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Dedicated to the memory of
Professor Ioan Silaghi-Dumitrescu (1950 – 2009)

^{1}H-NMR URINE METABOLIC PROFILING IN TYPE 1 DIABETES MELLITUS

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The metabolic profiling of urine for 19 Type 1 Diabetes Mellitus patients and 32 controls was obtained by ^{1}H-NMR spectroscopy at 400 MHz. The classical diagnosis was performed in the Craiova Clinical Emergency hospital, based on classical quantitative and semiquantitative clinical tests. Apart from glucose, the results indicate significant differences for the averaged relative concentrations (mmol/mol creatinine) between the two groups for valine (Val), lactate (Lac), alanine (Ala), γ-aminobutirate (GABA), pyruvate (Pyr), citrate (Cit), and formate (For). Currently, these metabolites are not considered in routine clinical Diabetes screenings.

INTRODUCTION

Diabetes Mellitus is a chronic life-threatening disease in which the body does not properly produce or respond to insulin, and is characterized by increased levels of blood glucose leading to severe damage to vital organs such as heart, eyes and kidneys. Diabetes Mellitus is among the most prevalent diseases in the world. A large proportion of the affected individuals remain undiagnosed because of lack of specific symptoms in the early stages of the disorder.

Type 1 diabetes mellitus, formerly known as insulin-dependent diabetes Mellitus (IDDM) or juvenile onset diabetes mellitus, is caused by autoimmune destruction of the β-cells of the pancreas, rendering the pancreas unable to synthesize and secrete insulin.[1]

In diabetes, there may also be metabolic alterations in urine other than the changes in the levels of glucose, and identification of such metabolites could be useful in the development of non-invasive methods for the early diagnosis of diabetes.[2]

^{1}H-NMR spectroscopy has already proven its power in urine analysis. Pioneering works have been carried out by J. K. Nicholson and P. J. Sadler in the mid 1980's once the 400 MHz NMR instruments widely penetrated the chemical community.[3,4] The potential of NMR for the

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diagnosis of diabetes was also realized in the early stages of development of this research field.[5,6] By late 1990's, the NMR urine analysis became an established technique for the diagnosis of metabolic disorders. In spite of the great number of published papers on NMR urine analysis, and of the existence of several groups around the world active in the field, there are only a few published results on metabolite ranges determined by NMR. Most of these data are being kept as in-house raw databases. To date, the reference work for normal values for metabolite concentrations in urine obtained by NMR have been published by the Zuppi's group.[7] The same group published data for Diabetes groups,[8,9] and also described the comparison of metabolite concentrations for control populations from two different geographical regions, South Europe (Rome) *versus* the Arctic scientific base of Ny-Alesund (Svaldbard).[10] We also published metabolite concentrations for normal and Type 2 Diabetes groups from Eastern Europe (Bucharest), and we discussed the results in comparison with previously published data.[11]

RESULTS AND DISCUSSION

The present study was conducted on a type 1 diabetes Mellitus (DM1) group of patients admitted in the Department of Diabetes, Nutrition and Metabolic Diseases of the Craiova Clinical Emergency Hospital. The study complied with the medical research ethical rules and was approved by the Head of Department of Diabetes, Nutrition and Metabolic Diseases. The DM1 group consisted of 19 cases (9 females and 10 males), with the average age of 35.9 (ranging between 22 and 64 years old). The control group consisted of 32 cases (21 females, 11 males), with the average age of 34.5 (ranging between 24 and 59 years old). The diagnosis was based on classical clinical chemistry assays, and the DM1 group was made of patients without albuminuria and renal complications. All DM1 patients presented glucosuria. In parallel with the classical clinical chemistry assay, a ^{1}H-NMR spectroscopy assay was conducted with the purpose of assessing changes in concentrations for low molecular weight metabolites in the DM1 group in comparison with the controls.

In order to asses the quantifying power of the NMR non-classical method, we compared the results for creatinine obtained by ^{1}H-NMR spectroscopy for ten urine samples from healthy individuals with those obtained by alkaline picrate standardized method on an Abbott Aeroset Analyzer. Deming regression of the concentrations (mmol/L) determined by ^{1}H-NMR vs. the expected concentrations gave for creatinine slope=1.05±0.05, y-intercept=-0.54±0.53, r^2=0.99, $S_{x/y}$=1.00 showing good agreement and indicating that the non-classical method is suitable for clinical practice.

Signal assignments for metabolites in the ^{1}H-NMR spectrum of a urine sample at 400 MHz are presented in figures 1 and 2, for the aliphatic and aromatic regions respectively.

In DM1 patients the region between 3.2-5.3 ppm in the NMR spectrum is dominated by the glucose and residual water signals. Thus, although additional metabolites can be assigned in this region, the quantitation is difficult and subjected to large errors. For this reason we decided to not include this spectral region in the present study.

The NMR spectra have been recorded with addition of 10% D_2O in order to ensure the lock signal. The added D_2O contained 5 mM sodium 3-(trimethylsilyl)-[2,2,3,3-d_4]-1-propionate (TSP) which was used both as an internal reference signal for chemical shifts and as a reference integral for quantitation of metabolites. In addition to absolute concentrations of metabolites (table 1) we also present the concentrations relative to the internal concentration of creatinine for the same metabolites (table 2).

By examining the results in tables 1 and 2, it appears that in addition to glucose, other metabolites exhibit significant averaged concentration differences in the DM1 group in comparison with controls. These effects are better seen in table 2 where the concentrations are internally referenced to creatinine. Thus, we found significant differences (higher than 10%) for relative concentrations in the cases of valine (increased 1.3 times in DM1 in comparison with controls), lactate (1.4 X), alanine (1.8 X), GABA (1.1 X), pyruvate (1.2 X), citrate (1.3 X) and formate (1.7 X).

The only other study reporting data for metabolites in urine of DM1 patients was published by Zuppi et. al.,[9] but the studied groups (both DM1 and control) where made of children with average age of 14 years and ranges from 6 to 18 years old. The previously reported study shown the same trend as in our case but with higher differences between groups: lactate (3.8 X), alanine (2.1 X), citrate (1.9 X) hippurate (1.8 X), with the other metabolites not reported or not measured (Val, 3OH-i-val, GABA, Pyr, DMA, For).[9]

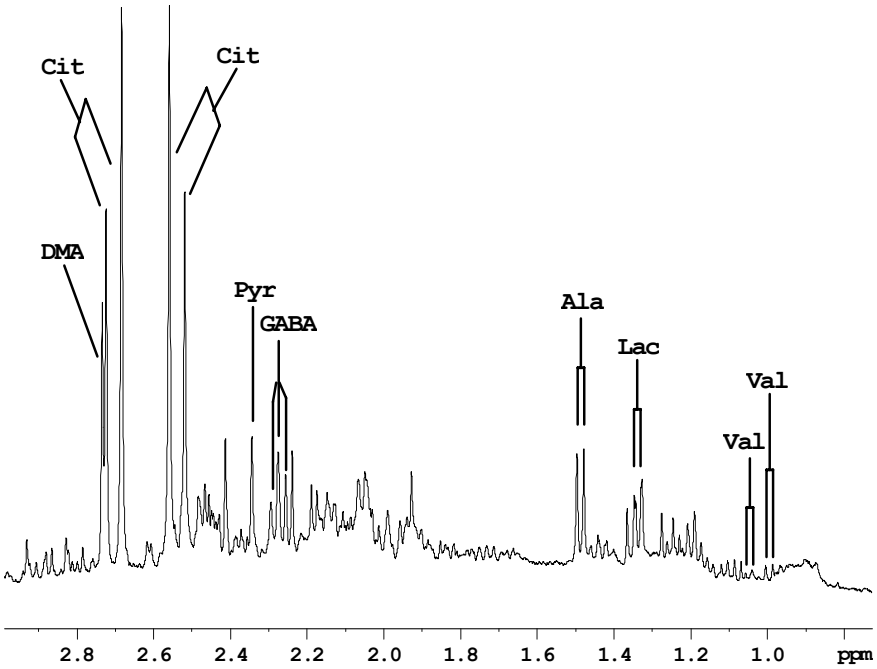

Fig. 1 – Signal assignments in the aliphatic region of the ^{1}H-NMR spectrum of a urine sample with 10% D_2O at 400 MHz.

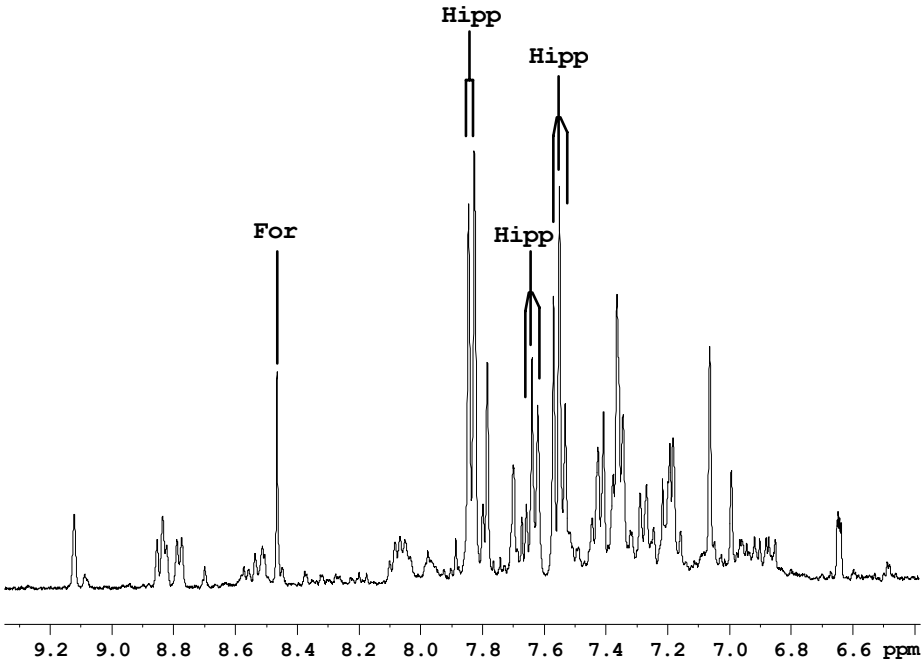

Fig. 2 – Signal assignments in the aromatic region of the ^{1}H-NMR spectrum of a urine sample with 10% D_2O at 400 MHz.

Table 1

Averaged absolute concentrations (mmol/L urine) and ranges for individual values (in brackets) for the selected metabolites as measured by ^{1}H-NMR spectroscopy

Metabolite	Averaged absolute concentration and ranges for the control group (mmol/L)	Averaged absolute concentrations and ranges for DM1 group (mmol/L)
Creatinine (Crn)	10.75 (2.42 – 33.61)	7.32 (0.96 – 14.40)
Valine (Val)	0.16 (0.08 – 0.28)	0.11 (0.03 – 0.20)
Lactate (Lac)	0.57 (0.16 – 1.38)	0.46 (0.12 – 0.87)
3-hydroxy-*iso*-valeriate (3OH-i-Val)	0.11 (0.03 - 0.30)	0.07 (0.02 – 0.14)
Alanine (Ala)	0.54 (0.11 – 1.58)	0.52 (0.13 – 1.26)

Table 1 (continued)

γ-aminobutyrate (GABA)	1.19 (0.25 – 4.65)	0.77 (0.15 – 1.40)
Pyruvate (Pyr)	0.48 (0.07 – 1.44)	0.28 (0.06 – 0.67)
Citrate (Cit)	2.36 (0.51 – 7.71)	2.17 (0.27 – 5.24)
Dimethylamine (DMA)	0.34 (0.05 – 0.95)	0.23 (0.04 – 0.44)
Hippurate (Hipp)	1.97 (0.38 – 5.84)	1.31 (0.04 -3.90)
Formate (For)	0.43 (0.19 – 0.92)	0.51 (0.13 – 2.09)
Glucose (Gluc)	0.00	76.40 (1.00 – 285.79)

Table 2

Averaged relative concentrations (mmol/mol creatinine) and ranges for individual values (in brackets) for the selected metabolites as measured by ^{1}H-NMR spectroscopy

Metabolite	Averaged relative concentration and ranges for the control group (mmol/mol Crn)	Averaged relative concentrations and ranges for DM1 group (mmol/mol Crn)
Valine (Val)	11.58 (8.51 – 15.04)	15.49 (4.79 – 30.71)
Lactate (Lac)	65.78 (21.37 – 249.66)	90.28 (23.35 – 424.10)
3-hydroxy-iso-valeriate (3OH-i-Val)	10.50 (7.02 – 14.38)	10.78 (4.94 – 21.07)
Alanine (Ala) 1.76	52.38 (14.39 – 136.02)	92.23 (30.81 – 397.76)
γ-aminobutyrate (GABA)	103.89 (72.43 – 169.51)	118.51 (63.24 – 247.72)
Pyruvate (Pyr)	38.50 (18.32 – 69.64)	45.58 (18.06 – 104.62)
Citrate (Cit)	242.34 (81.79 – 403.84)	304.51 (105.96 – 586.60)
Dimethylamine (DMA)	30.35 (16.30 – 50.36)	32.42 (4.89 – 61.25)
Hippurate (Hipp)	189.34 (60.44 – 521.82)	193.62 (5.52 – 731.67)
Formate (For)	55.42 (14.60 – 120.70)	92.45 (20.06 – 524.35)
Glucose (Gluc)	0.00	17,449.11 (137.28 – 129,930.33)

If we compare the results reported in *table 2* with the previously reported study,[9] apart from the smaller differences between groups in our case, and the higher number of metabolites reported by us, a significant difference is in the case of hippurate. Thus, the previous DM1 study reported a significant increase for relative concentrations of hippurate, whereas we did not find significant differences between groups. However, hippurate was previously reported in literature to have high variations of concentrations which are not related to the general state of health of the subject.

As both we and Zuppi's group used control and DM1 groups matching both age and geographical region, the differences mentioned above are most likely linked to the differences in the target groups (35 *versus* 14 years old respectively).

Another general observation is that, apart from glucose, no other metabolite alone may be used as a marker for DM1 diagnosis. This is due to the fact that although the averaged concentrations are significantly different for the two groups, the individual values overlap in all cases. Thus, in order to perform a non-glucose DM1 clinical diagnosis based on NMR urineanalysis, a chemometric approach accounting for all individual concentrations should be developed.

EXPERIMENTAL

The DM1 group with less than 5 years history of the disease, consisted of 19 cases (9 females and 10 males), with the average age of 35.9 (ranging between 22 and 64 years old). The control group consisted of 32 cases (21 females, 11 males), with the average age of 34.5 (ranging between 24 and 59 years old).

In order to select the DM1 patients for the present study, daily collection and investigation of the urine samples was performed by conventional clinical chemistry assays and microscopy method for DM patients hospitalized in the Diabetes, Nutrition and Metabolic Diseases Department of the Craiova Emergency Clinical Hospital. The diagnostic criteria for DM1 followed the methods established by the American Diabetes Association.[1]

Morning blood samples were collected and analyzed for glucose (glucose hexokinase HK enzymatic method), urea nitrogen (urea nitrogen modification of a totally enzymatic procedure) and creatinine (alkaline picrate method) using an Abbott Aeroset Analyzer. All patients showed reference plasma values (mean±SE) of blood urea nitrogen 12.42± 0.83 mmol/L (34.8±2.34 mg/dL) and creatinine 74.25± 2.65 μmol/L (0.84±0.03 mg/dL). Within the same day urine samples were collected, analyzed and stored as reported below. We measured creatinine 10.44±1.30 mmol/L (117.98±14.75 mg/dL) and urea nitrogen 712.53±59.46 mmol/L (1995.8±166.55 mg/dL) in all urine samples from DM1 patients on an Abbott Aeroset Instrument.

All urine samples were analyzed by semiquantitative biochemical determinations/strip tests of urobilinogen, glucose, bilirubin, ketones, SG, blood, pH, protein, nitrite, leukocytes, ascorbic acid and for the detection of albumin by

sulfosalycilic acid method. Also, we measured for 10 subjects urine creatinine by alkaline picrate reaction at 500 nm on the Abbott Aeroset Analyzer.

Urine samples were collected between 10 a.m and 2 p.m in sterile containers with tight-fitting covers and, after biochemical determinations, they were frozen and stored at -80°C until shipping them to the NMR laboratory. The transport to the NMR laboratory (typically 5 hours) was done in containers cooled at 0°C. In the NMR laboratory the samples were either analyzed the same day or stored at -20°C for a period ranging from 1-10 days.

The NMR spectra were recorded on a Bruker Avance DRX 400 MHz spectrometer, using a 5 mm inverse detection multinuclear probe equipped with gradients on the z-axis. The samples were run in 5 mm Wilmad 507 NMR tubes. Before NMR analysis, the samples were allowed to reach room temperature (typically one hour) and centrifuged at 7,000 rpm for 10 min. 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 pH was not adjusted. Most of the samples had pH values ranging from 5 to 6 with a few extremes between 6.5 and 7. 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 90º pulse, 30 s relaxation delay, 3 s CW irradiation, 4 s acquisition time, 4790 Hz spectral window, collecting 38 K data points, with a resolution of 0.13 Hz. Post-acquisition 0.5 Hz line broadening was applied, and manual baseline correction was performed.

CONCLUSIONS

The present study, provided a metabolic trend in urine NMR profiling of DM1 patients for 12 metabolites (including glucose). Comparison of our results with previously reported data for DM1, confirmed the trend for some metabolites, completed the trends for previously unreported metabolites, and underlined the need for larger studies, including extensive interlaboratory trials in order to asses the influence factors like age, sex and geographical region on the NMR diagnosis of diabetes. Non-glucose markers for DM1 are interesting in various respects, such as providing clues for the biochemistry and mechanisms of the disease, potential early diagnostic markers, and potential markers for DM patients without glucosuria.

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