Metabolic Profiling of Urine by ^{1}H-NMR Spectroscopy

A critical Assessment of Interpreting Metabolite Concentrations for Normal and Diabetes Groups

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The metabolic profile of urine from a control group has been obtained by ^{1}H-NMR spectroscopy at 400 MHz. Data have been processed both as absolute (mmol/L) and relative (mmol/mol of creatinine) concentrations. The normal values have been compared with data from type II diabetes mellitus (DM II). The average concentrations of various metabolites in urine for normal and DM II subjects are presented. Some of these values are not routinely obtained by classical urine analysis. Our data are in good agreement with some previously reported data but they are not identical. Possible explanations for the small variations are discussed in terms of NMR experimental parameters and lifestyle differences. Preliminary results indicate significant differences between the two groups for the averaged relative concentrations (mmol/mol creatinine) of valine (Val), lactate (Lac), γ-aminobutyrate (GABA), pyruvate (Pyr), and alanine (Ala). However, the interval over which the individual values are spread is overlapping for all metabolites, excepting glucose.

Keywords: NMR, MRS, Urine, Metabolites, Diabetes

^{1}H-NMR spectroscopy has already proven its power in the urine analysis. Pioneering works have been carried out by J. K. Nicholson and P. J. Sadler in mid 1980's once the 400 MHz NMR instruments widely penetrated the chemical community [1-7]. By late 1990's the NMR urine analysis became an established technique for diagnosis of metabolic disorders.

The major advantages of the NMR method are the provision of direct information, and a global biochemical profile, with minimum sample preparation. In contrast, classical methods require pre-selected conditions for the markers of interest. The technique is extremely powerful in tracing abnormal metabolites. However very little has been done until now in terms of establishing databases for healthy individuals, for quantifying interlaboratory accuracy, and for assessing normal population variations. In general, each NMR group is developing its own in-house reference spectral database and uses it to target abnormal cases. In this way, parameters are not standardized and the databases are not tested for populations outside the geographical region where it was developed.

To date the reference work for normal values for metabolite concentrations in urine obtained by NMR have been published by the Zuppi's group [8]. The same group also described the only comparison of metabolite concentrations for control populations from two different geographical regions [9].

We present below normal values for the same metabolites described by Zuppi for a control group in Bucharest. A preliminary comparison with a pathological group of type II Diabetes Mellitus in the same geographical region is also presented.

Experimental Part

The NMR spectra were recorded on a Bruker Avance DRX 400 MHz spectrometer (Bruker Biospin, Rheinstetten, Germany), using a 5 mm inverse detection multinuclear probehead equipped with gradients on the z-axis. The sample was run in 5 mm Norell 507 NMR tubes. To 0.9 mL urine, 0.1 mL of a stock solution of 5 mM sodium 3-(trimethylsilyl)-[2,2,3,3-d_4]-1-propionate (TSP) (Aldrich) in D_2O (Aldrich) was added. The chemical shifts are reported as δ values (ppm) referred to TSP as internal standard. The ^{1}H-NMR spectra were recorded with water presaturation. The pulse sequence used 32 scans, a 90° pulse, 30s relaxation delay, 3 s CW irradiation, 4s acquisition time, 4790 Hz spectral window, collecting 38 K data points, with a resolution of 0.13 Hz. The FID was processed for a line broadening of 0.5Hz, prior to the Fourier transformation.

The control group was made of 26 subjects, out of which 16 females and 10 males, characterized by the mean age of 36 years (ranging between 25 - 67 years old).

The type II DM group was made of 19 patients, out of which 9 females and 10 males, characterized by a mean age of 49 years (ranging between 38 and 63 years old). The DM group was selected so that all subjects present glucosuria.

In order to evaluate the daily variations, the interval of confidence for the NMR determinations, and the variability of the results introduced by different operators, 7 urine

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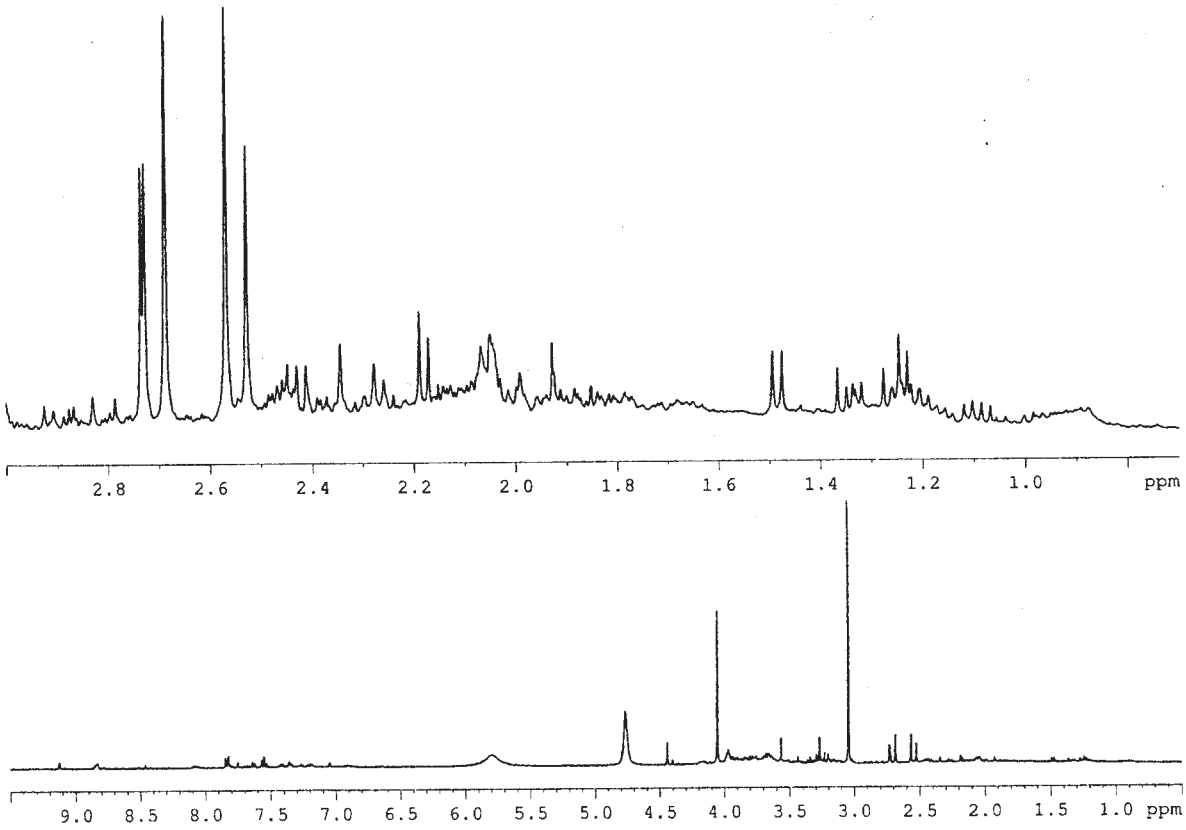

Fig. 1. The ^{1}H-NMR spectrum of urine from a control subject.

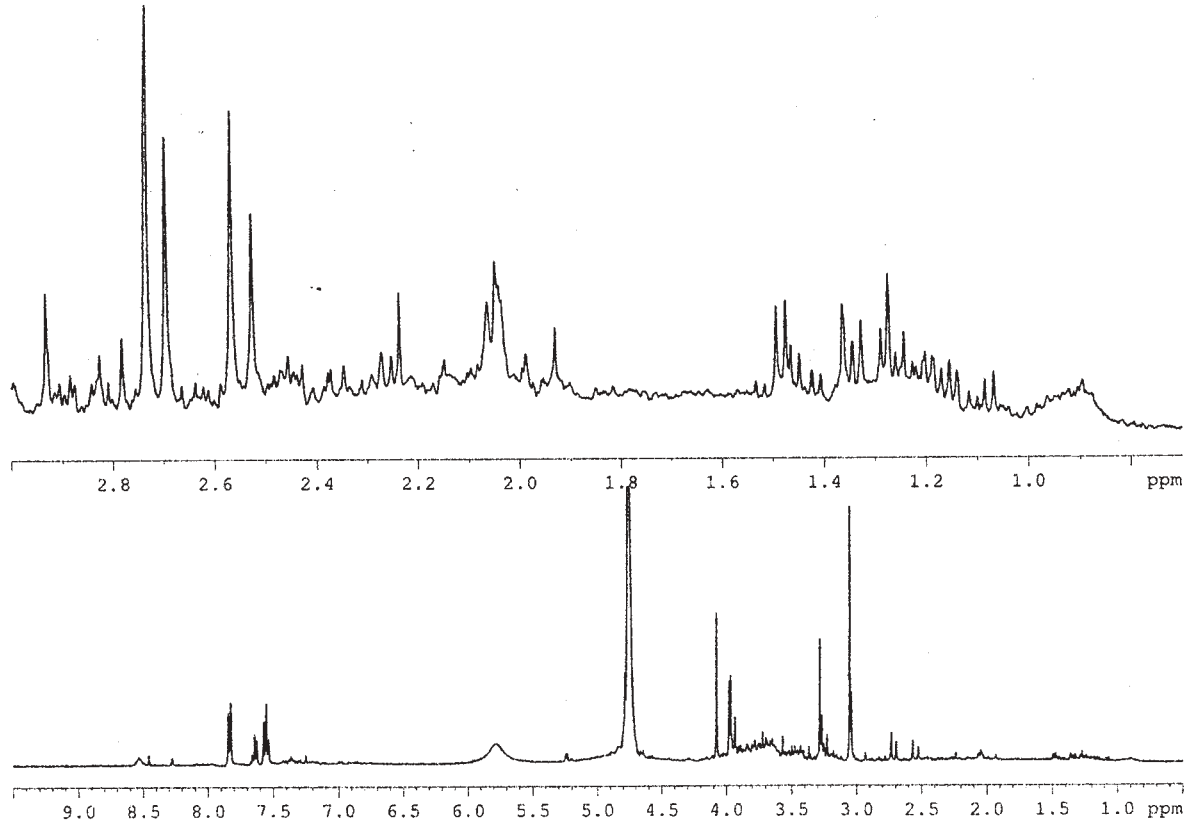

Fig. 2. The ^{1}H-NMR spectrum of urine from a diabetic patient with 2.4 mmol/L glucose.

samples from one healthy subject have been collected in different days and at different hours, showing reproducible results (95% IC).

Results and discussion

Figure 1 shows the ^{1}H-NMR spectrum of urine from a subject belonging to the control group. Figure 2 shows the ^{1}H-NMR spectrum of urine from a DM II subject with the level of glucose close to the low limit of detection. For such cases and for the DM cases without glucosuria, it is of great value to identify other metabolites as markers for the disease. Figure 3 shows ^{1}H-NMR spectrum of urine from a DM II subject with very high level of glucose. In this case, it is evident that the signals from glucose are masking almost all the other metabolites in the region between 3 – 4 ppm. For this reason, in the present paper we discuss only

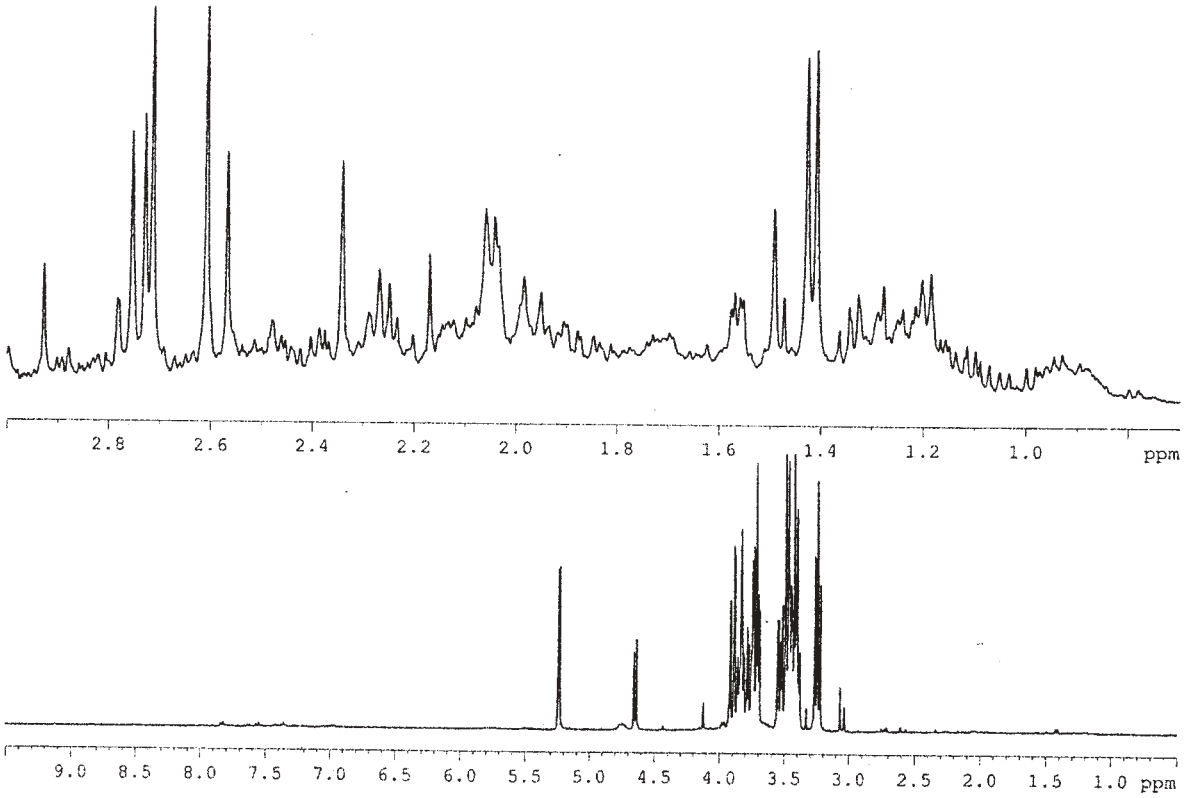

Fig. 3. The ^{1}H-NMR spectrum of urine from a diabetic patient with 325.4 mmol/L glucose.

metabolites that can be quantified based on signals outside the glucose region in the NMR spectrum.

For seven serial samples from the same healthy subject, the variations of the concentrations relative to creatinine where in the range of ±7 to ±12% for most of the metabolites, with the notable exceptions of the Pyr (±30%), Cit and DMA (-13% to +20%), and Hipp (±60%).

Although either no line broadening or 0.3 Hz line broadening are common post-acquisition FID processing, we have chosen 0.5 Hz line broadening. We found this value sufficient for assignment and resolution of the signals of interest. We ensured thus a better compensation for possible shim imperfections and variations between samples and we induced similar linewidths for all signals, enabling better comparison based on signal intensities instead of integrals.

In previous studies, urine has been subjected to NMR analysis both with and without *p*H adjustment. The *p*H adjustment has the advantage of making the peak assignments easier, as they would appear at the same chemical shift value. Analysis without *p*H adjustment would avoid supplementary manipulations of the sample (limiting the operator errors) and would provide an extra parameter (chemical shift variation) for possible chemometric treatments of the NMR data. For the present study, we have chosen not to adjust the *p*H.

In previous urine NMR analyses, depending on the author's choice, either absolute (mmol/L) or relative (mmol/mol of Creatinine) concentrations are reported. The absolute concentration provides a better understanding of the individual variation of metabolites. The relative concentration may have some advantages, as well. Thus, it would eliminate a possible source of error when preparing the standard solution and the sample solution (especially when more NMR operators are involved and in inter-laboratory trials). It would also work better in cases when small amounts of proteins are present in urine (TSP being known to complex with proteins, thus diminishing its signal intensity in such cases). As we ensured that we had no proteins in our urine samples, we tend to favor the absolute concentration. However, in order to enable easier comparisons with other studies, we present both absolute and relative concentrations for metabolites.

There are significantly different average concentration of some metabolites between the two groups (tables 1 and 2). We present values for several metabolites which have not been systematically studied for DM (For, GABA, Pyr, Val, 3-OH-i-Val), as they are easy to detect and do not interfere with saccharides in the spectrum. For all the other studied metabolites, we had good agreement with previously published data [10, 11], except for alanine for which we obtained higher values and for hippurate for which we obtained lower averaged concentrations. Hippurate is known to have high and not well understood variations. For the case of alanine, although each group (control and DM) exhibited higher concentrations in our case, when comparing the variations between groups the increase (2 times for DM) is in the same range with other published data [10, 11]. As there is only one other group that reported large NMR data sets on both control and diabetes groups, we should underline that there is good agreement in terms of effect of diabetes on the studied metabolite excretion. On the other hand, we report a slight shift in the average concentrations for both control and diabetes groups. Considering the dearth of available data to date any interpretation of these small differences should be made with great caution. We note that there are significant differences between our NMR experimental protocol and that of the Zuppi's group. Thus, Zuppi employed a very short recycling time, (3 or 4 s) which forced them to employ a short tip angle (40°) in order to compensate for T_1 relaxation and ensure quantitative results. Based on the short recycle time they employed a larger number of scans (120) than we did. In our experimental design we used an NMR spectrometer with a higher magnetic field (400 MHz instead of 300 MHz) and an inverse probehead, ensuring a much higher sensitivity

Table 1
AVERAGED ABSOLUTE CONCENTRATIONS (MMOL/L URINE) AND RANGES FOR INDIVIDUAL VALUES (IN BRACKETS) FOR THE STUDIED METABOLITES AS MEASURED BY ^{1}H-NMR

Metabolite	Averaged Absolute Conc and Range of Values Control (mmol/L)	Averaged Absolute Conc and Range of Values DMII (mmol/L)
Creatinine (Crn)	15.51 (2.16 – 42.03)	9.29 (3.26 – 22.85)
Valine (Val)	0.12 (0.02 – 0.21)	0.12 (0.04 – 0.21)
Lactate (Lac)	0.50 (0.09 – 1.35)	0.86 (0.16 – 3.49)
3-hydroxy-iso-valeriate (3OH-i-Val)	0.14 (0.02 – 0.33)	0.11 (0.01 – 0.31)
Alanine (Ala)	0.63 (0.09 – 1.28)	0.68 (0.16 – 1.37)
γ-aminobutyriate (GABA)	1.59 (0.16 – 4.77)	1.48 (0.28 – 3.69)
Pyruvate (Pyr)	0.62 (0.08 – 1.99)	0.60 (0.06 – 2.18)
Citrate (Cit)	2.95 (0.68 – 5.87)	2.41 (0.67 – 6.02)
Dimethylamine (DMA)	0.41 (0.06 – 1.40)	0.32 (0.10 – 0.73)
Hippurate (Hipp)	3.78 (0.53 – 27.80)	2.00 (0.31 – 8.97)
Formate (For)	0.38 (0.09 – 1.11)	0.26 (0.05 – 0.62)
Glucose (Gluc)	0.00	126.45 (2.37 – 364.99)

for ^{1}H nuclei. We employed a long pulse (90°) with long recycling time (37 s) in order to ensure quantitative results. Based on the higher spectrometer sensitivity and also on the longer pulse length, we were able to obtain good signal to noise ratios using only 32 scans. In order to explain the small differences in the averaged values one could consider that the different experimental setups could induce a small systematic shift of the results. On the other hand, the same shift could be explained by the variations in diet, environmental factors and other lifestyle factors between the population considered in the present study and those considered in previously published results. There is only one paper reporting differences in the metabolite excretion for two geographical populations (Rome, Italy and from the Arctic Scientific Base in Svaldbard) [9]. When we considered the previously published data for subjects in Rome and Svaldbard, we found that our dataset from Bucharest, although slightly different, it is much closer to the group from Rome than that from Svaldbard. This could be explained by the fact that Bucharest and Rome are located at almost similar latitude and also the cooking traditions for these two Latin countries (Romania and Italy) have many common elements. As it is acknowledged that diet habits play an important role in excretion rates of various metabolites, there is a strong need for large inter-laboratory and inter-country trials for establishing normal ranges and geographical variations of metabolite concentrations in urine. For such trials, NMR proved to be the method of choice in terms of speed and costs (being able to spot several metabolites and the global profile in "one shot"). Although there are already several groups around the world who developed their own "in house" databases of urine NMR spectra, there is no way to avoid future large inter-laboratory trials allowing for both large geographical variations and magnetic field strength variations, in order to validate medical diagnosis based on NMR spectra of bodyfluids. In conclusion we can say that each NMR group seems to be able to assess the state of health based on its in-house database. It is still an open question how data can be transferred from one database to another and also how good is the prediction of the state of health for people belonging to geographical populations very different to the one for which the database has been built.

In addition to this, we have an even more critical assessment of the interpretation of urine metabolite concentrations (other than glucose) as a diagnostic tool for diabetes. Glucose can be easily spotted by NMR even at mmolar levels making the NMR diagnosis of DM with glucosuria, rapid and accurate. A preliminary comparison of the normal values with those from DM II patients, indicated that the averaged concentrations for other

Table 2

AVERAGED RELATIVE CONCENTRATIONS (MMOL/MOL CRN) AND RANGES FOR INDIVIDUAL VALUES (IN BRACKETS) FOR THE STUDIED METABOLITES AS MEASURED BY ^{1}H-NMR

Metabolite	Averaged Relative Conc and Range of Values Control (mmol/mol)	Averaged Relative Conc and Range of Values DMII (mmol/mol)
Valine (Val)	8.26 (5.39 – 12.07)	14.01 (7.27 -28.49)
Lactate (Lac)	39.83 (18.37 – 60.40)	100.15 (31.00 – 432.16)
3-hydroxy-iso-valeriate (3OH-i-Val)	10.01 (6.32 – 19.69)	12.91 (2.78 – 21.49)
Alanine (Ala)	45.74 (18.38 – 91.01)	79.25 (32.59 – 172.33)
γ-aminobutyriate (GABA)	108.05 (54.36 – 249.39)	167.61 (78.50 – 316.88)
Pyruvate (Pyr)	42.93 (19.73 – 94.24)	64.42 (17.47 – 134.36)
Citrate (Cit)	235.55 (45.81 – 394.38)	275.54 (64.07 – 643.03)
Dimethylamine (DMA)	28.67 (10.26 – 57.81)	36.05 (15.94 – 72.35)
Hippurate (Hipp)	246.76 (37.40 – 766.16)	239.91 (21.41 – 854.88)
Formate (For)	32.31 (10.55 – 78.90)	34.95 (9.55 – 124.73)
Glucose (Gluc)	0.00	15,018.65 (725.10 – 64,670.91)

metabolites are significantly different. However, excepting glucose, the interval over which the individual values are spread overlaps for all metabolites. Thus, NMR could be confidently used for studies where statistical tendencies are evaluated (e.g. for gathering information on the biochemistry and mechanisms associated with DM). However, the interpretation of any other metabolite values (except glucose) as an early medical diagnosis for DM should be avoided. In order for this approach to be transferred to clinical applications, a robust statistical classifier must be developed. We are currently investigating methods to develop this classifier.

Conclusion

The average concentration of various metabolites in urine from DM II patients is significantly different for that of the Control Group. As food habits and other lifestyle factors affect the metabolites profile in urine, the present study indicates that when both the control and DM groups are chosen from the same geographical region, the tendencies can be interpreted with confidence. A glucose based NMR clinical diagnosis for diabetes is accurate and reliable. A non-glucose NMR clinical diagnosis is not possible based only on several discrete metabolite concentrations. Such a non-glucose diagnosis might be possible based on a statistical classifier which uses the entire digitized NMR spectrum.

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