Guanidinoacetate and Creatine plus Creatinine Assessment in Physiologic Fluids: An Effective Diagnostic Tool for the Biochemical Diagnosis of Arginine:Glycine Amidinotransferase and Guanidinoacetate Methyltransferase Deficiencies

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Background: Disorders of creatine metabolism arise from genetic alterations of arginine:glycine amidinotransferase (AGAT), guanidinoacetate methyltransferase (GAMT), and the creatine transporter. We developed a strategy for the detection of AGAT and GAMT defects by measurement of guanidinoacetate (GAA) and creatine plus creatinine (Cr+Crn) in biological fluids.

Methods: Three patients with AGAT deficiency from the same pedigree and their eight relatives, as well as a patient affected by a GAMT defect and his parents were analyzed by a new HPLC procedure in comparison with 90 controls. The method, which uses precolumn derivatization with benzoin, separation with a reversed-phase column, and fluorescence detection, has shown good precision and sensitivity and requires minimal sample handling.

Results: In the three AGAT patients, plasma GAA was 0.01–0.04 μmol/L [mean (SD) for neurologically normal controls was 1.16 (0.59) μmol/L], Cr+Crn was 15–29 μmol/L [reference limit in our laboratory, 79 (38) μmol/L]. Urinary GAA was 2.4–5.8 μmol/L [reference, 311 (191) μmol/L], and Cr+Crn was 2.1–3.3 mmol/L [reference, 9.9 (4.1) mmol/L]. We found a smaller decrease in GAA and Cr+Crn in some carriers of an AGAT defect.

In the patient with GAMT deficiency, plasma and urine GAA was increased (18.6 and 1783 μmol/L, respectively), and Cr+Crn was decreased in plasma (10.7 μmol/L) and urine (2.1 mmol/L). GAA was increased in the parents’ plasmas and in the mother’s urine.

Conclusion: The assessment of GAA is a new tool for the detection of both GAMT and AGAT deficiencies.

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Creatine (Cr)4 and creatine phosphate (CrP) play essential roles in the storage and transmission of phosphate-bound energy. Cr is synthesized mainly in the liver and pancreas by two reactions. The first, catalyzed by arginine:glycine amidinotransferase (AGAT; EC 2.1.4.1), transfers the amidino group from arginine to glycine, forming ornithine and guanidinoacetate (GAA). The second, catalyzed by guanidinoacetate methyltransferase (GAMT; EC 2.1.1.2), transfers a methyl group from S-adenosylmethionine to GAA, forming Cr (Fig. 1). Cr is not used in these organs but is concentrated in tissues with high activities of Cr kinase, such as muscle, by means of an active Na+–energy-dependent Cr transporter system (CT1). Cr kinase catalyzes the phosphorylation and dephosphorylation of Cr and CrP and thus provides a high-energy phosphate buffering system during states of ATP synthesis and ATP use. At constant fractional rates, Cr and CrP

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*Nonstandard abbreviations: Cr, creatine; CrP, creatine phosphate; AGAT, arginine:glycine amidinotransferase; GAA, guanidinoacetate; GAMT, guanidinoacetate methyltransferase; CT1, creatine transporter 1; Crn, creatinine; 1H-MRS, proton magnetic resonance spectroscopy; and S/N, signal-to-noise ratio.
Disorders of Cr metabolism have been discovered at the level of Cr synthesis (AGAT and GAMT deficiency) (3–11) and transport (CT1 defect) (12, 13). The accumulation of GAA in biological fluids in GAMT deficiency was demonstrated to be a reliable diagnostic marker of this disease (4, 14). In contrast, for AGAT and CT1 the diagnosis relies on in vivo proton magnetic resonance spectroscopy (1H-MRS), although decreased GAA has been reported in the urine of patients affected by AGAT deficiency (11), and increased plasma Cr has been reported in all patients studied with CT1 defect (12, 13).

The aims of this study were (a) to examine the biochemical pattern of Cr metabolism derangement in AGAT deficiency, (b) to evaluate whether GAA and/or Cr+Crn concentrations tested by a new HPLC method might constitute specific and sensitive markers for the biochemical diagnosis of the disease, (c) to compare the patterns of biochemical alterations in AGAT and GAMT deficiencies, and (d) to test the sensitivities of these biochemical markers for the detection of heterozygous carriers of AGAT and GAMT deficiency traits.

**Materials and Methods**

**AGAT-DEFICIENT PATIENTS AND THEIR RELATIVES**

Three related patients affected by AGAT deficiency, two sisters [5 and 10 years of age; the index cases for this disorder (11)] and an 8-year-old second-degree cousin (unpublished case), as well as eight healthy adult relatives were enrolled in the study after we had obtained informed consent. The clinical presentation, neuroradiologic findings, and enzymatic and genetic analyses of the index cases have been reported elsewhere (11). All three patients suffered from early psychomotor delay and presented severe to moderate mental and language retardation. Slow somatic growth was noticed in the two sisters. Although brain magnetic resonance imaging was normal, brain 1H-MRS demonstrated the lack of a Cr/CrP peak in all three patients. A low GAA concentration in urine was reported in the two index cases (11). Cr monohydrate therapy, starting from a dose of 400 mg·kg⁻¹·day⁻¹, almost completely restored brain Cr in all three patients a few months after the beginning of the therapy. Clinical improvement paralleled the restoration of the brain Cr pool, which also produced a return to normal somatic growth in the two sisters with delayed growth (11).

**GAMT-DEFICIENT PATIENT AND THEIR RELATIVES**

A 6-year-old child, the only son of healthy nonconsanguineous parents, presented neurologic regression, movement disorders, and epilepsy during the first year of life. Brain magnetic resonance imaging revealed pallial and periaqueductal alterations. Cr depletion was detected by brain 1H-MRS, and the marked increase of GAA in biological fluids (blood, urine, and cerebrospinal fluid) as well as molecular genetic analysis (15) confirmed the diagnosis of GAMT deficiency (8). A prompt clinical, biochemical, and neuroradiologic improvement was observed after Cr supplementation.

**BIOCHEMICAL METHODS**

We obtained first morning urine and plasma samples from the individuals enrolled in the study. Plasma and urine specimens were ultrafiltered at 5000g for 30 min using 10 000 mass cutoff Ultrafree-MC filters (Millipore). To obtain GAA concentrations within the range appropriate to the calibration curves, urine filtrates were diluted 1:1000 [10 μL of urine filtrate was added to 990 μL of 1× Dulbecco phosphate-buffered saline (HyClone Laboratories), then 100 μL of this solution was added to 900 μL of Dulbecco phosphate-buffered saline]. The filtrates of urine samples in which GAA was not detectable in the 1:1000 dilution (as in the case of AGAT deficiency) were diluted 1:2 with phosphate-buffered saline and reanalyzed. GAA and Cr+Crn in the filtrates were then determined according to a previously described method for the determination of GAA in dried-blood spots based on automated precolumn derivatization with benzoin, chromatographic separation, and fluorometric detection (16). The samples were subjected to a completely automated derivatization procedure as reported previously (16), with an ASPEC XL sample processor (Gilson). The analytical system was composed of two Model 2248 pumps and a Model 2252 controller (Pharmacia Biotech), a Croco-Cil column oven (Cil-Cluseau), and a FP1520 fluorescence detector (Jasco Corp.). The column was a TSK gel Super-
ODS [100 × 4.6 mm (i.d.); 2-μm particle size; TosoHaas], in conjunction with an ODS Hypersil guard column [20 × 4 mm (i.d.); Hewlett-Packard]. The chromatographic separation conditions were reported previously (16). Data were stored and processed using HP ChemStation Chromatographic software. This method was tested for the determination of GAA and Cr+Crn in plasma and urine samples.

Fig. 2. Chromatograms of samples from healthy individuals and patients with AGAT and GAMT deficiencies. (A), calibration sample (GAA, 0.8 μmol/L; Cr+Crn, 80 μmol/L); (B), plasma sample obtained from a healthy individual (GAA, 0.67 μmol/L; Cr+Crn, 62 μmol/L); (C), plasma sample obtained from a patient with AGAT deficiency (GAA, 0.02 μmol/L; Cr+Crn, 18 mmol/L); (D), urine sample obtained from a healthy individual (GAA, 458 μmol/L; Cr+Crn, 9.8 mmol/L); (E), urine sample obtained from a patient with AGAT deficiency (GAA, not detectable; Cr+Crn, 3.3 mmol/L); and (F), urine sample obtained from a patient with GAMT deficiency (GAA, 1783 μmol/L; Cr+Crn, 2.1 mmol/L).
### Table 1. Plasma and urine reference values of GAA and Cr+Crm.

<table>
<thead>
<tr>
<th>Age, years</th>
<th>n</th>
<th>Plasma GAA, µmol/L</th>
<th>Plasma Cr+Crm, µmol/L</th>
<th>Age, years</th>
<th>n</th>
<th>Urine GAA, µmol/L</th>
<th>Urine Cr+Crm, mmol/L</th>
<th>Urine GAA/(Cr+Crm), mmol/mol</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25–4</td>
<td>19</td>
<td>1.08 (0.39)</td>
<td>[0.55–1.91]</td>
<td>0.5–3</td>
<td>30</td>
<td>283 (168)</td>
<td>[68–718]</td>
<td>7.6 (3.3) [2.8–14.3]</td>
</tr>
<tr>
<td>5–10</td>
<td>22</td>
<td>1.16 (0.59)</td>
<td>[0.41–2.31]</td>
<td>4–8</td>
<td>20</td>
<td>311 (191)</td>
<td>[74–712]</td>
<td>9.9 (4.1) [3.3–19.9]</td>
</tr>
<tr>
<td>11–20</td>
<td>20</td>
<td>1.48 (0.51)</td>
<td>[0.62–2.46]</td>
<td>9–16</td>
<td>14</td>
<td>365 (232)</td>
<td>[74–809]</td>
<td>8.1 (3.6) [2.7–16.4]</td>
</tr>
<tr>
<td>21–64</td>
<td>38</td>
<td>1.83 (0.45)</td>
<td>[0.79–2.9]</td>
<td>17–64</td>
<td>16</td>
<td>297 (195)</td>
<td>[54–793]</td>
<td>6.8 (4.3) [1.6–16.2]</td>
</tr>
<tr>
<td>0.25–64</td>
<td>All</td>
<td>1.47 (0.58)</td>
<td>[0.41–2.9]</td>
<td>0.5–64</td>
<td>All</td>
<td>313 (199)</td>
<td>[54–809]</td>
<td>8.1 (3.9) [2.7–19.9]</td>
</tr>
</tbody>
</table>

*Values are shown as mean (SD) [minimum-to-maximum interval].

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**REFERENCE VALUES AND STATISTICAL ANALYSIS**

We collected 99 plasma samples (age range of the individuals was 3 months to 64 years) and 80 urine samples (age range of the individuals was 6 months to 64 years) from neurologically healthy individuals. Children’s plasma and children’s and adults’ urine samples were obtained from hospitalized Caucasian patients on a typical diet (after informed consent). The above patients were hospitalized for diagnostic removal of skin nevi (n = 40), cosmetic correction of congenital squint (n = 12), removal of congenital skin cavernous hemangioma (n = 7), tonsillectomy and adenoidectomy (sleep apnea syndrome; n = 12), or traumatic corneal injury (n = 9). All plasma and urine samples were collected before the surgical procedures. We obtained 38 adult plasma samples from anonymous blood donors. The effects of age and gender on the biochemical variables (GAA and Cr+Crm concentrations) were evaluated. Relationships between variables were examined by correlation analysis and ANOVA; multiple comparisons were examined by the Spjotvolla–Stoline test. Statistical analysis was performed with the use of Statistica software (StatSoft).

**Results**

**MATERIAL VALIDATION**

The separation of GAA and Cr+Crm from other guanidino compounds and interfering arginyl-containing oligopeptides was achieved in 20 min. Fig. 2 shows a chromatogram of a calibrator sample (panel A) and chromatograms of plasma (panel B) and urine (panel D) samples from a healthy control. The fluorescent derivatives of Cr and Crn produced by benzoin reaction in an aqueous 2-methoxyethanol–potassium hydroxide solution have identical 2-substituted amino-4,5-diphenylimidazole chemical structures and therefore cannot be separated by this method.

To test whether Cr and Crn contribute equally to the fluorescence, we analyzed a 200 µmol/L Cr solution and a 200 µmol/L Crn solution seven times each and compared the chromatographic peak areas obtained that did not show a significant difference (P = 0.76626, two-tailed t-test). The limit of detection was 80 fmol for GAA and 2 pmol for Cr+Crm at a signal-to-noise ratio (S/N) of 5.

The linear correlation between peak area and concentration was assessed in the 0.02–4 µmol/L range for GAA and the 2–400 µmol/L range for Cr+Crm. The correlation coefficients were 0.9998 and 0.9999, respectively, and the equations were: GAA concentration (µmol/L) = 0.0047 + 0.000006 × GAA area (S_{adj} = 0.008732 µmol/L; SD of slope < 0.00001; and SD of intercept, 0.00245); and Cr+Crm concentration (µmol/L) = −0.5412 + 0.0020 × Cr+Crm area (S_{adj} = 0.401436 µmol/L; SD of slope, 0.000006; and SD of intercept, 0.1148).

Precision was assessed in several ways. In 10 consecutive analyses of a solution containing 0.08 µmol/L GAA and 8 µmol/L Cr+Crm, the CVs were 3.9% and 3.2%, respectively. The within-run precision was evaluated by performing 10 analyses of a plasma sample (1.5 µmol/L GAA; 83 µmol/L Cr+Crm) and 10 analyses of a urine sample (318 µmol/L GAA; 9.1 mmol/L Cr+Crm) on the same day. The CVs obtained were, respectively, 4.0% and 4.2% for GAA and 3.6% and 4.0% for Cr+Crm. The between-run precision was assessed by analyzing 10 aliquots of a plasma sample and 10 aliquots of a urine sample stored at −20°C on different days within a period of 3 weeks. The CVs obtained were, respectively, 4.4% and 4.8% for GAA and 4.1% and 4.7% for Cr+Crm.

Recovery was evaluated by adding GAA and Cr to a sample of plasma and to a sample of urine to obtain concentrations of 2 µmol/L GAA and 200 µmol/L Cr+Crm in plasma and 1000 µmol/L GAA and 10 mmol/L Cr+Crm in urine. The samples obtained were analyzed in triplicate, and the range of the mean recoveries was 98–102%.

**VALUES IN HEALTHY CONTROLS**

The relationships of age and concentrations of GAA and Cr+Crm were analyzed. Plasma GAA was significantly correlated with age (r = 0.57; P < 0.0001), and the scatter plot showed a more pronounced effect of age in children and adolescents than in adults. This effect was confirmed by ANOVA [F(3,95) = 15.02; P < 0.00001] with metabolite concentrations grouped according to age (Table 1). Multiple range analysis (Spjotvolla–Stoline test) showed that the first group was significantly (P < 0.05) different from the third and fourth groups and that the second group was significantly different from the fourth group. The effect of age on Cr+Crm was less evident than on GAA: the Spjotvolla–Stoline test showed a significant difference only between the first, third, and fourth groups, respectively. Urinary GAA and Cr+Crm concentrations did not seem to be affected by age. Table 1 shows the GAA and...
RESULTS IN AGAT- AND GAMT-DEFICIENT PATIENTS AND CARRIERS

The method was applied to the analysis of plasma and urine samples from the individuals affected by AGAT and GAMT deficiencies and their relatives. Fig. 2 shows chromatograms of plasma (panel C) and urine (panel E) obtained from a patient with AGAT deficiency in comparison with the chromatograms of plasma (panel B) and urine (panel D) from a healthy control and the chromatogram of urine (panel F) from a GAMT-deficient patient. Fig. 3 shows plots of plasma and urine concentrations of GAA, Cr+Crm, and urinary GAA/(Cr+Crm) vs the genotypes of individuals from the pedigree affected by AGAT deficiency. We found a marked decrease in Cr in the plasma and urine of the affected individuals. Plasma and urine Cr+Crm values were also markedly decreased, although they seemed less altered in comparison with GAA [urinary GAA/(Cr+Crm) ratio was markedly decreased]. The results of biochemical assessment of carriers for the AGAT-deficient trait (Fig. 3) showed a decrease in plasma GAA in one and plasma Cr+Crm in two of the four individuals examined; decreased GAA and Cr+Crm concentrations in urine were found in one of the two individuals examined.

Shown in Table 2 are the genotypes (15) and the results of GAA and Cr+Crm assessments in plasma and urine samples of a patient affected by GAMT deficiency (8) and his parents. We found a marked increase in GAA concentrations in the plasma and urine of the propositus, coupled with a marked decrease in Cr+Crm concentration. Surprisingly, we also found increased GAA in the parents’ plasmas and in the mother’s urine. In the last sample, a high concentration of GAA was coupled with a very high concentration of Cr+Crm, leading to a GAA/(Cr+Crm) ratio that was within reference values.

Discussion

Patients with brain Cr deficiency present nonspecific neurologic symptoms, including mental retardation, language disorders, epilepsy, autistic-like behavior, neurologic deterioration, and movement disorders. The positive results of Cr treatment (in AGAT and GAMT deficiencies) and the observation that fetal and early postnatal development are normal in these patients support the hypothesis that earlier diagnosis and treatment can substantially improve the final prognosis of these diseases. Brain 1H-MRS examination is a reliable and minimally invasive technique to assess brain Cr disorders. Because of its limited availability and high cost, the 1H-MRS technique cannot be proposed for all children whose clinical condi-
ion suggests the diagnosis of brain Cr depletion. Our work suggests a biochemical strategy that can be applied for a wider examination of symptomatic patients.

Several methods for the diagnosis of GAMT deficiency by determination of GAA concentrations in biological fluids have been reported (14, 17–21), including methods based on liquid chromatography with postcolumn derivatization with ninhydrin (14, 21), gas chromatography–mass spectrometry (18, 19), and tandem mass spectrometry (20). We used a HPLC method that we had developed for the determination of GAA in dried-blood spots (16) and applied it to plasma and urine samples. This method has high sensitivity and can detect values below and above those found in healthy individuals. Particularly in AGAT deficiency, high sensitivity is an essential characteristic for the diagnosis of this disease. The distribution of GAA concentrations was incept for the values obtained by tandem mass spectrometry (19) and HPLC with postcolumn derivatization with ninhydrin (10 pmol; S/N = 2; injection volume, 150 μL) (21).

We also used our method to measure GAA and Cr+Crn concentrations in neurologically healthy individuals. The distribution of GAA concentrations was in agreement with those published previously (19, 22) except for the values obtained by tandem mass spectrometry (20), which were higher than the others [mean (SD) = 5.02 (1.84) μmol/L in the adults’ plasma and 3.91 (0.76) μmol/L in the children’s plasma]. We found a correlation between age and GAA concentrations in the plasma, whereas in urine, GAA concentrations did not correlate with age, as reported previously (18).

We assessed GAA and Cr+Crn concentrations in the only three patients affected by AGAT deficiency currently known [Ref. (11), and unpublished results], showing that the concentrations of GAA were markedly decreased in both the plasma and urine samples of the patients compared with healthy individuals. This result confirms and extends the preliminary data from Item et al. (11), who reported extremely low urinary GAA excretion in the two index cases. We also found marked decreases in the Cr+Crn concentration in plasma and urine, although of smaller magnitude than GAA alteration. The different sensitivities of the two markers (GAA vs Cr+Crn) were also evident when looking at the ratio between GAA and Cr+Crn, which was markedly decreased in all three of the patients. These results support the hypothesis that an exogenous source of Cr partially replenishes the endogenous pool of Cr, whereas GAA is mainly synthesized by the organism.

We had the opportunity to analyze only a few carriers for AGAT-deficient trait (Fig. 3). Although limited, our data suggest that, in some individuals, heterozygosity alters both GAA and Cr+Crn concentrations. Our data support the conclusion that GAA and Cr+Crn in biological fluids, as detected by the method we developed, are candidate markers for the biochemical diagnosis of AGAT deficiency in symptomatic patients, although it is currently not possible to establish whether they can be of help for the detection of carriers.

We also evaluated GAA concentrations in biological fluids from a patient with GAMT deficiency (8), and our method was able to clearly detect the increase of GAA expected in this disorder as already reported by others (5, 14). The most intriguing result concerns the alteration we found in the obligate heterozygotes who had increased plasma GAA, especially the mother (GAA > mean + 3 SD), where we also found extremely high urinary excretion of GAA and Cr+Crn. That the GAA/(Cr+Crn) ratio was within reference values suggests that the increases in urinary GAA and Cr+Crn could arise from the competition between the two guanidino compounds for the kidney transporter (1).

Although there are still not sufficient data to establish a genotype/phenotype correlation in this disease, it is noteworthy that, among the two obligate carriers, the mother carries a severe splicing mutation whose transcript is not detectable in either the proband (the paternal origin allele is expressed) or the mother (only the wild-type allele is expressed) (15). To our knowledge, these are the first data concerning the metabolic alteration in carriers of GAMT deficiency.

To select symptomatic patients for brain 1H-MRS examination, the first step could be assessment of GAA and Cr in biological fluids. If GAA is increased, a deficit of GAMT can be suspected, whereas if GAA is decreased, AGAT deficiency should be suspected. A normal GAA coupled with increased Cr should suggest a CT1 defect (13). Because no specific treatment is available for the latter disorder, an early diagnosis is less urgent. All patients having one of the biochemical patterns described above should undergo brain 1H-MRS assessment and

### Table 2. Plasma and urine values of GAA and Cr+Crn according to GAMT genotype.

<table>
<thead>
<tr>
<th>Individual</th>
<th>Age, years</th>
<th>Plasma GAA, μmol/L</th>
<th>Plasma Cr+Crn, μmol/L</th>
<th>Urine GAA, μmol/L</th>
<th>Urine Cr+Crn, mmol/mol</th>
<th>Urine GAA/(Cr+Crn), mmol/mol</th>
<th>GAMT genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 4</td>
<td>18.6 a</td>
<td>10.7 a</td>
<td>1783 a</td>
<td>2.1 a</td>
<td>849 a</td>
<td>C.491insG/IVS5-3C→G</td>
<td></td>
</tr>
<tr>
<td>Father</td>
<td>49</td>
<td>2.34</td>
<td>68</td>
<td>300</td>
<td>10.0</td>
<td>30</td>
<td>C.491insG</td>
</tr>
<tr>
<td>Mother</td>
<td>45</td>
<td>3.65 a</td>
<td>94</td>
<td>1304 a</td>
<td>18.9 a</td>
<td>69</td>
<td>IVS5-3C→G</td>
</tr>
</tbody>
</table>

a Outside minimum-to-maximum interval of reference values.
molecular analysis. Other investigations, such as enzymatic analysis of GAMT (23) and AGAT (11) and the study of Cr transport in cultured fibroblasts (13), should follow, being of minor practical relevance to the aim of an early diagnosis.

In conclusion, we suggest a low-cost strategy for the biochemical investigation of patients suspected of having the recently reported AGAT defect. The approach may be most useful when patients’ clinical conditions require that a defect in Cr synthesis be excluded.

References