Chapter 8

Screening for X-linked creatine transporter (SLC6A8) deficiency via simultaneous determination of urinary creatine to creatinine ratio by tandem mass-spectrometry


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Abstract

High urinary creatine to creatinine ratio (U-CrCrtR) is a potential diagnostic marker of X-linked creatine transporter (SLC6A8) deficiency. We developed a tandem mass-spectrometry method to simultaneously determine urinary creatine and creatinine in 975 individuals (0–18 years). U-CrCrtR increased up to 8 years and decreased thereafter. U-CrCrtR was 2.29 and 2.12 (99th percentile: 1.87) in two males with subsequently confirmed SLC6A8 mutations. The frequency of SLC6A8 deficiency was 2.3% in 157 males at risk.

Keywords:
Cerebral creatine deficiency
X-linked mental retardation
Age dependent normal values

Methods and results

Analytical procedure

Urine or aqueous standard:methanol (1:10) solutions containing [3H] creatine and [3H] creatinine (Cambridge Isotopes USA) as internal standard were derivatized with butanol and 10% acetylchloride, incubated at 65 °C, dried, and dissolved in water/acetonitrile/formic acid (2500:2500:1). Samples were injected in a Perkin Elmer Sciex API 2000 triple quadrupole mass spectrometer and measured in positive ion mode using a turbo ion spray ionization probe source at voltage 5500 V, gas temperature 300 °C and flow rate 150 µl/min. The transitions chosen for the quantification of creatine, [3H] creatine, creatinine and [3H] creatinine were m/z 188.1 → 90, m/z 191.1 → 93, m/z 114.0 → 44, and m/z 117.0 → 47. For quantitation the ratio of the unlabeled metabolite to the labeled internal standard was used in a linear regression analysis. Calibration curves revealed a linear increase for creatine and creatinine within physiological ranges. Urine samples spiked with 0.76–4.4 mmol/l creatine and creatinine revealed recoveries between 93.0% and 97%. Intra-assay variation of the SID-ES-MS-MS quantitative step was 5.0–6.0%. Inter-assay variation of the entire procedure was 10.8% for creatine and 6.1% for creatinine. Correlation with the Jaffe method was 0.81 for creatine.

Samples and patients

We used random urine samples from 975 individuals (557 males, 418 females) between 0 and 18 years (median 19.5 months); (0–1 year: n = 537; 1–3 years: n = 148; 3–8 years: n = 162; 8–18 years: n = 128) sent to our laboratory for selective metabolic screening.

Statistical analysis

OneWay ANOVA was applied to compare obtained values between age groups. Homogeneity of variances-test was significant. ANOVAs for creatine, creatinine and U-CrCrtR were
significant (p < 0.001) showing significant differences between one or more age groups (data not shown). As variances between age groups were not equal (p < 0.0001), we used Dunnett T3 test for post hoc test.

Age dependent normal values

We selected 4 age groups (0–1; 1–3; 3–8; 8–18 years) according to physiological changes in growth velocities and body mass index. U-CrCrtR increased up to 3 years and decreased thereafter (Fig. 1a). Age dependent changes of creatine and creatinine see Fig. 1b and c. T-test variances were equal between males and females.

The 99th percentiles for U-CrCrtR were 1.3; 1.85; 1.87 and 1.80 for the respective age groups. The 3–97th range was 0.01–1.32 for U-CrCrtR, 1.06–23.33 mmol/L for creatine; and 0.04–8.84 mmol/L for creatinine. U-CrCrtRs were explicitly above the 99th percentile for creatinine excretion. Differences are statistically significant (p < 0.0001) between all age groups except between age groups 0–1 and 8–18 years. (a) Age dependent changes of mean and 99th percentile U-CrCrtR. Differences are statistically significant (p < 0.001) between all age groups except between age groups 0–1 and 8–18 years. (b) Age dependent changes of mean and 99th percentile urinary creatine excretion. Differences are statistically significant (p < 0.001) between age group of 0–1 year and age groups of 1–3, 3–8 and 8–18 years, whereas there is no statistical significance between age groups of 1–3; 3–8 and 8–18 years. (c) Age dependent changes of mean and 99th percentile urinary creatine excretion. Differences are statistically significant (p < 0.001) between all age groups.

Screening results

In 29 out of 975 individuals, U-CrCrtR was >97th. In two out of 8 samples available for recall, U-CrCrtR was >99th percentile (2.29 and 2.5) in 2 positive controls with confirmed SLC6A8 deficiency. Positive results were confirmed by current standard procedures using stable isotope dilution gas chromatography–mass spectrometry (SID-GC–MS) for creatine, and using colorimetric (Jaffe) determination of creatinine [3].

Sequencing of the SLC6A8 gene revealed disease-causing mutations [4] in both individuals with U-CrCrtR >99th and wild type in the remainder. An 8-year-old male with severe mental retardation, speech delay, behavioral problems and epilepsy had a novel missense mutation (c.1145 C > T; p.Pro382Leu) which was confirmed by reduced creatine uptake in cultured fibroblasts. A 6-year-old male with moderate mental retardation, hyperactivity, speech delay and ataxia had a known pathogenic one amino acid deletion in exon 2 (c.321_323delCTT; p.Phe107del) [5].

According to the clinical information given in the requisition, we retrospectively identified one or more symptoms at risk for SLC6A8 deficiency (developmental delay/mental retardation associated with speech delay, seizures, autistc behavior) in 87 out of 157 males >1 year. With the two confirmed diagnoses, the frequency of SLC6A8 deficiency was 2.3% in this cohort at risk.

Discussion

Urinary creatine and creatinine are usually determined with two separate methods. Gas chromatography–mass spectrometry [6] or tandem mass–spectrometry [7] based methods are currently applied for accurate and sensitive determination of urinary creatine excretion, but creatinine is measured separately e.g. by the colorimetric Jaffe method [6,7]. Our SID-ES-MS-MS method allows for simultaneous determination of both creatine and creatinine. This is possible because of sample pretreatment with butanol, derivatizing creatine into its butyl-ester while creatinine remains undervatized. The reason for the relatively high inter-assay variation for creatine (10.8%) in the presence of only 6.1% inter-assay variation for creatinine is likely due to this selective derivatization reaction. The moderate correlation of the SID-ES-MS-MS method with the Jaffe method for creatinine is most likely due to interferences of the Jaffe method with non-creatinine chromogens such as glucose, ketone bodies and pharmacological substances. In contrast, such interferences are not to be expected with our tandem mass-spectrometry based method.

Existing age dependent normal values for U-CrCrtR are limited. Almeida et al. [3] found an age dependent decrease of U-CrCrtR in 140 samples supporting our findings in the older age groups. Our study is more comprehensive in terms of sample size covering also younger age groups. Whereas the observed increase of U-CrCrtR in the younger age groups was mainly due to a strong increase of urinary creatine, its decrease in the older age groups was mainly determined by an increasing creatinine excretion. Potential causes of these changes include growth dependent increase in muscle mass as the major site of creatinine formation, as well as changes in dietary creatine intake.

The U-CrCrtR was clearly above the 99th percentile in the 2 positive control samples and in the 2 patients identified in the study. More patients need to be studied in order to determine whether a cut off value at or higher than the 99th can be used for screening male subjects in future. This strategy will not allow detection of female carriers as in most of them U-CrCrtR has been found within the normal range [2].

In the cohort of males with symptoms at risk, the frequency of SLC6A8 deficiency was 2.3%. This finding is consistent with results from others who found SLC6A8 deficiency in 2.1% (6 out of 288) of males with non-syndromal X-linked mental retardation [5], and in 2.2% (2 out of 92) of males with global developmental delay and mental retardation [8]. Other investigators found 0.8% (4 out of 478) and 3.5% (4 out of 114) of males with mental retardation of unknown causes and negative for fragile X syndrome [9,10] to be positive for SLC6A8 deficiency.
SLC6A8 deficiency is a frequent but still an under-diagnosed cause of mental retardation. Therefore, determination of U-CrCrR should be routinely included into selective screening test panels for inborn errors of metabolism and mental retardation. Our SID-ES-MS-MS method for the simultaneous determination of creatine and creatinine is valuable for screening and high throughput analysis.

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References


