Supplemented Creatine Induces Changes in Human Metabolism of Thiocompounds and One- and Two Carbon Units

(Shortened version of the title: Supplemented Creatine Induces Changes in Human Metabolism)

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Summary

One month's administration of creatine (CR) in the dose of 5 g/day to 11 young active sportsmen affected their daily amount of CR, creatinine, and thiodiglycolic acid (TDGA) excreted into urine, and homocysteine, vitamin B_{12} and folates in blood. The probands were divided into 4 groups, according to the amount of CR found in urine, and of folates and vitamin B_{12} determined in blood. The changes of folates and vitamin B_{12} were mutually reciprocal. Each group utilized CR as donor of one- and two-carbon (1C and 2C) units by means of homocysteine (HoCySH), folates, and vitamin B_{12}, in different metabolic pathways. In 10 men the CR administration was accompanied by an increase of HoCySH level in blood, while for the last man, with accidentally discovered hyperhomocysteinemia, the HoCySH level dropped by 50 %. Differences between initial and terminal TDGA levels indicate that CR disturbs equilibria of redox processes. Creatinine excretion into urine changed in dependence on the extent of metabolic disturbances.

Key Words: Creatine (CR), Creatinine, Folates, Homocysteine (HoCySH), Vitamin B_{12}, Thiodiglycolic Acid (TDGA), Voltammetry, Urine, Blood

Introduction

Creatine (CR) is an amino acid (methyl guanidine acetic acid), discovered in 1832. It represents one of the most important nitrogen containing compounds playing role in energetic metabolism. At the end of 19^{th} century, it was found that certain part of exogenously applied CR stays retained in human body (Webber 2006).

CR is not an essential component of food, because it is formed naturally in human body. It represents about 0.17 % of body mass (e.g., 120 g from 70 kg), of which 95 % is in bone muscles and 5 % in brain, kidneys, liver, testicles (Persky and Brazeau 2001), and a small part in blood plasma (Webber 2006). Two thirds of CR are present in the human body as creatine phosphate (PCR), the rest as free CR (Webber 2006). In normal, healthy man, the turnover of CR is about 1-2 g daily, which is covered by its endogenous synthesis from amino acids (arginine, glycine, methionine) in liver and kidneys (Murray et al. 2003), and by food (from animal sources as meat). Under identical conditions the same amount (about 2 g daily) of CR is degraded by non-enzymatic dehydration to creatinine and excreted into urine (Murray et al. 2003).

It is recommended to apply CR as food supplement in amount corresponding to its natural level in meaty food. It is supposed that the human organism uses it for formation of CR phosphate, which is necessary as energetic source for muscular work.

Exogenously applied CR is used in treatment of neurodegenerative diseases - dystrophy, myalgia, rheumatoid arthritis, etc. (Felber et al. 2000, Petr 2007, Tarnopolsky and Martin 1999). More than 5 million kg of CR is sold yearly through the world (Hespel et al. 2001), mostly for purposes of food supplement for sportsmen.

CR has been used for more than 15 years intensively as food supplement as well as remedy; however, up to now the question of hazardousness (safeness) of its application has not been answered. Each of the previously published studies concentrates on a certain specific problem of its application. Their results and conclusions are influenced by the extent and arrangement of experiments. On the basis of published results it is possible to unambiguously conclude that CR application increases the muscle mass and volume of body fluid, formation of ATP and body performance connected with short-time load (Petr 2007). With its increasing application many metabolic questions have to be answered to clear the risks and consequences of CR supplementation.

We have proved in other experiments, which will be presented in the prepared paper (Navratil et al. 2009), that in 4 to 6 hours after CR supplementation the level of thiodiglycolic acid (TDGA) in urine rapidly increases and then decreases again to the original level. At the same time the pH-values of urine increase by 1.5 units (Navratil et al. 2009). The changes in TDGA excretion proved that exogenous application of CR affects metabolic pathways of thiocompounds and of two carbon (2C) units. The effect of CR on TDGA excretion differs from those recorded after vitamin B_{12}, and betaine applications (Navratil et al. 2007).

At present, the voltammetric determination of TDGA in urine (among other methods, e.g. (Chylkova and Fadrna 2004, Samcova et al. 1999)) is being used in toxicology (Dlaskova et al. 2003). It helps to monitor the exposure of workers in chemical plants to certain carcinogenic compounds, e.g., to vinylchloride monomer (VCM) or ethylene dichloride (EDC) in factories producing polyvinyl chloride (Dlaskova et al. 2003, Senholdova-Dlaskova 2002). Similarly as in the case of VCM exposure, the TDGA level in urine increases after intake of some remedies (Navratil et al. 2004), victuals (Navratil et al. 2004), of compounds with organically bound sulfur (Steventon 1999) or of compounds, which affect oxidative metabolic pathways accompanied by release of 2C units (e.g., ethanol, VCM (Navratil et al. 2004), mustard gas (Ermakova et al. 2002, Lee et al. 2000)). In intoxicated humans, these 2C units, which take part in formation of TDGA, originate from xenobiotics. The oxidative

degradation of xenobiotics via TDGA formation decreases the cell pool of glutathione (GSH). In some critical cases all disposable GSH can be exhausted (Murray et al. 2003). This endangers all metabolic pathways dependent on the presence of GSH.

The TDGA concentrations determined in samples of urine in healthy individuals (Dlaskova et al. 2003) do not exceed 20 mg L^{-1}. We suppose that in healthy humans the source of 2C units is serine or glycine. The thiolic part of the TDGA molecule comes from cysteine. The urinary level of TDGA increases only by imbalance among oxidative release of 2C-units, and in supply of thio-compounds and oxygen (Ermakova et al. 2002). TDGA is merely one of the intermediates in this oxidative pathway of 2C unit bound to cysteine. TDGA is excreted into urine, due to imbalance in its formation and in its further degradation. One of the final oxidative products of TDGA is sulfate, which might be later used (after reaction with ATP) as active sulfate (PAPS), e.g., for the synthesis of collagen.

The present study is intended to shed more light on some of the mentioned questions, to search for relationship among levels of various compounds in blood and in urine, and of body parameters connected with CR metabolism.

In our study we want to show how CR, given as food supplement, affects a) its related metabolic pathways and b) the TDGA excretion into urine.

From the analytical point of view, this illustrates the necessity of using wide range of analytical techniques (electrochemistry, chromatography, spectrophotometry, etc.) in order to gain complete, reasonable and reliable results.

Methods

Apparatus

The analysis of TDGA was carried out by the computer-controlled Eco-Tribo Polarograph using the software “Polar 5.1” version for Windows (Polaro-Sensors, spol. s r. o., Czech Republic), on pen type hanging mercury drop electrode (HMDE) (Polaro-Sensors, spol. s r. o., Czech Republic), on mercury meniscus modified silver solid amalgam electrode (e.g., (Barek et al. 2003, Barek et al. 2006, Yosypchuk and Novotny 2002a, Yosypchuk and Novotny 2002b))**,** or on solid composite electrode (e.g., (Barek et al. 2007, Navratil and Kopanica 2002a, Navratil and Kopanica 2002b, Navratil et al. 2003, Sebkova et al. 2004, Sebkova et al. 2005, Yosypchuk et al. 2007)). The results, achieved using all three above mentioned working electrodes, were equivalent. More precisely, the calculated confidence intervals of results overlapped with probability higher than 95 %. The shapes of recorded curves with all three tested electrodes were similar; the peak positions and peak shapes were

similar as well. Nevertheless, the determinations realized with electrode containing liquid mercury, i.e., HMDE exhibited the highest sensitivity and the lowest background. HMDE was the most "user friendly", its surface was the easiest and the fastest renewable, the repeatability of results achieved by it was the best. Smaller amount of mercury on the electrode surface (mercury meniscus, mercury film, polished amalgam surface or amalgam composite surface) causes smaller sensitivity to TDGA concentration. Responses of such electrodes are lower and worse reproducible. The highest background and the lowest sensitivity were recorded with composite electrodes. Therefore, in most experiments presented in this manuscript, the HMDE was used (Navratil et al. 2009).

CR and creatinine were determined using Specord 200. pH was measured by digital laboratory pH-meter Inolab (Benella CZ, Praha).

Compound levels in blood were determined in a commercial laboratory by usual methods.

Procedures

More than 35 parameters were evaluated for each person (Petr 2007). However, in this paper we evaluated only:

In blood: folates, vitamin B_{12}, homocysteine (HoCySH).

In urine: TDGA, CR, creatinine, pH, specific mass. Corrected parameters: a) per specific gravity: TDGA, CR and creatinine; b) per creatinine: TDGA and CR.

The corrections for specific gravity were calculated by means of Eq. (1) (Bardodej et al. 1989, Pristoupilova et al. 2005). In calculations it is assumed that the mean reference value of the specific gravity of human urine is 1.020 $g.mL^{-1}$.

$$X_{cor} = X \frac{0.020}{s.g. - 1} \quad (1)$$

where X_{cor} denotes the corrected value (TDGA/s.g. [$mg.L^{-1}$], CR/s.g. [$g.L^{-1}$], etc. respectively), X denotes the value without corrections (TDGA [$mg.L^{-1}$], CR [$g.L^{-1}$], creatinine [$g.L^{-1}$] respectively), s.g. denotes the urine specific gravity [$g.mL^{-1}$]. If not otherwise specified, the values of TDGA, creatinine and CR in the whole text are given after corrections per specific gravity.

Special attention was paid to voltammetric determinations of TDGA, which are not common in medical laboratories.

Urine samples were analyzed by the voltammetric technique described in our previous paper (Dlaskova et al. 2003): The preparation of the sample was realized in a column of powdered PVC, the urine sample was transferred to the top of the column and eluted by 0.2M perchloric

acid. The resulting eluate was introduced into the electrolytic cell, deaerated by a stream of nitrogen (purity 99.999 %), and then subjected to direct current (D.C.) voltammetric analysis. The measurement was started by accumulation for 10 s under stirring at the initial potential of –800 mV vs. Ag/AgCl/1 mol L^{-1} KCl, followed by rest period of 15 s, and then by potential scan at the rate of -10 $mV.s^{-1}$ to the final potential of -1200 mV. The values of potentials given in this paper are referred to that of Ag/AgCl reference electrode, which is at 25 ^{o}C by 9 mV more negative than the SCE. All experiments were carried out at room temperature (25 ± 2 ^{o}C). For quantitative evaluation the method of double standard addition was used.
Creatinine was determined according to standard laboratory determination (Bardodej et al. 1989). Afterwards the CR present in sample was transformed to creatinine by acidification and boiling (1-2 hours) in a water bath. Total creatinine in the sample was determined again and the difference between the second and the first result corresponds to the CR concentration (Bardodej et al. 1989, Kraml 1999).
All parameters in blood were determined in a commercial laboratory using commonly applied methods (the laboratory has certificate ISO 9001:2000).
Vitamin B_{12} was determined using the competitive binding immune-chemiluminescence "Access® vitamin B12 set" supplied by the company Beckman Coulter, Inc. (Beckman_Coulter 2008). This determination in human serum and plasma (heparin) was based on the use of paramagnetic particles. All vitamin B_{12} is transformed into cyanocobalamine form before the determination. The applied procedure cannot distinguish among different forms of vitamin B_{12} (total, bound on the binding proteins (transcobalamin and haptocorrin (Wickramasinghe and Fida 1993))), because all binding proteins are denatured before start of the determination. Hence, estimation of "active Vitamin B-l2"-Holotranscobalamin has not been used). The paramagnetic particles are impregnated by goat binding protein against mice immuno globulins with mice monoclonal antibody against intrinsic factor. Vitamin B_{12} conjugates with intrinsic factor and competes in binding on the solid phase (Beckman_Coulter 2008).
Folates were determined using similar principle - the competitive binding immune-chemiluminescence "Access® Folate set" supplied by the company Beckman Coulter, Inc. (Beckman_Coulter 2008). Folates from the sample after reaction with alkali phosphatase compete with mice immunoglobulins for binding sites on goat binding protein for folates. (Beckman_Coulter 2008).
Homocysteine was determined according to the method described in (Dou et al. 2005). "Diazyme" method of determination is based on co-substrate conversion of the determined

substance in a circle. Oxidized HoCySH is transformed to the free, which reacts firstly with co-substrate *S*-adenosylmethionine (S-AM) using enzyme homocysteine-S-methyltransferase to methionine and S-adenosylhomocysteine (S-AH). S-AH is hydrolyzed to adenosine and homocysteine using S-AH hydrolase. Newly formed homocysteine from co-substrate S-AM represents a part of cyclic enzymatic system. Adenosine is decomposed to inosine and ammonium, which flowingly reacts with glutamate dehydrogenase under transformation of NADH to NAD^+. HoCySH concentration in the sample is proportional to the NADH transformed to NAD^+.

Reagents and Materials

"CR-monohydrate – a special nutritional supplement for athletes" (Plutino, Czech Republic) was administered as described in the present study.

Chemicals used were of analytical reagent purity grade. Doubly distilled water was applied throughout the work (conductivity < 1 $\mu S.cm^{-1}$). All measurements were carried out at room temperature.

Proband Group Characterization, Sampling and CR Supplementation

Group of volunteers consisted of 11 young men, students of Faculty of Physical Education and Sport, of the age from 21 to 28 (on average 24.6 year). Women were not subjected to the present study due to complicated hormonal changes during month, which affect cell water content substantially. All probands were young, healthy, physically active persons, dealing with sportive activities (ice hockey, football, horsemanship, and athletics) on professional level. The sportive activities were very carefully observed, however, they were not consistent. All probands signed the informed consent. None of the volunteers applied any other food supplements, stimulants, drugs, vitamin preparations, etc., during this study.

Because all the sportsmen, who were subjects of the reported study, took part in some similar (short time) experiments (results of which were reported in earlier published studies) (Navratil et al. 2009), we could evaluate the changes in their body weights in course of a few last months. We can therefore conclude that regardless of sport activities, the body weight of the sportsmen was practically constant before the start of the testing.

CR was administered per os (p. o.) in 5 g doses, diluted in tepid water. These doses were administered every morning (at about 8 a.m.) during 30 days. Blood and urine of each proband were sampled, and body impedance was measured one day in the morning before the first CR application and the day after the last dose application. All collected urine samples were immediately frozen to -18 ^oC and were then defrosted just before analysis (Navratil et al.

2004, Senholdova-Dlaskova 2002). Δ-value, used for individual characterization of probands, was calculated for each parameter as difference between the values determined in the samples of urine and blood on the last and on the first day.

During the days of CR supplementation and one day before, none of the volunteers consumed victuals containing onion, garlic etc., alcohol (Dlaskova et al. 2003, Chylkova and Fadrna 2004, Navratil et al. 2004), remedies containing carboxymethyl cysteine (ACC 100 etc.) or higher content of organically bound sulfur, and B_{12} vitamin, folic acid (Navratil et al. 2007, Pristoupilova et al. 2005), etc. They drank about 2 liters of fluids per day. We can almost characterize their nutrition as standardized.

For evaluation of the results plenty of statistical characterizations were used: average, standard deviation, skeewness, excess, test of distribution normality, two-sample F-Test for Variances; two-sample t-test for equal variances; two-sample t-test for equal means, paired two sample t-test for means, correlation. The outlying points (diagnosed by Dean-Dixon and Grubbs tests) could not be eliminated, because each result has its importance and must be taken into account. The calculations were realized using MS Excel 2003 (Microsoft Corporation, USA), software Adstat 2.0 and QC Expert (both TriloByte, Czech Republic), Statistica (StatSoft CR, s.r.o., Czech Republic).

Results

After a month of CR application it was possible to divide the 11 volunteers into 4 groups, A, B, C, D, according to changes of the levels of vitamin B_{12} and of folates in blood, and of CR in urine, irrespective of the absolute values (Tab. 1). The men in groups were numbered from 1 to 11. In the group A (probands No. 1, 2, and 3), in the group B (proband No. 4) and in the group C (probands No. 5, 6, and 7) the content of unutilized CR in urine increased. On the contrary, in the group D (probands No. 8, 9, 10 and 11) the CR was decreasing or even disappeared from urine, into which it had been excreted before the experiment begun. In blood of the group A the level of folates increased, while the level of vitamin B_{12} decreased, in contrast to groups B, C and D, where the levels changed in the opposite direction.

The values presented in Figures 1. - 5. enable to show how one month of regular consumption of CR affected the enzymatic systems concerning vitamin B_{12}, folates, HoCySH and TDGA, and how it affected the excretion of creatinine into urine.

Group A

In volunteers of group A after one month of regular CR administration only a small amount of unutilized CR was excreted into urine. The high uptake of CR was accompanied by higher

consumption of vitamin B_{12}, therefore its level in blood decreased on average by 14 % (Fig. 1). Levels of folates and of HoCySH in blood increased within +31 - +64 % and +79 - +127 % respectively (decreased consumption of folates and HoCySH) (Fig. 2, 3).
The amount of TDGA increased proportionally to the amount of unused CR excreted into urine (Fig. 4), similarly as did creatinine (from 8.4 to 41.6 $mg.L^{-1}$, and 0.01 to 0.78 $g.L^{-1}$) (Fig. 5).

Group B

This group, represented by a single man (No. 4) with high initial levels of both vitamins (B_{12} 670 $ng.L^{-1}$ and folates 16.3 $\mu g.L^{-1}$), is characterized by increased levels of vitamin B_{12} (+13 %) and of folates (+4.3 %) after CR administration (Tab.1, Fig. 1 and Fig. 2). The HoCySH level increased only moderately (Fig. 3). There was more unused CR washed out into urine than in group A (by +1.79 $g.L^{-1}$) (Fig. 5). Although the levels of TDGA (Fig. 4) and of creatinine increased, they did not exceed normal values.

Group C

The men No. 5, 6 and 7, forming group C, reacted to CR administration by opposite changes of vitamin B_{12} and folate levels than did the men of group A (Tab. 1, Fig. 1 and Fig. 2). The average level of vitamin B_{12} increased (+13 %), that of folates decreased (-34.1 %). The increase of HoCySH level was about one half on average (+45 %) of that found in group A (+96 %) (Fig. 3).
The proband No. 5 exhibited the lowest content of unused CR (0.5 $g.L^{-1}$) in urine and the highest level of vitamin B_{12} in blood (Fig. 1).
The proband No. 6, whose vitamin B_{12} level did not change and the folate level decreased only slightly (Fig. 1 and Fig. 2), had the highest CR increase in urine from all the 11 men (from 0 to 1.9 $g.L^{-1}$). The level of HoCySH increased considerably (from 7.9 to 11.5 $\mu mol.L^{-1}$, i.e., by 45.6 %) in blood, and that of creatinine and TDGA in urine increased from 1.05 to 1.45 $g.L^{-1}$, and from 0.00 to 25.3 $mg.L^{-1}$ respectively (Fig. 4, Fig. 5).
A different reaction to administered CR was observed in the proband No. 7. His high content of CR in urine before the first administration of CR (4.38 $g.L^{-1}$) increased to 5.27$g.L^{-1}$. This was accompanied by the highest decrease of folates (-51 %) among all the 11 volunteers (Fig. 2). The already low level of creatinine decreased from 0.87 to 0.59 $g.L^{-1}$ (Fig. 5) and the level of TDGA/sp. g. increased from 0 to 25.3 $mg.L^{-1}$ (Fig. 4).

Group D

The CR administration paradoxically decreased or completely prevented excretion of CR into urine and did not affect markedly the metabolism of folates (Fig. 1). The men of group D reacted most notably by change of HoCySH levels (from –48 % to +238 %) (Fig. 3) on the decreased consumption of vitamin B_{12} (more of it went over to blood) (from 3.8 % to 25 %). The D men also differed from each other most markedly (Fig. 1-Fig. 5).

The CR application had a relatively small effect on man No. 8. Little change was shown by CR excretion into urine (CR/sp. g. from 1.53 to 1.05 $g.L^{-1}$), by the level of folates (from 5.1 to 4.8 $\mu g.L^{-1}$), of vitamin B_{12} (from 846 to 865 $ng.L^{-1}$) and of HoCySH (from 5 to 7.6 $\mu mol.L^{-1}$) in blood. The low level of creatinine increased, from 0.69 to 0.85 $g.L^{-1}$, and even the TDGA/sp. g. went up from 0.0 to 26.0 $mg.L^{-1}$.

For man No. 9, with accidentally discovered hyperhomocysteinemia, the CR administration caused a small decrease of CR (from 0.59 to 0.3 $g.L^{-1}$), accompanied by the highest increase of vitamin B_{12} level (by 25 %), the highest decrease of HoCySH level (from 33.3 to 17.1 $\mu mol.L^{-1}$) (Fig. 1, Fig. 3) and those of creatinine and TDGA in urine from 1.69 to 1.23 $g.L^{-1}$ and from 22.5 to 0.00 $mg.L^{-1}$ respectively (Fig. 4, Fig. 5).

For man No. 10 the strong decrease of CR in urine (CR/sp. g. from 1.62 $g.L^{-1}$ to 0.00 $g.L^{-1}$) was accompanied by the highest increase of HoCySH level in blood of all the 11 tested probands (from 5.0 to 16.9 $\mu mol.L^{-1}$, i.e., by +238 % (Fig. 3), which is beyond the limit of mild hyperhomocysteinemia).

In men No. 9 and 10 the conditions for TDGA formation did not occur (Fig. 4). This took place in man No. 11 with the greatest loss of CR in urine (CR/sp. g. from 5.2 to 0.0 $g.L^{-1}$). No. 11 had the highest increase of TDGA level in urine (TDGA/sp. g. from 0 to 51.4 $g.L^{-1}$), the greatest decrease of creatinine level among all the 11 probands (Fig. 5), and only a small increase of HoCySH and vitamin B_{12} levels with a small decrease of folates (Fig. 1, Fig. 2, Fig. 3).

Discussion

CR administered regularly as a food supplement in course of one month, had disturbed the equilibrium among the compounds participating in its endogenous formation. Those are glycine and arginine in kidneys, and the precursors of S-adenosylmethionine (S-AM) in liver (Murray et al. 2003). The detailed scheme of suggested metabolic pathways, describing the transformation of arginine, glycine, and S-Adenosyl methionine (S-AM) to CR was published by us in (Navratil et al. 2009), Fig. 6. The scheme describes the oxidative pathway of CR into

urea, involving vitamins B_2, B_6, tetrahydrofolate (THF) and glutathione, with urea and TDGA (besides others) as final products. The other (non oxidative) metabolic pathways aim one to the non-enzymatic creatinine formation (excreted from the human body) and the other one to creatine phosphate. Up to 70 % of the generated S-AM is used for methylation of guanidinoacetate, which process terminates the formation of CR in liver. ATP is thereby used as energy source, and HoCySH and adenosine are set free. In the following, both compounds participate again in generation of cell energy. HoCySH as source of cysteine mediates the transport of protons and electrons from nutrients into the respiratory chain (Murray et al. 2003).

Each man, followed within the groups A, B, C and D, utilized in his own way the compounds, by means of which he would normally generate CR. That is made possible by the high number of enzymatic reactions, which can affect the endogenous synthesis of CR. The interaction between folates, vitamin B_{12} and HoCySH is fundamental for the regulative system that monitors utilization of serine, the source of 1C and 2C units, necessary for processes catalyzed by them. Glycine and serine are interchangeable by means of folates. An inexhaustible source of serine is D-3-phosphoglycerate. Whether it will produce serine or not, depends on suitable redox conditions in the metabolism of sugars.

We presume that due to administered CR the molecules of glycine, arginine and S-AM, utilized for endogenous CR synthesis, could enter into other enzymatic reactions, and alter the physiological coordinated recyclation of compounds in phospholipid metabolism and in other connected cycles. The pathways differ in lower or higher participation of vitamin B_{12}, HoCySH and folates.

The results have shown that administered CR does not merely replace the endogenous synthesis of CR and that its surplus is not excreted by urine as creatinine. When the amount of creatinine excreted at the beginning of the experiment differed markedly from that excreted at the end, then the administered CR evidently caused considerable discrepancies in the metabolic pathways connected with synthesis or degradation of CR.

It followed from the results that everybody is sensitive in a different way to changes in nutrition, represented in our case by daily administration of CR, and that the equilibrium in metabolic processes, which are the basis of respiratory activity and of energy formation, is adjusted for everyone in a different way. The individual character of metabolic pathways of each person has to be respected in the search for cure of the anomalies.

Importance of HoCySH for formation and utilization of CR

The regular consumption of CR increased the HoCySH level of all men with exception of one man from Group D with accidentally found hyperhomocysteinemia, in whom it got decreased. An increase in HoCySH level can be explained by inability of the men to utilize it completely in other processes than in the CR synthesis. There exist 3 enzymatic reactions for utilization of HoCySH. One of them, catalyzed by methionine synthetase (E.C. 2.1.1.13), occurs under very complicated conditions, and requires participation of folates and vitamin B_{12}. It was suppressed by administered CR in group A, as could be judged from high increase of HoCySH and of N^5-methyltetrahydrofolate (N^5-methyltetrahydrofolate is the only folate form present in human blood (Pristoupilova and Pristoupil 1997)). The other one, catalyzed by betaine-homocysteine S-methyltransferase (E.C. 2.1.1.5), takes place without vitamin B_{12} and without direct participation of folates, the level of both increased in blood in the group B. Methionine, formed in these two enzymatic reactions, becomes the donor of methyl group after reaction with ATP. The S-AM, produced in this way, is used, among others, for CR formation. Both methionine synthetases are playing part in formation and degradation of phospholipids and/or of choline respectively (Murray et al. 2003). A third enzymatic reaction, into which HoCySH enters together with serine, is catalyzed by cystathionine beta-synthetase (E.C. 4.2.1.22). It changes HoCySH into cystathionine, which is decomposed to cysteine and homoserine. Further processing of homoserine needs the presence of vitamin B_{12}, for transformation of methylmalonyl-CoA from homoserine into succinyl-CoA in mitochondria. This last step, catalyzed by vitamin B_{12}, is common with degradation of fatty acids with odd number of carbon atoms. This is also the only way how fatty acids can be utilized for glycogenesis (Murray et al. 2003). The original inability of the man with hyperhomocysteinemia from D group to utilize HoCySH, was probably due to a defect of the system providing serine for reaction with HoCySH without participation of vitamin B_{12}. The administered CR supported transformation of more HoCySH into cysteine and homoserine through cystathionine synthase, and into methionine through betaine-homocysteine methyltransferase.

The only mild increase of HoCySH level in groups B and C was probably due to lack of folates in proper coenzymatic forms. In group D it varied depending on vitamin B_{12} consumption. The absolute value of the initial levels of folates, vitamin B_{12} and HoCySH did not affect utilization of CR in any of the mentioned groups.

Relation between the way of CR processing, thiocompound metabolism and changes in oxidation reactions

Oxidation of sulfur atom, which is contained in TDGA molecule and whose source can also be HoCySH (Navratil et al. 2007, Navratil et al. 2004) , can proceed up to inorganic sulfate. However, the increase of TDGA level in urine was not always parallel with the increase of HoCySH level in blood.

In Fig. 1, 2 and 4 it can be seen that in each group of men HoCySH, folates and vitamin B_{12} participated to a different extent on the increase of TDGA level in urine, caused by the administered CR. That represented regular replacement of glycine, arginine and ATP, consumed in endogenous formation of CR. At the same time it decreased reutilization of HoCySH, methyltetrahydrofolate, tetrahydrofolate and vitamin B_{12}, which are also indispensable for CR synthesis.

For men of group A, the more unutilized CR got excreted into urine, the more unutilized HoCySH and folates (Fig. 2) went over to blood, and the more TDGA appeared in urine (Fig. 4). In group A in TDGA formation participated to a higher extent vitamin B_{12}, as its level in blood decreased (Fig. 1).

In groups B and C the CR administration produced only a small increase of TDGA level (Fig. 4) and a small increase of HoCySH level (Fig. 3). The higher were the equalizing changes between the levels of folates and vitamin B_{12} (Fig. 1, Fig. 2).

In the group D, the more CR was processed under participation of vitamin B_{12} and HoCySH, i.e., the less of them was in blood and of CR in urine, the more TDGA was there. On the contrary, the less of HoCySH and of vitamin B_{12} participated in processing of CR, the higher were their levels in blood and the less the CR levels in urine, the less TDGA appeared there (Fig. 1, Fig. 2).

Conclusions

In human body, CR is endogenously synthesized and, in addition, it is taken in food as part of meat victuals. In that case, its endogenous production adjusts itself to the exogenous intake with the help of vitamin B_{12} and folates. This can be deduced from reciprocal changes of their levels in blood and changes of the utilization of HoCySH in various metabolic pathways. The results, gained by TDGA determination in urine, agree with our assumption that the main source of 2C units, from which TDGA is formed in healthy individuals, is degradation of glycine or serine respectively.

According to changes of the levels of vitamin B_{12} and folates in blood and of CR in urine, the tested men were divided into 4 groups (A, B, C and D). This separation was connected with increase of the HoCySH level in blood and, independently, also with increase of TDGA level in urine. In addition to that there were differences established within the 4 groups in the other followed parameters (Petr 2007).

Our study has demonstrated that healthy individuals – sportsmen – include CR in their metabolic pathways individually according to their state of metabolism. In some cases such differences can markedly affect their health and physical fitness.

Group D reacted in most sensitive way by changing levels of vitamin B_{12} and HoCySH to administered CR. In that group, containing accidentally a man with hyperhomocysteinemia, the thorough utilization of the administered CR resulted in decrease of the HoCySH level in blood by one half and in disappearance of TDGA from urine.

In agreement with our results published earlier (Navratil et al. 2007, Pristoupilova et al. 2005), the values of creatinine in spot urine changed according to metabolic conditions. Hence the generally recommended recalculation of the levels of compounds excreted into urine per creatinine concentration does not seem to be suitable for all cases, and it can be replaced by correction per specific gravity of urine.

Very simple procedures of voltammetric determinations of TDGA and of common CR and of creatinine, all in urine, i.e. without necessity of blood taking, can yield very interesting and important information on changes in human metabolism.

Acknowledgments

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References

BARDODEJ Z, DAVID A, SEDIVEC V, SKRAMOVSKY S, TEISINGER J: *Expoziční testy v průmyslové toxikologii*. Avicenum Prague, 1989.

BAREK J, DODOVA E, NAVRATIL T, YOSYPCHUK B, NOVOTNY L, ZIMA J: Voltammetric determination of N,N-dimethyl-4-amine-carboxyazobenzene at a silver solid amalgam electrode. *Electroanalysis* **15**: 1778-1781, 2003.

BAREK J, FISCHER J, NAVRATIL T, PECKOVA K, YOSYPCHUK B: Silver solid amalgam electrodes as sensors for chemical carcinogens. *Sensors* **6**: 445-452, 2006.

BAREK J, FISCHER J, NAVRATIL T, PECKOVA K, YOSYPCHUK B, ZIMA J: Nontraditional electrode materials in environmental analysis of biologically active organic compounds. *Electroanalysis* **19**: 2003-2014, 2007.

BECKMAN_COULTER: *Access® Vitamin B12*. http://www.beckman.com/.

DLASKOVA Z, NAVRATIL T, HEYROVSKY M, PELCLOVA D, NOVOTNY L: Voltammetric determination of thiodiglycolic acid in urine. *Anal Bioanal Chem* **375**: 164-168, 2003.

DOU C, XIA DY, ZHANG LQ, CHEN XR, FLORES P, DATTA A, YUAN C: Development of a novel enzymatic cycling assay for total homocysteine. *Clinical Chemistry* **51**: 1987-1989, 2005.

ERMAKOVA IT, STAROVOITOV II, TIKHONOVA EB, SLEPEN'KIN AV, KASHPAROV KI, BORONIN AM: Bioutilization of thiodiglycol, the product of mustard detoxification: isolation of degrading strains, study of biodegradation process and metabolic pathways. *Process Biochemistry* **38**: 31-39, 2002.

FELBER S, SKLADAL D, WYSS M, KREMSER C, KOLLER A, SPERL W: Oral creatine supplementation in Duchenne muscular dystrophy: A clinical and 31P magnetic resonance spectroscopy study. *Neurological Research* **22**: 145-150, 2000.

HESPEL P, OP'T EIJNDE B, DERAVE W, RICHTER EA: Creatine supplementation: Exploring the role of the creatine kinase/phosphocreatine system in human muscle. *Canadian Journal of Applied Physiology-Revue Canadienne De Physiologie Appliquee* **26**: S79-S102, 2001.

CHYLKOVA J, FADRNA R: Procedure for Isotachophoretic Determination of Thiodiglycolic Acid in Urine Using Desalination of the Analyzed Sample. *Chem Listy* **98**: 260-263, 2004.

KRAML J: *Návody k praktickým cvičením z lékařské biochemie*. Karolinum Prague, 1999.

LEE TS, CHAN SH, WEIGAND WA, BENTLEY WE: Biocatalytic transformation of [(2-hydroxyethyl)thio]acetic acid and thiodiglycolic acid from thiodiglycol by Alcaligenes xylosoxydans ssp xylosoxydans (SH91). *Biotechnol Prog* **16**: 363-367, 2000.

MURRAY RK, GRANNER KD, MAYES PA, RODWELL VW: *Harper's Biochemistry*. McGraw-Hill Companies USA, 2003.

NAVRATIL T, KOHLIKOVA E, PETR M, HEYROVSKY M, PELCLOVA D, PRISTOUPILOVA K, PRISTOUPIL TI, SENHOLDOVA Z: Contribution to

Explanation of the Effect of Supplemented Creatine in Human Metabolism. *Food Chem* **112**: 500-506, 2009.

NAVRATIL T, KOPANICA M: Analytical application of silver composite electrode. *Crit Rev Anal Chem* **32**: 153-166, 2002a.

NAVRATIL T, KOPANICA M: Lead determination on silver composite electrodes using the effect of underpotential deposition. *Chem Listy* **96**: 111-116, 2002b.

NAVRATIL T, KOPANICA M, KRISTA J: Anodic stripping voltammetry for arsenic determination on composite gold electrode. *Chemia Analityczna* **48**: 265-272, 2003.

NAVRATIL T, PETR M, SENHOLDOVA Z, PRISTOUPILOVA K, PRISTOUPIL TI, HEYROVSKY M, PELCLOVA D, KOHLÍKOVA E: Diagnostic Significance of Urinary Thiodiglycolic Acid as a Possible Tool for Studying the Role of Vitamins B12 and Folates in the Metabolism of Thiolic Substances. *Physiological Research* **56**: 113-122, 2007.

NAVRATIL T, SENHOLDOVA-DLASKOVA Z, HEYROVSKY M, PRISTOUPILOVA K, PRISTOUPIL TI, PELCLOVA D: Excretion of thiodiglycolic acid in urine affected by some victuals and cetirizine. *Anal Lett* **37**: 1093-1102, 2004.

PERSKY AM, BRAZEAU GA: *Clinical pharmacology of the dietary supplement creatine monohydrate*. Department of Pharmaceutics. College of Pharmacy. University of Florida., Florida, 2001.

PETR M: *Metabolic pathways leading to excretion of Thiodiglycolic acid into urine affected by creatine supplementation.* PhD Thesis, Charles University, Prague, 2007.

PRISTOUPILOVA K, PRISTOUPIL TI: *Role of Folates in Metabolic Pathways*. ACADEMIA Prague, 1997.

PRISTOUPILOVA K, PRISTOUPIL TI, NAVRATIL T, HEYROVSKY M, SENHOLDOVA Z, PELCLOVA D: Daily rhythm of urinary excretion of thiodiglycolic acid (TDGA) in humans under different health conditions and treatment. *Anal Lett* **38**: 613-627, 2005.

SAMCOVA E, KVASNICOVA V, URBAN J, JELINEK I, COUFAL P: Determination of thiodiglycolic acid in urine by capillary electrophoresis. *Journal of Chromatography A* **847**: 135-139, 1999.

SEBKOVA S, NAVRATIL T, KOPANICA M: Silver composite electrode for voltammetric determination of halogenides. *Anal Lett* **37**: 603-628, 2004.

SEBKOVA S, NAVRATIL T, KOPANICA M: Graphite composite electrode in voltammetry. *Anal Lett* **38**: 1747-1758, 2005.

SENHOLDOVA-DLASKOVA Z: *Polarographic/Voltammetric Determination of Thiodiglycolic Acid as the Indicator of Exposure to Vinylchloride.* Ph.D. Thesis, University Pardubice, Pardubice, 2002.

STEVENTON GB: Diurnal variation in the metabolism of S-carboxymethyl-L-cysteine in humans. *Drug Metab Dispos* **27**: 1092-1097, 1999.

TARNOPOLSKY M, MARTIN J: Creatine monohydrate increases strength in patients with neuromuscular disease. *Neurology* **52**: 854-857, 1999.

WEBBER M: *Creatine Supplementation.* http://www.faccioni.com/Reviews/creatine.htm, 2.4.2006.

WICKRAMASINGHE SN, FIDA S: Correlations between Holo-Transcobalamin-Ii, Holo-Haptocorrin, and Total B-12 in Serum Samples from Healthy-Subjects and Patients. *Journal of Clinical Pathology* **46**: 537-539, 1993.

YOSYPCHUK B, NAVRATIL T, LUKINA AN, PECKOVA K, BAREK J: Solid Amalgam Composite Electrode as a New Sensor for the Determination of Biologically Active Compounds. *Chemia Analityczna (Warsaw)* **52**: 897-910, 2007.

YOSYPCHUK B, NOVOTNY L: Electrodes of nontoxic solid amalgams for electrochemical measurements. *Electroanalysis* **14**: 1733-1738, 2002a.

YOSYPCHUK B, NOVOTNY L: Nontoxic electrodes of solid amalgams. *Critical Reviews in Analytical Chemistry* **32**: 141-151, 2002b.

Table 1. Changes in levels of vitamin B_{12}, folates in blood and CR in urine after 30 days of CR application

Group	Proband No.	Changes in levels of		
		Vitamin B_{12}	Folates	CR
A	1, 2, 3	-	+	+
B	4	+	+	+
C	5, 6, 7	+	-	+
D	8, 9, 10, 11	+	-	-

Legends for Figures:

Figure 1.

ΔB_{12} in dependence on Δcreatine/sp. g. levels (Δ = Value $_{\text{Last day}}$ – Value $_{\text{Fist day}}$) as a result of 30days' creatine supplementation.

Figure 2.

Δfolates in dependence on Δcreatine/sp. g. levels (Δ = Value $_{\text{Last day}}$ – Value $_{\text{Fist day}}$) as a result of 30days' creatine supplementation.

Figure 3.

Δhomocysteine in dependence on Δcreatine/sp. g. levels (Δ = Value $_{\text{Last day}}$ – Value $_{\text{Fist day}}$) as a result of 30days' creatine supplementation.

Figure 4.

ΔTDGA/sp. g. in dependence on Δcreatine/sp. g. levels (Δ = Value $_{\text{Last day}}$ – Value $_{\text{Fist day}}$) as a result of 30days' creatine supplementation.

Figure 5.

Δcreatinine/sp. g. in dependence on Δcreatine/sp. g. levels (Δ = Value $_{\text{Last day}}$ – Value $_{\text{Fist day}}$) as a result of 30days' creatine supplementation.

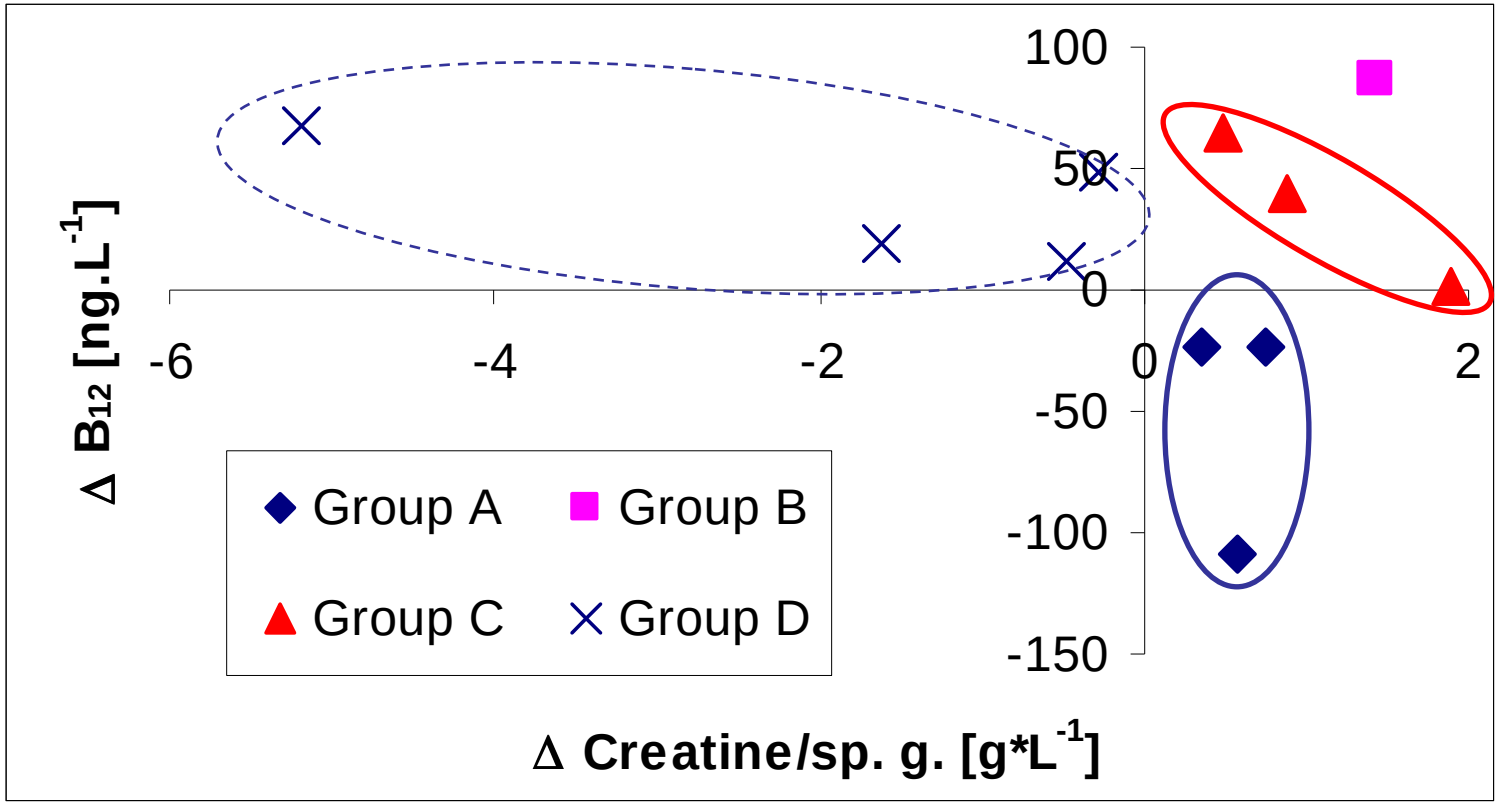
100
50
0
-50
-100
-150
-6
-4
-2
0
2
Δ B12 [ng.L-1]
Δ Creatine/sp. g. [g*L-1]
Group A
Group B
Group C
Group D

Figure 1.

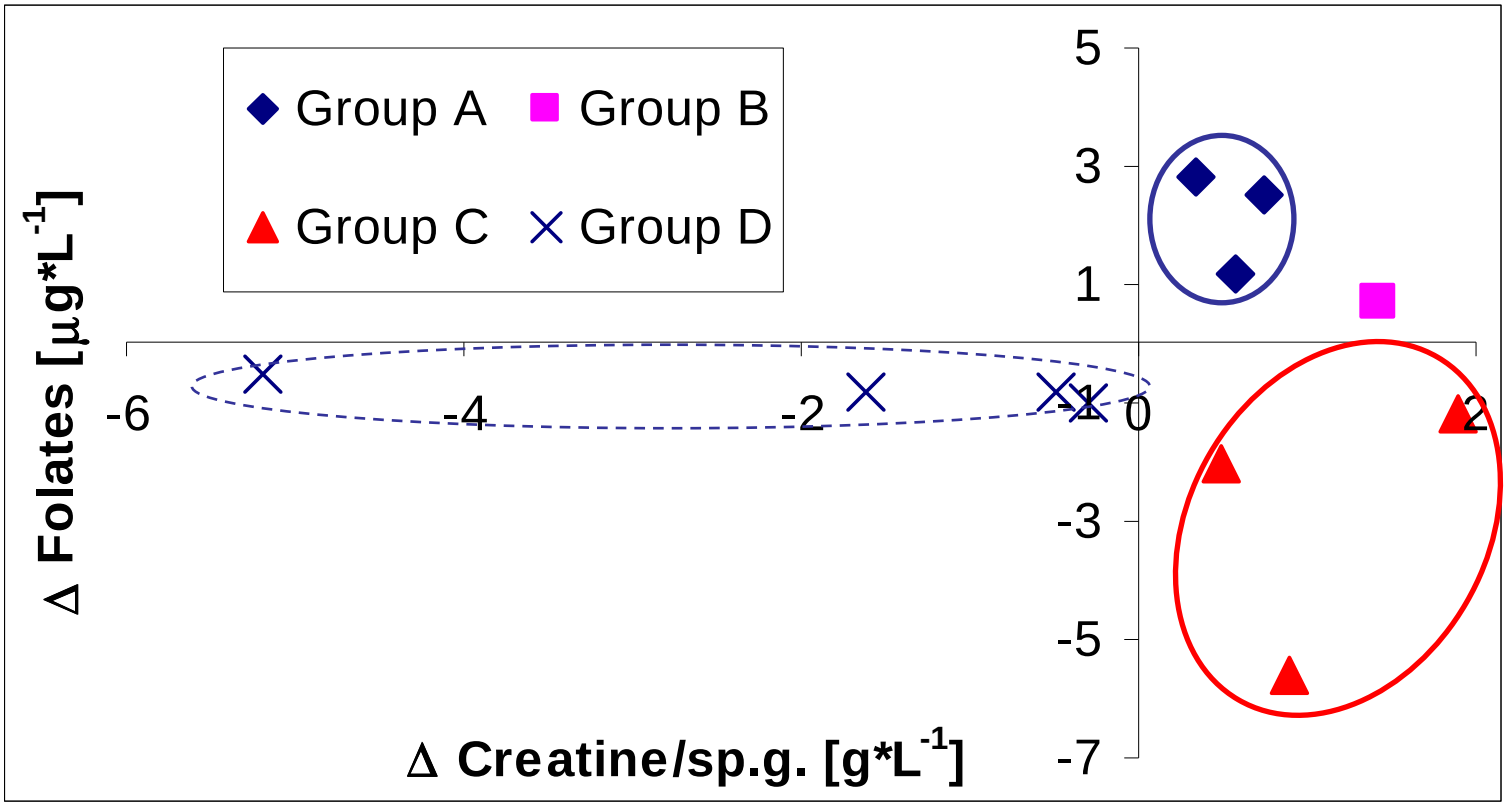
Group A
Group B
Group C
Group D
Δ Folates [µg*L-1]
Δ Creatine/sp.g. [g*L-1]
5
3
1
-1
-3
-5
-7
-6
-4
-2
0
2

Figure 2.

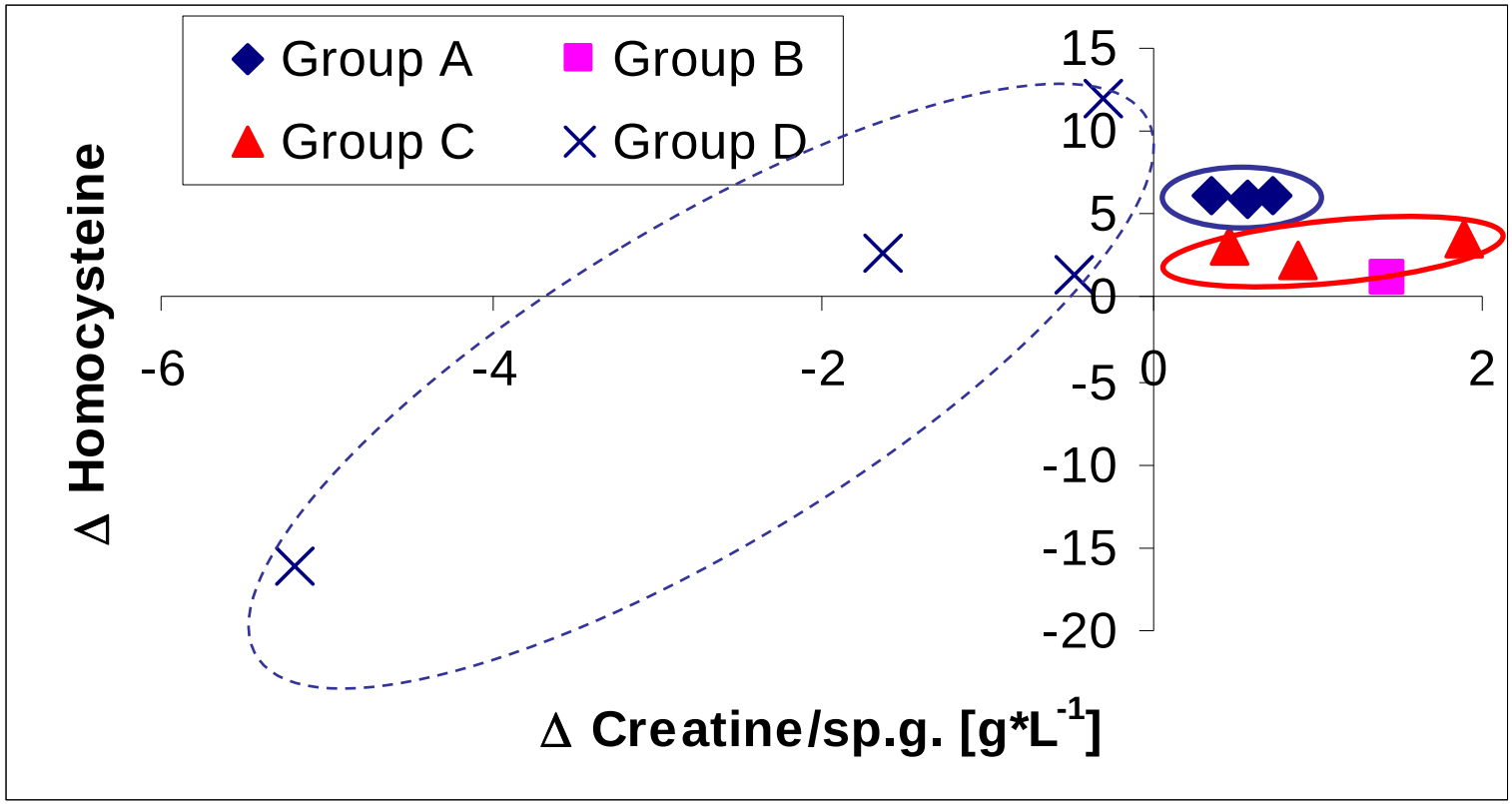
Group A
Group B
Group C
Group D
Δ Homocysteine
Δ Creatine/sp.g. [g*L-1]
15
10
5
0
-5
-10
-15
-20
-6
-4
-2
0
2

Figure 3.

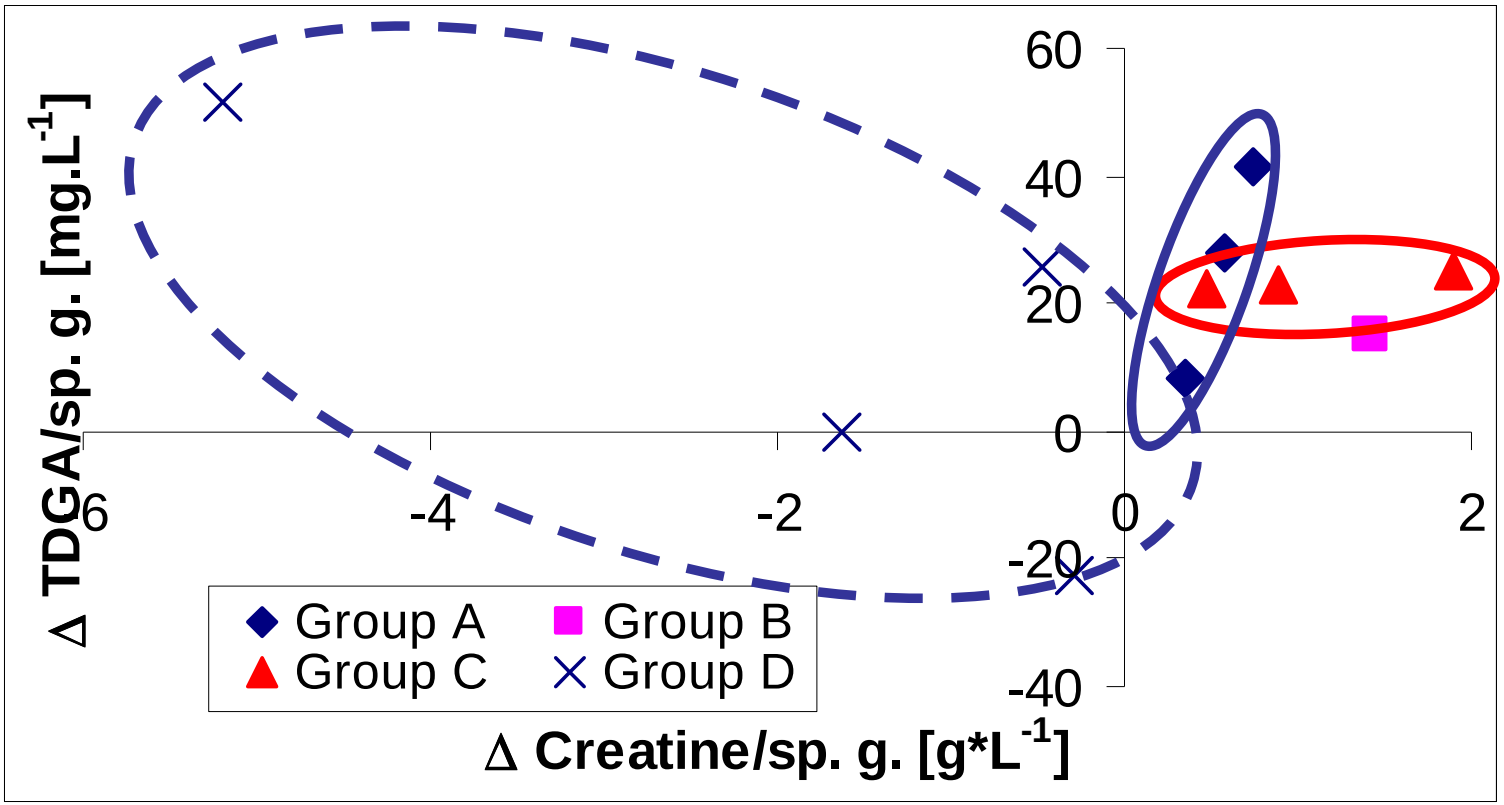
60
40
20
0
-20
-40
-6
-4
-2
0
2
Δ TDGA/sp. g. [mg.L-1]
Δ Creatine/sp. g. [g*L-1]
Group A
Group B
Group C
Group D

Figure 4.

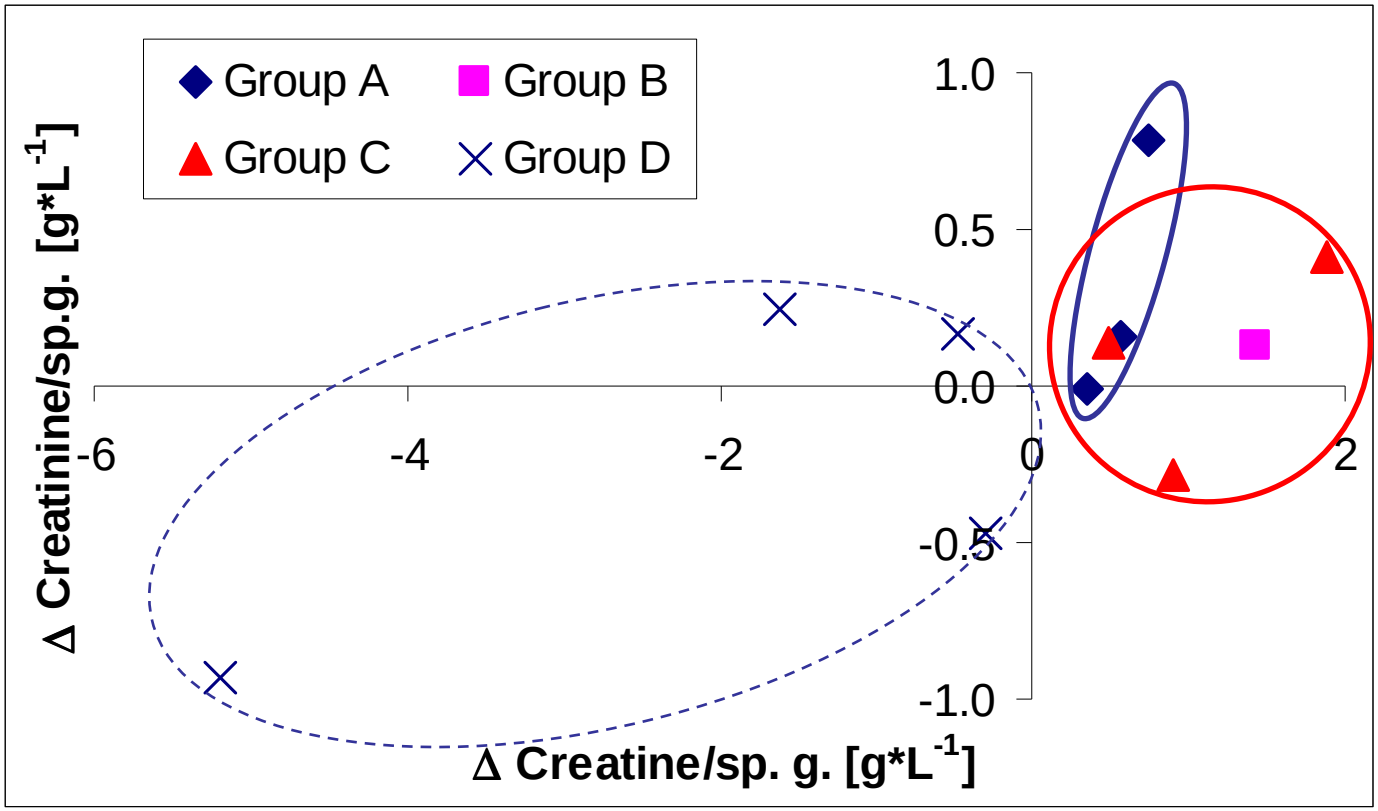
Group A
Group B
Group C
Group D
1.0
0.5
0.0
-0.5
-1.0
-6
-4
-2
0
2
Δ Creatinine/sp.g. [g*L-1]
Δ Creatine/sp. g. [g*L-1]

Figure 5.