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RESEARCH REPORT

Guanidinoacetate Methyltransferase Activity in Lymphocytes, for a Fast Diagnosis

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Abstract *Introduction*. Guanidinoacetate methyltransferase (GAMT) deficiency is an inborn error of metabolism (IEM), clinically characterized by intellectual disability, developmental delay, seizures, and movement disorders. Biochemical diagnosis of GAMT deficiency is based on the measurement of creatine and guanidinoacetate in urine, plasma, or CSF and is confirmed genetically by DNA analysis or by enzyme assay in lymphoblasts or fibroblasts. To obtain enough cells, these cells need to be cultured for at least 1 month. A less time-consuming diagnostic functional test is needed, since GAMT deficiency is a candidate for newborn screening (NBS) programs, to be able to confirm or rule out this IEM after an initial positive result in the NBS.
Methods. Stable-isotope-labeled $^{13}C_2$-guanidinoacetate and $^{2}H_3$-*S*-adenosylmethionine (SAM) were used, which are converted by GAMT present in lymphocyte extracts into $^{2}H_3$-$^{13}C_2$-creatine. The formed $^{2}H_3$-$^{13}C_2$-creatine was butylated and subsequently measured by liquid chromatography tandem mass-spectrometry (LC-MS/MS).
Results. We measured GAMT enzyme activity in lymphocyte extracts of 24 controls, 3 GAMT deficient patients and of 2 parents proven to be carrier. Because GAMT activity decreases when isolation time after venipuncture increases, reference values were obtained for 2 control groups: isolation on the day of venipuncture (27–130 pmol/h/mg) and 1 day afterwards (15–146 pmol/h/mg). Deficient patients had no detectable GAMT activity. The two carriers had GAMT activity within the normal range.
Conclusion. We designed a fast, less invasive, and valid method to measure GAMT activity in lymphocytes using LC-MS/MS analysis without the need of time-consuming and laborious cell culture.

Nonstandard Abbreviations

AGAT	Arginine:glycine amidinotransferase
EDTA	Ethylenediaminetetraacetic acid
GAMT	Guanidinoacetate methyltransferase
HBSS	Hanks balanced salt solution
LC-MS/MS	Liquid-chromatography tandem mass-spectrometry
MRS	Magnetic resonance spectroscopy
NBS	Newborn screening
SAM	S-adenosylmethionine
tris	Tris(hydroxymethyl)aminomethane
WES	Whole exome sequencing

Introduction

Guanidinoacetate methyltransferase (GAMT; EC 2.1.1.2; MIM 601240) is one of the enzymes involved in creatine biosynthesis. Creatine is synthesized in liver, pancreas, and kidney in a two-step enzymatic pathway. First, arginine and glycine are converted into ornithine and guanidinoacetate by arginine:glycine amidinotransferase (AGAT; EC 2.1.4.1; MIM 602360), taking place mainly in the kidney. Next step is the formation of creatine out of guanidinoacetate by GAMT as enzyme, using S-adenosylmethionine as methyl-donor, which takes place mainly in the liver (Wyss and Kaddurah-Daouk 2000; Almeida et al. 2004; Mercimek-Mahmutoglu and Salomons 2009; Joncquel-Chevalier Curt et al. 2015; Hanna-El-Daher and Braissant 2016).

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Creatine is known for its use as energy buffer in muscle and brain in the form of phosphocreatine. Creatine kinase (EC 2.7.3.2) converts creatine into phosphocreatine, after creatine is transported into the cell by the creatine transporter (MIM 300036). Phosphocreatine is a phosphagen which can convert ADP to ATP with creatine kinase as enzyme when energy demand is high (Joncquel-Chevalier Curt et al. 2015; Wallimann et al. 2011; Wyss and Kaddurah-Daouk 2000).

GAMT deficiency is an autosomal recessive disorder, with an estimated incidence varying from 1:114,000 newborns in Utah (US), to 1:250,000 newborns in the Netherlands and in Australia of 1:≥770,000 (Pitt et al. 2014; Mercimek-Mahmutoglu et al. 2016). It results in the accumulation of guanidinoacetate and depletion of creatine.

Patients with GAMT deficiency can suffer from mental retardation, developmental delay, seizures, and movement disorders. After diagnosing GAMT deficiency, treatment consists of supplementation of creatine and a protein-, or arginine-restricted diet to decrease guanidinoacetate in plasma and CSF. If treated at birth, the symptoms can be prevented (El-Gharbawy et al. 2013), therefore newborn screening (NBS) for GAMT-deficiency is advised by the Ministry of Health in the Netherlands (Health Council of the Netherlands 2015) and is implemented in the Utah (US) newborn screening program since July 2015. Newborn screening will most likely be done by measuring guanidinoacetate/creatine ratio in dried blood spots with flow injection-MS/MS, with a second tier test using LC-MS/MS (Pasquali et al. 2014) followed by genetic/enzymatic confirmation (Verhoeven et al. 2004). Our current enzyme assay measures GAMT activity in lymphoblasts and fibroblasts. To obtain sufficient amount of cells, lymphoblasts and fibroblasts need to be cultured for at least 1 month, which is time consuming and delaying the enzymatic confirmation. Since GAMT deficiency is a candidate to be included in the NBS, an enzymatic confirmation method in readily available cells, i.e. lymphocytes is needed. We developed an enzyme assay in lymphocytes for the detection of GAMT activity by using tandem mass spectrometry (LC-MS/MS).

Materials and Methods

Chemicals and Reagents

Tris(hydroxymethyl)aminomethane (tris), and methanol (99.8%), butanol (99.5%), acetylchloride and acetonitrile (99.5%) were purchased from Merck (Darmstadt, Germany). $^{13}C_2$-guanidinoacetate was manufactured by Dr. H.J. ten Brink (Organic Synthesis Laboratory VU Medical Center, Amsterdam). $^{2}H_3$-S-adenosylmethionine; 99 atom-% D) and $^{2}H_3$-creatine (99 atom-% D) were purchased from C/D/N isotopes (Quebec, Canada). Leucosep™ tubes with Histopaque were purchased from Greiner Bio-One. Bicinchoninic acid protein assay kit was purchased from Sigma-Aldrich (St. Louis, MO). All other solvents and chemicals were of analytical grade.

Sample Collection

Lymphocytes were isolated from venous blood samples in ethylenediaminetetraacetic acid (EDTA) containing tubes. Collection of venous blood was done at the venipuncture unit in VU medical center. Written consent was obtained from each participant. Material was then sent to the metabolic laboratory. Frozen lymphocyte cell pellets of three known patients with GAMT deficiency were available at the metabolic laboratory of VU medical center, as well as from two parents from one of the patients who were proven to be heterozygous for a pathogenic mutation. The three patients were diagnosed by measurement of metabolites and brain magnetic resonance spectroscopy (MRS) and the diagnosis was confirmed by genetic analysis. Lymphocytes of these patients and the two parents were isolated within 1 day after venous blood collection.

Sample Preparation

Isolating lymphocytes was performed using Leucosep™-tubes with Histopaque. 3–8 mL blood was gently transferred into the Leucosep™ tube which was centrifuged 20 min at 1,000 g with switched-off brakes. Then the enriched cell fraction was pipetted into another tube and washed twice with 5 mL of Hanks balanced salt solution (HBSS) and centrifuged 6 min at 340 g. An erythrocyte shock was performed by dissolving the cell pellet in 1 mL HBSS, after which 3 mL cold distilled water was added, and after 1.5 min 1 mL 3.6% NaCl was added. Then the tube was centrifuged again for 6 min at 340 g. Pellets were dissolved in 1 mL HBSS, and centrifuged 3 times at 10,000 g for 8 s. The dry pellets were stored at −80°C. Cell pellets were dissolved in 250 µL of 200 mmol/L tris-HCl buffer (pH 8.5) and sonicated 3 times 10 s on ice. Lysates were centrifuged for 5 min at 4°C and 10,000 g, then protein concentration within the supernatant was measured with bicinchoninic acid protein assay kit.

An adapted method of Verhoeven et al. (2004) was used to form labeled creatine from stable isotope labeled substrates in the lymphocyte extract, which is a direct measure for GAMT activity. In short, 25 µL 8 mmol/L $^{13}C_2$-guanidinoacetate, and 12.5 µL 25 mmol/L $^{2}H_3$-SAM were used as substrates. 0.1 mg protein of cell homogenate was used. Finally, 200 mmol/L tris-HCl buffer (pH 8.5) was added till a final volume of 0.2 mL. The reaction mixture

was incubated for 2 h at 37°C. At the start of incubation and after 2 h, an 85 µL sample was taken and stored at −20°C until preparation for LC-MS/MS analysis. This preparation method and LC-MS/MS analysis was derived from the method used by Mercimek-Mahmutoglu et al. (2016) to measure guanidinoacetate and creatine in dried bloodspots. An assay blank was prepared with 100 µL distilled water instead of cell homogenate. To quantify the formation of $^{2}H_3$-$^{13}C_2$-creatine, 6.25 pmol of $^{2}H_3$-creatine was added to the samples as internal standard. Samples were deproteinized with 330 µL methanol and were centrifuged 2 min at 5,000 g. The mixture was transferred to a vial and evaporated to dryness with nitrogen at 40°C. Then 100 µL butylating-reagens (consisting of acetylchloride and *n*-butanol in a ratio of 1:17) was added. Derivatization took place for 15 min at 60°C, after which it was evaporated with nitrogen again. After dissolving in 50 µL acetonitrile, samples were injected on the LC-MS/MS.

Liquid Chromatography

Chromatography was performed using an LC-30AD UPLC-system (Shimadzu, Kyoto, Japan). Chromatographic separation was achieved by using a Symmetry-C_8 column: 4.6 * 100 mm, 3.5 µm (Waters, Milford, USA) with isocratic elution of 30% acetonitrile (with 0.4% formic acid) at a flow rate of 0.35 mL/min. 5 µL of sample was injected into the column and the total run time was 5 min.

Mass Spectrometry

Tandem mass spectrometry experiments were carried out using an API5000 triple quadrupole mass spectrometer (Applied Biosystems-Sciex, Toronto, Canada) equipped with a Turbo Ion Spray Source. The mass spectrometer operated in ESI-positive mode with a needle potential of 5,500 V and a source temperature of 300°C. Optimized collision energy and voltages were used and the compound of interest was analyzed using multireaction monitoring mode (*m/z* 193.0 → 95.0 for $^{2}H_3$-$^{13}C_2$-creatine and *m/z* 191.0 → 93.0 for $^{2}H_3$-creatine).

Performance Characteristics

To optimize the enzyme assay in lymphocytes, a protein curve was made (0–25–50–75–100–150–200 µg protein), as well as a sequence of incubation time (for 0–30–60–90–120–180 min). To determine the stability of GAMT enzyme in blood samples, an experiment was performed using each three blood samples of two adult controls which were isolated immediately, 24 or 48 h after venipuncture. After this, they were prepared for enzyme assay as described above.

The intra- and inter-assay variation were determined. The limit of detection (LOD) and limit of quantitation (LOQ) were determined based on the signal to noise (S/N) ratio. LOD was determined as 3 times (S/N)-ratio, LOQ was determined as 10 times (S/N)-ratio. Obtained values were the mean of three independent experiments. Since GAMT appeared to be instable, reference values were obtained for lymphocytes isolated directly after venous blood collection and 1 day afterwards. Lymphocytes from three established GAMT deficient patients and two carriers were used to determine the enzymatic activity.

Statistical Analysis

Statistical analysis, such as calculating intra- and inter-assay variation, was performed using the software of Microsoft Excel 2010. Linear regression analysis was used to calculate GAMT activity.

Results

The protein curve showed an optimum of 100 µg, which was used in further experiments. The incubation curve was performed in duplicate, which showed an optimum of 120 min (data not shown).

The stability of the GAMT enzyme in blood was tested in two different cell lines (A and B) and was as follows. When isolated immediately after venipuncture, the activities were 31 pmol/h/mg and 130 pmol/h/mg, respectively; after 24 h, the activities were 17 pmol/h/mg and 50 pmol/h/mg; after 48 h, the activities were 12 pmol/h/mg and not detectable in cell lines A and B, respectively.

Table 1 GAMT activity in lymphocytes from controls and individuals with GAMT deficiency and carriers. Controls were isolated on the day of venipuncture and 1 day afterwards

	N	Days after venipuncture	Range (pmol/h/mg)	Mean ± sd (pmol/h/mg)
Lymphocytes of adult controls	13	0	27–130	58 ± 18
Lymphocytes of adult controls	11	1	15–146	62 ± 46
GAMT deficient patients	3	0–1	Not detectable	Not detectable
GAMT carriers	2	0–1	106; 115	

The limit of detection is 3.8 pmol/h/mg.

Reference values were determined by measuring GAMT activity in lymphocytes of unaffected adult controls (Table 1). Because of the decline of GAMT activity with increased isolation time, reference values were obtained for two groups: isolation on the day of venous blood collection and 1 day afterwards. The intra-assay variation ($n = 8$) was 6.3%. The inter-assay variation ($n = 6$) was 6.7%.

The LOD and LOQ were 3.8 pmol/h/mg and 12.7 pmol/h/mg, respectively, and were sufficient to diagnose patients with GAMT deficiency. Measurement of three known GAMT deficient patients showed no detectable GAMT activity. The two parents tested who were carrier showed GAMT activity within the normal range.

Discussion

Historically, the first enzymatic assays of GAMT were performed in liver biopsies, since GAMT is mainly active in the liver (Stöckler-Ipsiroglu et al. 1996) while later, assays were developed for lymphoblasts and fibroblasts (Verhoeven et al. 2004; Ilas et al. 2000), which were less invasive, but due to the need to have enough protein, cell culturing was necessary. Since quick diagnosis and treatment is of great importance in patients with GAMT deficiency, and because of the advice of the Ministry of Health in the Netherlands to include GAMT deficiency in the NBS program (Health Council of the Netherlands 2015), we developed a method to measure GAMT activity in readily obtainable lymphocytes. Also with the increased findings of genetic variants by whole exome sequencing (WES), it is important to functionally confirm, by enzymatic (functional) tests, whether these variants are clinically relevant.

The different isolation times showed that GAMT activity decreases when the venous blood sample is older. Therefore, two groups were made for reference values: immediately after collecting the venous blood sample, and 1 day afterwards. Blood isolated after 48 h is not reliable to use for lymphocyte analysis for GAMT enzymatic analysis.

The measurement of known deficient patients showed no detectable GAMT activity, as suspected based on results in lymphoblasts and fibroblasts. Our new method for measurement of GAMT activity in lymphocytes is validated according to ISO15189 accreditation for medical laboratories and is therefore valid for diagnostics.

Future Perspectives

Because GAMT deficiency is a candidate to be included in the NBS program, an enzyme assay measuring GAMT activity in dried blood spots would be ideal. In the current enzyme assay, the sensitivity was enhanced by changing the original described workflow for GAMT activity in fibroblasts and lymphoblasts from GC-MS to LC-MS/MS. Measuring GAMT activity in dried bloodspots will require even more sensitivity compared to our here presented GAMT enzyme assay in lymphocytes, and will be our next goal to achieve.

Conclusion

We developed a fast, less invasive, reproducible, reliable, and sensitive method to measure GAMT activity in lymphocytes.

Synopsis

A new, fast, less invasive and valid method to measure GAMT activity in lymphocytes using LC-MS/MS analysis of a $^{2}H_{3}$-$^{13}C_{2}$-creatine butyl derivative was designed.

Compliance with Ethics Guidelines

Lisette M. Berends, Eduard A. Struys, Birthe Roos, Ulbe Holwerda, Erwin E. W. Jansen, Gajja S. Salomons, and Mirjam M. C. Wamelink declare that they have no conflict of interest.

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000 (5). Informed consent was obtained from all patients for being included in the study.

Author Contribution

Lisette M. Berends: conception and design; analysis and interpretation of data; drafting the article; revising it critically.

Eduard. A. Struys: analysis and interpretation of data; revising it critically.

Birthe Roos: conception and design; analysis and interpretation of data; revising it critically.

Ulbe Holwerda: analysis and interpretation of data; revising it critically.

Erwin E. W. Jansen: analysis and interpretation of data; revising it critically.

Gajja S. Salomons: conception and design; revising it critically.

Mirjam M. C. Wamelink: conception and design; analysis and interpretation of data; revising it critically, guarantor.

References

Almeida LS, Verhoeven NM, Roos B et al (2004) Creatine and guanidinoacetate: diagnostic markers for inborn errors in creatine biosynthesis and transport. Mol Genet Metab 82(3): 214–219

El-Gharbawy AH, Goldstein JL, Millington DS et al (2013) Elevation of guanidinoacetate in newborn dried blood spots and impact of early treatment in GAMT deficiency. Mol Genet Metab 109(2): 215–217

Hanna-El-Daher L, Braissant O (2016) Creatine synthesis and exchanges between brain cells: what can be learned from human creatine deficiencies and various experimental models? Amino Acids 48(8):1877–1895

Health Council of the Netherlands (2015) Neonatal screening: new recommendations. Health Council of the Netherlands, The Hague. Publication No. 2015/08. ISBN 978-94-6281-050-1

Ilas J, Mühl A, Stöckler-Ipsiroglu S (2000) Guanidinoacetate methyltransferase (GAMT) deficiency: non-invasive enzymatic diagnosis of a newly recognized inborn error of metabolism. Clin Chim Acta 290(2):179–188

Joncquel-Chevalier Curt M, Voicu PM, Fontaine M et al (2015) Creatine biosynthesis and transport in health and disease. Biochimie 119:146–165

Mercimek-Mahmutoglu S, Salomons GS (2009) Creatine deficiency syndromes. Genereviews. ISSN: 2372-0697. https://www.ncbi.nlm.nih.gov/books/NBK3794/. Accessed 31 Oct 2016

Mercimek-Mahmutoglu S, Pop A, Kanhai W et al (2016) A pilot study to estimate incidence of guanidinoacetate methyltransferase deficiency in newborns by direct sequencing of the GAMT gene. Gene 575(1):127–131

Pasquali M, Schwarz E, Jensen M, Yuzyuk T, Debiase I, Randall H, Longo N (2014) Feasibility of newborn screening for guanidinoacetate methyltransferase (GAMT) deficiency. J Inherit Metab Dis 37(2):231–236

Pitt JJ, Tzanakos N, Nguyen T (2014) Newborn screening for guanidinoacetate methyl transferase deficiency. Mol Genet Metab 111(3):303–304

Stöckler-Ipsiroglu S, Isbrandt D, Hanefeld F, Schmidt B, von Figura K (1996) Guanidinoacetate methyltransferase deficiency: the first inborn error of creatine metabolism in man. Am J Hum Genet 58(5):914–922

Verhoeven NM, Roos B, Struys EA, Salomons GS, Van der Knaap MS, Jakobs C (2004) Enzyme assay for diagnosis of guanidinoacetate methyltransferase deficiency. Clin Chem 50(2):441–443

Wallimann T, Tokarska-Schlattner M, Schlattner U (2011) The creatine kinase system and pleiotropic effects of creatine. Amino Acids 40(5):1271–1296

Wyss M, Kaddurah-Daouk R (2000) Creatine and creatinine metabolism. Physiol Rev 80(3):1107–1213