Quebec Cooperative Study
of Friedreich's Ataxia

Quantitative Metabolic Profiling of α-Keto Acids in Friedreich's Ataxia

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SUMMARY: *The plasma distribution of α-keto acids was measured in 26 subjects including 8 patients with Friedreich's ataxia, 8 with the recessive spastic ataxia of Charlevoix-Saguenay and 10 healthy volunteers. The groups were matched with regards to age, sex, weight and the study was conducted under standardized dietary intake. The results indicate significant differences in α-keto acids distribution between the groups.*

RÉSUMÉ: *Nous avons étudié la distribution plasmatique d'acides α-cétonés chez 26 sujets, incluant 8 patients atteints de l'ataxie de Frioedreich, 8 atteints de l'ataxie spastique récessive de Charlevoix-Saguenay ainsi que 10 volontaires sains. Les groupes étaient appariés pour l'âge, le sexe, le poids et l'apport diététique était normalisé pour tous les sujets durant l'étude. Les résultats indiquent des différences significatives dans la distribution des acides α-cétonés entre les groupes.*

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INTRODUCTION

The investigation of Friedreich's ataxia over the last few years has revealed several biochemical anomalies in patients suffering from this disease. Abnormal handling of pyruvate in blood during glucose tolerance test has been reported (Barbeau, 1975; Barbeau et al, 1976; D'Angelo et al, 1980).

Furthermore, anomalies related to enzymatic deficiencies in pyruvate dehydrogenase PDH (Blass et al, 1976) and glutamate-dehydrogenase GDH (Barbeau et al, 1980) coupled with defects in aspartic and glutamic acids (Huxtable et al, 1979) have incited us to examine the possibility of generating metabolic mapping in Friedreich's ataxia. Since Barbeau (1980) has recently suggested the presence of an energy deprivation state in mitochondria in order to explain the biochemical defects underlying the development of the main symptoms and since the precited reports reflect directly or indirectly on certain α-keto acids, which play a major role as intermediates in metabolic pathways leading to cellular energy production, it appeared important to quantify these acids. This report presents the results obtained in a quantitative mapping of plasma α-keto acids in patients with Friedreich's ataxia from Rimouski, Quebec (Bouchard et al, 1979)and patients with the recessive spastic ataxia of Charlevoix-Saguenay (Bouchard et al, 1978).

SUBJECTS AND METHODS

Subjects

The study was carried out in a total of 26 subjects including 8 patients with Friedreich's ataxia from Rimouski, Quebec (Bouchard et al, 1979), 8 patients with the recessive spastic ataxia of Charlevoix-Saguenay (Bouchard et al, 1978) and 10 healthy volunteers. The subjects received a complete physical and biochemical examination and groups were matched with regards to age (mean 25,5) sex and weight. Indications of the presence of diabetes was a reason for rejection. Furthermore, during and prior to the period of the study none of the subjects were known to take medication. The clinical staging of patients according to Pourcher et al (1980) was as follows: 1 stage II, 6 stage IV and 1 stage V for the Friedreich and 4 stage II, 3 stage III and 1 stage IV for the patients with recessive spastic ataxia of Charlevoix-Saguenay.

The subjects were hospitalized and were standardized with respect to their dietary intake for a period of 72 hours before sampling was done. Meals were prepared based on the customary eating habits of the subjects (beef, chicken) and followed the Canadian nutritional standards (1977) with a daily limit of 2800 calories. The actual food intake was weighed after every meal. Dietary standardization of the subjects is essential in order to obtain meaningful results in such a study since many factors can influence the distribution of α-keto acids. Throughout the duration of the study all subjects were under strict medical supervision.

Sample collection

During the second day of the study, a 24 hour urine collection was obtained from subjects and blood samples were collected on the third morning. Upon venipuncture, the first 5 ml of blood were discarded in order to eliminate artefacts and the following 10 ml were collected in vacutainer tubes without

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silicone and additives. The samples were immediately centrifuged at 0°C and fresh plasma was transferred into culture tubes and frozen at -35°C. The samples, kept in dry ice, were brought to the laboratory and remained frozen until analysis.

Sample preparation

All reagents and solvents were of analytical grade and solvents were redistilled before use. All α-keto acids as well as 1,2 diaminobenzene dihydrochloride were obtained from Sigma Chemicals (St-Louis, Mo., U.S.A.). Silylating reagents such as N-methyl-N-trimethyltrifluoro-acetamine (MSTFA), trimethylchlorosilane (TMCS) and silylation grade pyridine were from Pierce (Rockford, Il., U.S.A.). Pure quinoxalinol standards were prepared by reaction of the appropriate α-keto acids with 1,2 diaminobenzene as described by Morrison (1954).

Untreated plasma samples are defrozen and a 0.50 ml aliquot is placed in a screw capped test tube with 100 μl of the internal standard solution (1 mM α-keto-valeric acid in 2 N HC1). The mixture is then adjusted to 2N with respect to HC1 and 50 μl of a freshly prepared 1% solution, of 1,2 diaminobenzene dihydrochloride in HC1 2N is added. The tube is then incubated for 1 hour at 70°C on an aluminum bloc. After this period of time, the formation of the quinoxalinol derivatives is quantitative (Bertrand et al, 1982). The sample is cooled and the pH adjusted to 1.0 with addition of 2 gr of ammonium sulfate in order to optimize recovery yields. The solution is then extracted with 5 ml of ethyl acetate for 10 minutes on an Eberback mechanical agitator. After centrifugation at 3000 rpm for 5 minutes, the organic layer is carefully transferred into a clean dry reaction tube and evaporated to dryness under a stream of nitrogen 99.99 Zero gas, (Cryogas, Montreal, Can.) The dry residue is then taken up in 50 μl of pyridine to which 50 μl of MSTFA containing 1% TMCS is added and the mixture sealed under nitrogen is incubated for 30 minutes at 70°C. The sample is then ready for analysis.

Sample analysis

All analyses were performed using independently capillary gas-chromatography (GC) and gas-chromatography-mass spectrometry (GC/MS) and quantitative results obtained by both techniques were the same, within experimental error. The GC analysis were performed using a Perkin-Elmer Sigma 2B gas chromatograph equipped with a flame ionisation detector (FID). The chromatographic conditions were as follows: a 10 meter fused silica (0.23 mm I.D.) capillary column SP-100 (Hewlett-Parkard) coated with methylsilicone was maintained at 120°C for 2 minutes, then temperature programmed at 8°C/min to a final temperature of 240°C where it was held for 10 minutes. The reaction mixture was injected using a Perkin-Elmer all glass split injector maintained at 250°C. The capillary injector contains a glass liner packed with silanized glass beads. This precolumn ensures complete vaporisation and mixing with the carrier gas prior to splitting and traps undesirable substances before they reach the column. For all analysis the precolumn flow was 20 ml/min giving a 2 ml/min flow of He through the column and a split ration of 9:1. The make up gas was helium at 30 m/min and H_2 and air at the detector (250°C) were respectively 20 and 450 ml/min. GC analysis data acquisition and reduction was done by the use of a Sigma 15 data station linked to the chromatograph and computerized reports of analysis were obtained using the internal standard method. Peak quantification was based on molar response factor to the internal standard (Bertrand and Gauthier, 1982).

When using GC/MS, the analyses were done on an MS-25 (Kreatos Inc.) mass spectrometer coupled to a Perkin-Elmer Sigma 3 gas-chromatograph. The chromatograph was equipped with a 1.83 meter 2 mm I.D. glass column packed with 3% OV-1 on chromatosorb W. The column was maintained at 70°C for 2 minutes, then temperature programmed at 3°C/min to a final temperature of 250°C and held there for 10 minutes. The chromatograph was interfaced to the mass spectrometer through a jet separator interface. The mass spectrometer was operated in the selective ion monitoring mode which offers a specific response to α-keto acid quinoxalinol derivatives (Gauthier et al, 1980). The quantification of α-keto acids was effected through a DS-50S data system using the quinoxalinol derivative of α-keto-valeric acid as an internal mass reference standard (Bertrand et al, 1982).

During analysis using both techniques a calibration mixture of the quinoxalinol derivatives of the α-keto acids was injected after every four samples to verify calibration and instrumental parameters.

RESULTS AND DISCUSSION

Quantitative profiles of α-keto acids in plasma have been obtained in 26 subjects. Results for six α-keto acids: pyruvic (PYR), 2-oxo-isocaproic (KICAP), 2-oxo-isovaleric (KIVAL), 2-oxo-glutaric (KGLU), 2-oxo-3-methyl-valeric (KMVAL) and 3-phenyl-pyruvic (PHPYR) are presented. The individual plasma concentrations for PYR and KICAP, KGLU and KMVAL, KIVAL and PHPYR are illustrated in Fig. 1, 2 and 3 respectively.

Table 1 gives mean concentrations, standard deviation and statistical significance for the plasma distribution of α-keto acids for controls, Friedreich's and Charlevoix-Saguenay. The results indicate that the quantitative distributions vary between groups and that the concentrations of some acids are significantly different. All p values appearing in Table 1 were obtained using a two-tailed unpaired t-test.

The mean plasma concentration measured for pyruvic acid in the controls (7.18 mg/1) agrees well with the value of 6.6 recently reported by Rocchiccioli et al (1981), using a similar technique, in a study of α-keto acids distribution in several metabolic diseases. The same applies for the mean value for plasma concentration of the other α-keto acids reported in Table I. As for the plasma con-

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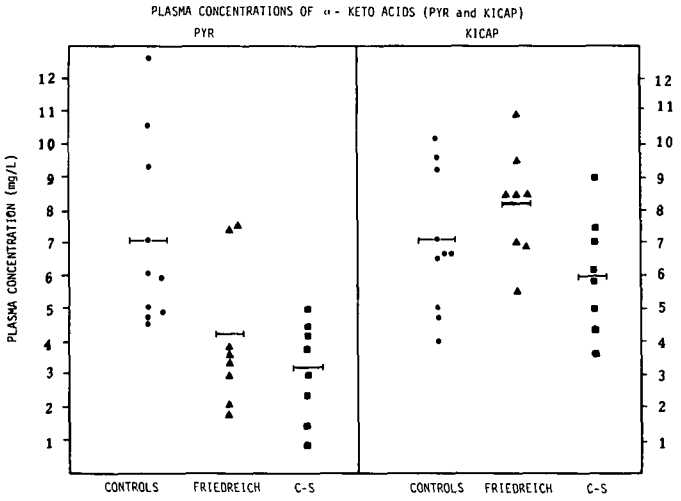

Figure 1 — Individual plasma concentration of Pyruvic and 2-oxo-isocaproic acids for controls (n=10), Friedreich's ataxia (n=8) and ataxia of Charlevoix-Saguenay (n=8).

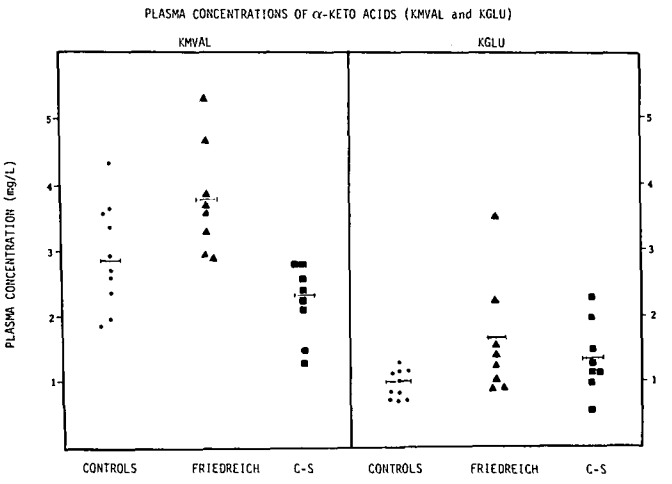

Figure 2 — Individual plasma concentration of 2-oxo-3-methyl-valeric and 2-oxo-glutaric acids for controls (n=10), Friedreich's ataxia (n=8) and ataxia of Charlevoix-Saguenay (n=8).

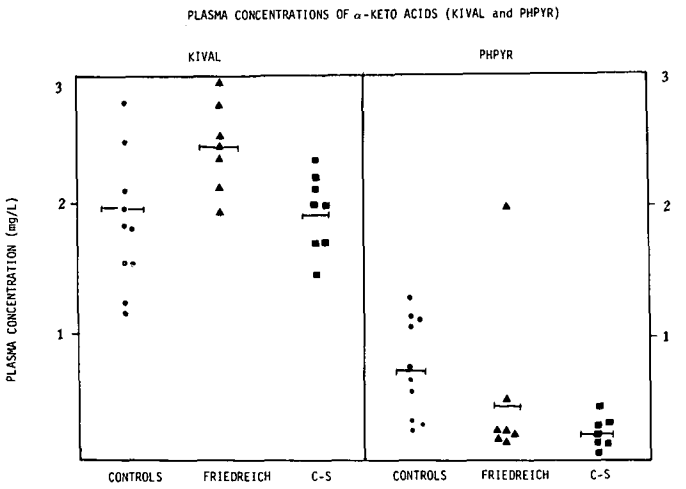

Figure 3 — Individual plasma concentration of 2-oxo-isovaleric and 3-phenyl-pyruvic acids for controls (n=10) Friedreich's ataxia (n=8) and ataxia of Charlevoix-Saguenay (n=8).

TABLE I

MEAN PLASMA CONCENTRATION OF α-KETO ACIDS FOR CONTROLS, FRIEDREICH AND CHARLEVOIX-SAGUENAY (mg/L)

α-Keto Acid	Controls (n = 10)	C-S (n = 8)	Friedreich (n = 8)
Pyruvic (PYR)	mean = 7.18 S.D. = 2.82	mean = 3.19 S.D. = 1.44 p = 0.002	mean = 4.02 S.D. = 2.24 p = 0.02
2-oxo-isovaleric (KIVAL)	mean = 1.92 S.D. = 0.51	mean = 1.90 S.D. = 0.30 N.S.	mean = 2.41 S.D. = 0.39 p = 0.05
2-oxo-3methyl valeric (KMVAL)	mean = 2.86 S.D. = 0.95	mean = 2.25 S.D. = 0.58 N.S.	mean = 3.78 S.D. = 0.86 p = .05
2-oxo-isocaproic (KICAP)	mean = 7.11 S.D. = 2.12	mean = 6.11 S.D. = 1.75 N.S.	mean = 8.34 S.D. = 1.65 N.S.
2-oxo-glutaric (KGLU)	mean = 1.00 S.D. = 0.28	mean = 1.37 S.D. = 0.56 N.S.	mean = 1.70 S.D. = 1.03 p = .05
2-phenyl-pyruvic (PHPYR)	mean = 0.73 S.D. = 0.38	mean = 0.25 S.D. = 0.08 p = 0.007	mean = 0.48 S.D. = 0.67 N.S.

centration of individuals acids, quite a few significant values are observed. The mean plasma concentration of pyruvic acid is significantly lower for Friedreich ($p<.02$) and Charlevoix-Saguenay ($p<0.002$). As for 3-phenyl-pyruvic the plasma concentration for both types of ataxia is lower but a significant value is only observed for Charlevoix-Saguenay ($p=.007$). In the case of 2-oxo-isovaleric, 2-oxo-3methyl-valeric and 2-oxo-isocaproic no significant difference are found between Charlevoix-Saguenay and controls but a contrary situation occurs for Friedreich. In this case, the three acids are seen to have mean values higher than the controls with significant values for 2-oxo-isovaleric and 2-oxo-3-methyl-valeric. Finally the mean plasma concentration of 2-oxo-glutaric acid is higher for both Friedreich and Charlevoix-Saguenay with a significant value for Friedreich ($p<.05$).

As has been stated, 24 hour urine collections were made during the study in order to verify the potential loss of α -keto acids in urine. The excretion values for these acids are given in Table II.

For Charlevoix-Saguenay, significant lower excretion values are observed for pyruvic, 2-oxo-isocaproic and 3-phenyl-pyruvic ($p<.005$). The same table indicates significant lower excretion values for 2-oxo-isocaproic and 3-phenyl-pyruvic (p .002) for Friedreich. These results indicate that the plasma values cannot be explained by enhanced urinary excretion. It is worthwhile to mention significant differences in the excretion of hippuric acid and creatinine over a 24 hour period. It was observed that both Charlevoix-Saguenay and Friedreich

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TABLE II

MEAN URINARY EXCRETION OF α-KETO ACIDS FOR 24 HOURS (mg per 24 hours) FOR CONTROLS, FRIEDREICH AND CHARLEVOIX-SAGUENAY

α -Keto acid	Controls (n = 10)	C-S (n = 8)	Friedreich (n = 8)
Pyruvic (PYR)	mean = 18.53 S.D. = 8.24	mean = 11.26 S.D. = 3.23 p = 0.042	mean = 13.24 S.D. = 6.84 p = N.S.
2-oxo-isovaleric (KIVAL)	mean = 3.48 S.D. = 1.74	mean = 2.30 S.D. = 0.79 N.S.	mean = 3.38 S.D. = 1.94 N.S.
2-oxo-3-methyl-valeric (KMVAL)	mean = 2.22 S.D. = 0.98	mean = 1.82 S.D. = 0.37 N.S.	mean = 2.20 S.D. = 1.05 N.S.
2-oxo-isocaproic (KICAP)	mean = 2.30 S.D. = 1.15	mean = 0.85 S.D. = 0.29 p = 0.003	mean = 1.08 S.D. = 0.51 p = 0.019
2-oxo-glutaric (KGLU)	mean = 85.87 S.D. = 52.26	mean = 54.97 S.D. = 21.22 N.S.	mean = 48.79 S.D. = 29.82 N.S.
3-phenyl-pyruvic (PHPYR)	mean = 0.81 S.D. = 0.33	mean = 0.35 S.D. = 0.14 p = 0.005	mean = 0.29 S.D. = 0.17 p = 0.002

had significant lower excretion values ($p<.05$) for hippuric acid relative to controls, the mean values being respectively 212.4, 163.4 and 298.5 mg. Other results further indicated that creatinine excretion over 24 hours for Friedreich was significantly different from controls ($p<.01$), with mean values of 0.84 and 1.48 mg respectively.

In summary, we have demonstrated that α-keto acid mapping can be a useful tool in distinguishing subtle differences in various forms of ataxia. In Friedreich's ataxia it appears that all α-keto acids measured can be indicative of possible metabolic disturbances, with the proviso that plasma concentrations can be altered by many factors. Nevertheless Friedreich patients appear to have a defect in pyruvate metabolism and a deficiency in GDH. We find significant differences in the concentration of 2-oxo-glutaric acid, a substance not related only to the GDH complex but also recognized as an important factor in transamination. The plasma concentration of 2-oxo-isovaleric, 2-oxo-3-methyl-valeric and 2-oxo-isocaproic acids, related to the metabolism of corresponding amino-acids, is also different from controls in Friedreich's ataxia. At this stage no final explanation of these differences can be assigned, but the results as a whole appear to reflect perturbed metabolism in mitochondria and are in line with the hypothesis of energy deprivation suggested by Barbeau (1980). The results will be discussed, in perspective, further in this issue.

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