nachtegaal for their invaluable help and assistance. We also gratefully acknowledge the help of the coworkers of the Laboratory for Paediatrics and Neurology (laboratory head, J. M. F. Trijbels) for their advice and for the samples they kindly provided. This work was made possible by a grant from "Het Fonds Academische Profilering," Nijmegen, The Netherlands.

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