



NMR-Based urine screening delivers the most comprehensive overview of newborn health

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Introduction

Bruker is participating in projects to establish NMR-based screening of newborns in Germany with Greifswald University Hospitals and in Turkey with so far 12 hospitals coordinated by Infai GmbH. Newborn urine samples are collected together with relevant metadata. The projects are fully supported by ethics committee vote. NMR screening of urine has been chosen because of its significant benefits over other methods:

- Non-invasive sample collection
- Straightforward sample preparation without the need for derivatization
- Highest reproducibility detects even the smallest variations in concentration of all relevant compounds found in urine
- High throughput with full automation
- Access to more compounds than plasma
- Targeted and non-targeted screening in one experiment

The NMR procedure is based on reference cohort urine spectra. Reference models must be extensively validated and then tested against further samples. Various statistical models can be developed beyond the reference model, enabling the prediction of overall status of the newborn in terms of growth, maturity, recognition of common diseases, relativity to parameters not directly visible by NMR and more. In addition to the non-targeted approach using statistical methods, a substantial set of compounds can be quantified using the same experiment run for statistical

evaluations. Compounds can be safely identified using a rapid 2D-experiment (2D-J-Resolved), implemented within the measurement sequence before quantification is executed. No internal standard is needed for quantification; as long as values are determined for a reference sample, all other compounds can be quantified. With modern digital NMR receiver technology, a dynamic range of compound concentrations of at least $2 \cdot 10^5$ is possible within a single experiment.

Reproducibility

High reproducibility is essential for the detection of the smallest variations in the spectra. Figure 1 shows 30 replicates of the same sample, each prepared separately. The

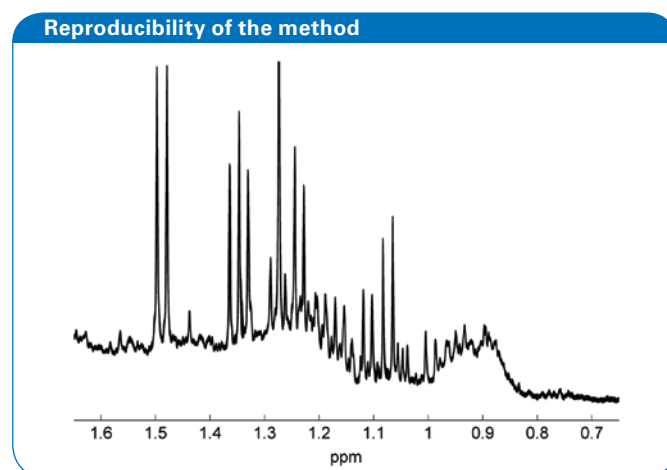


Figure 1: 30 replicate urine samples in overlay

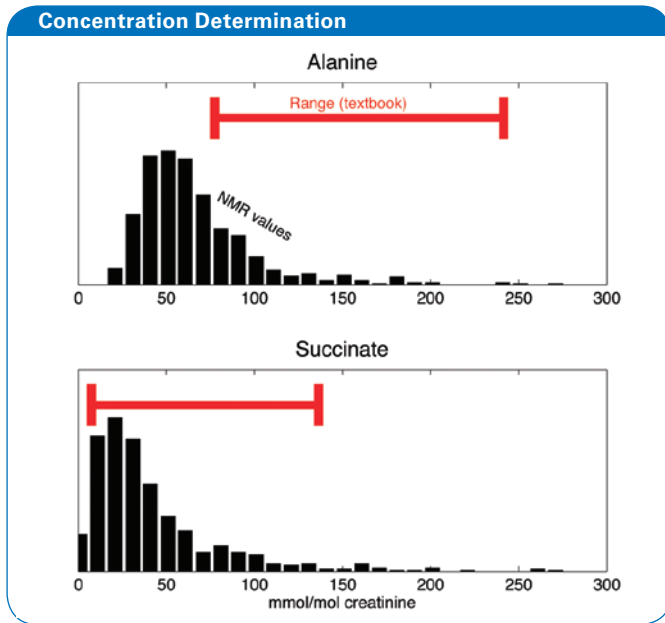


Figure 2: Comparison of Alanine and Succinate concentrations by NMR to textbook ranges (red bars).

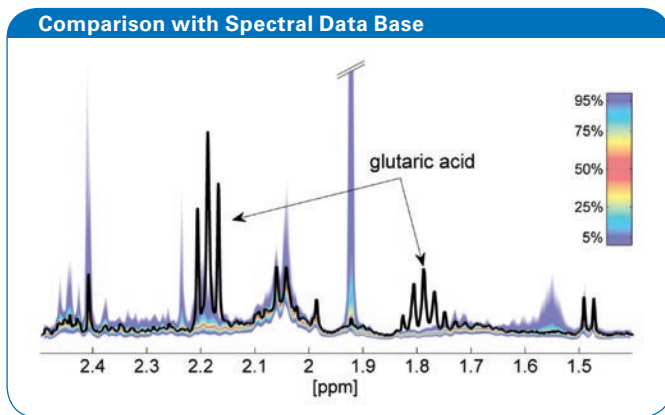


Figure 3: Projection of the urine spectrum of an inborn error sample into the German normal model.

| | |
|------------|------------------------|
| P < 0.0001 | Gender |
| | Day of life |
| | Gestational age |
| | Petrussa Score |
| | Head circumference |
| | Birth weight / -length |
| | Spontaneous / Section |
| P < 0.005 | Mother → BMI increase |
| | Anesthesia (yes / no) |
| | Anesthesia method |
| P < 0.05 | Age of mother |

Figure 4: Probability values for testing the hypothesis that the above listed metaparameters do not influence the urine NMR spectra

measurements were performed on 3 different instruments and then all was overlaid. Spectral databases of pure reference compounds and knowledge bases derived from thousands of urine samples support the project. The reference compound spectral base consists of over 600 compounds occurring in urine, including compounds indicative of inborn errors of metabolism. The ability to quantify a large number of compounds in every sample allows valid concentration distributions to be accumulated. Some were found to be different from the typical value sets published in textbooks and literature about inborn error analysis, where the number of samples previously used had been typically limited to small cohorts.

Non-targeted Screening

Non-targeted screening enables the detection of all NMR-visible deviations from normative references, whether known or unknown. The figure below illustrates the identification of deviations from normality. All NMR spectra included in the reference model are combined in a so-called quantile plot, shown as a color band over the NMR spectrum. At each point in the spectrum the intensity distribution is visualized, where red represents the 50% average. The resulting blue indicates that only a few samples in the model show such intensities. A spectrum from a new sample can be overlaid and tested for consistency with the model, i.e. all resonances will fit into the envelope defined by the model. This testing can be performed automatically in a uni- or multivariate fashion. The sample spectrum in figure 3 represents a glutaric aciduria case. In the expansion of the overall NMR spectrum, signs of Glutaric Acid are clearly visible. The spectral segment shown represents only about 15% of the overall spectrum. As part of the reference compound database, Glutaric Acid can be identified automatically; however statistics would also reveal the existence of previously unseen deviations with the same certainty.

Influence of Metadata

Beside the use of normal models, it is also possible to investigate the influence of metadata on the NMR spectra. In newborn screening, typical metadata are gender, birth weight, head circumference, Petrussa score, age of the mother at birth, and age of the newborn when the sample was collected. The hypothesis that such parameters do not influence the NMR spectrum can be tested with statistical tools, calculating a probability value. If this value is very small, the hypothesis has to be rejected. Figure 4 shows the results obtained from the German cohort under investigation, where it is obvious that multiple parameters have been found that influence the NMR spectrum.

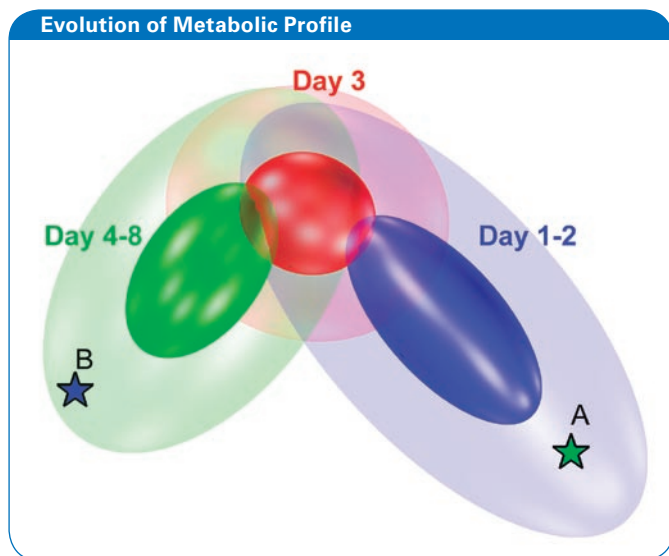


Figure 5: Trajectory of the first days of life as obtained from the NMR analysis of urine, containing 2 new samples projected onto the trajectory

Evolution of Metabolic Profile during First Days of Life

The influence of the day of life after birth on the NMR spectra was especially investigated. The sample set was divided into three groups. Group one contained samples from days 1 and 2 after birth, group two contained all samples from day 3 and group three contained all samples from days 4 to 8. This then created a trajectory for the first days of life, representing the rapid changes that occur in the first days of life, when organ functions are developed, jaundice peaks and the influence of the mother recedes. When new samples are projected onto this trajectory, it is possible to determine whether the babies' development follows the model pattern. Figure 5 shows the trajectory, with 2 new samples projected onto it. The color of the stars represents the age group in which the babies should. It is clear that sample B corresponds to a baby who has developed further than usual. Sample A is interesting because it comes from day 5 but falls into the group of day 1 and 2. This could indicate retardation in development and therefore advise further testing, as retardation could be the first sign of an inborn error not yet manifested by the usual biomarkers.

Figure 6 summarizes the outliers found in a screening of 700 newborns at Greifswald University Hospital, tested against the normal model. 2 inborn errors were confirmed, which was surprising, since the accumulated rate of inborn errors in Germany is believed to be 1 out of 1100 babies. The other outliers demonstrate the power of NMR in testing the general health of a baby.

Conclusion

The potential for NMR in the analysis of inborn errors is comprehensively described in the Handbook of ^1H -NMR Spectroscopy in Inborn Errors of Metabolism, published by the R.A. Wevers group in Nijmegen (SPS, ISBN-10: 3936145024), which details approximately 80 different inborn errors detected by NMR.

This potential can be applied in a push-button high throughput screening mode, once the normative reference model has been established. Reference models will not apply on a worldwide basis as there are differences in race, phenotype, nutrition and many more parameters. Therefore the applicability of a regional model must be tested in the surrounding regions; eventually a different model will have to be developed.

1 MCAD (medium chain acyl-CoA dehydrogenase) deficiency

- Cametine, acetyl-, octanoyl-carratine
- 3-aminobutyric acid

4 Transient galactosemia?

- Galactose

1 Orotic aciduria, galactosemia?

- Orotic acid
- Galactose and lactose

11 Ketosis?

- 3 Hydroxybutyric acid, ...

13 Abnormal gut colonization, increased intestinal permeability?

- Acetoine

40 Other outliers

- Dicarboxylic acids, butyric acid, 2-aminobutyric acid, glycine, taurine, ...
- Lactate, acetate, ethanol, formate, succinate, 2-oxoglutarate, ...

Figure 6: Results on a cohort of 700 German Newborns screened against the normal model, resulting in 11.5% outliers

References

The following organizations are involved in the newborn screening development project. ZIM financially supports the project in Turkey.



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