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An accurate stable isotope dilution gas chromatographic–mass spectrometric approach to the diagnosis of guanidinoacetate methyltransferase deficiency

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Abstract

A gas chromatography–mass spectrometry (GC–MS) method is described for the quantification of guanidinoacetate in different body fluids, using a two step derivatisation procedure which involves a reaction with hexafluoroacetylacetone to form a bis(trifluoromethyl)pyrimidine ring structure followed by a reaction with pentafluorobenzyl bromide. $^{13}C_2$-labelled guanidinoacetate is used as an internal standard. Bis(trifluoromethyl)pyrimidine pentafluorobenzyl derivatives were separated on a polar capillary GC-column and were quantified using negative chemical ionisation mass fragmentography. The detection limit of the method is 1 pmol guanidinoacetate in a 100 μl sample. Control values were obtained for urine (53.9 ± 25.9 mmol mol^{-1} creatinine), plasma (1.08 ± 0.31 μmol l^{-1}), cerebrospinal fluid (CSF) (0.114 ± 0.068 μmol l^{-1}) and amniotic fluid (3.44 ± 0.64 μmol l^{-1}). The applicability of the method is illustrated by the determination of guanidinoacetate in urine, plasma and CSF of a patient affected with guanidinoacetate methyltransferase deficiency. In all body fluids of this patient, guanidinoacetate was highly elevated.

Keywords: Guanidinoacetate; $^{13}C_2$-guanidinoacetate; GAMT-deficiency; GC–MS; Stable isotope dilution; Control values

1. Introduction

Four years ago, a new defect in the biosynthesis of creatine was discovered. The underlying biochemical feature of this disease is a deficiency of the enzyme guanidinoacetate methyltransferase (GAMT) [1]. Deficiency of GAMT results in a low creatine and creatinine formation. Patients clinically exhibit extrapyramidal movement disor-

Abbreviations: GC–MS, Gas chromatography–mass spectrometry; NCI, Negative chemical ionization; HPLC, High performance liquid chromatography; PFB-Br, Pentafluorobenzylbromide; GAMT, Guanidinoacetate methyltransferase.

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ders, hypotonia, autism, severe epilepsy and retarded psychomotor development. The diagnosis of GAMT-deficiency can not reliably be made by the quantification of creatinine in urine and plasma alone since the levels in some cases are within the normal range. The biochemical marker for GAMT-deficiency is the precursor of creatine, guanidinoacetate (*N*-amidinoglycine, $C_3H_7N_3O_2$). As this compound cannot be metabolised, accumulation in different body fluids occurs. Therefore, quantification of guanidinoacetate in urine, plasma and CSF is a powerful diagnostic tool for GAMT-deficiency. The diagnosis can be confirmed by in vivo proton magnetic resonance spectroscopy (^{1}H-MRS) of the brain of the patient whereby a decreased creatine signal is observed. Furthermore, a deficiency of GAMT can be demonstrated in lymphocytes and cultured skin fibroblasts.

The determination of guanidinoacetate has been performed using several analytical techniques: cation-exchange chromatography combined with post-column derivatization with ninhydrin [2], thin layer chromatography followed by a Sakaguchi colorimetric detection [3] and gas chromatography–mass spectrometry (GC–MS) [4]. No stable isotope dilution GC–MS method for the quantification of guanidinoacetate has been described yet. In this paper, we present a new approach for the quantification of guanidinoacetate in different body fluids with the advantage of high selectivity, sensitivity and accuracy.

2. Experimental

2.1. Sample collection

Age matched control values were determined in urine, plasma and CSF samples of hospitalized children who were unaffected with a metabolic disease. The samples were stored at −25fl°C in the dark. Control values in amniotic fluid were obtained in samples taken in weeks 18–20 of pregnancy. The reason for amniocentesis in all cases was advanced maternal age.

2.2. Patient

The patient affected with GAMT-deficiency was diagnosed on the basis of elevated concentrations of guanidinoacetate in urine, plasma and CSF. The diagnosis was confirmed by non detectable GAMT-activity in cultured skin fibroblasts (Dr Stöckler, Vienna, Austria). In addition, in vivo ^{1}H-MRS of the patient's brain revealed the absence of the creatine signal. Clinical and biochemical features concerning this patient will be published elsewhere.

2.3. Materials

Hexafluoroacetylacetone was obtained from Sigma (St. Louis, MO). Pentafluorobenzylbromide was purchased from Pierce (Rockford). [$^{13}C_2$]glycine (99% $^{13}C_2$) was from Isotec (Miamisburg), and both cyanamide (50 wt.% solution in water) and guanidinoacetate were from Aldrich (Milwaukee). The SGE BPX-70 analytical GC column was purchased from Bester (Amstelveen, The Netherlands). The Supelcosil LC-18S analytical reversed phase column came from Supelco (Bellafonte). All other solvents and chemicals were of analytical grade.

2.4. Synthesis $^{13}C_2$-labelled guanidinoacetate

Guanidino-[$^{13}C_2$]acetate was synthetized following the described method [5](method A) treating [$^{13}C_2$]glycine (0.50 g, 6.6 mmol) with cyanamide (11 mmol) in water (3 ml), and by the addition of two drops of concentrated ammonia. The white suspension which resulted after 5 days stirring at room temperature, was centrifuged for 5 min at 4000 rpm after which the supernatant was separated. The residue was washed with ice-cold water (three times in 5 ml) and with acetone (three times in 10 ml), and dried under a stream of nitrogen. The total yield was 0.63 g (82%). Chemical purity was checked by ^{1}H-NMR (D_2O, δ 3.76, dd, J_{CH} 140 Hz, J_{HH} 4 Hz, CH_2), ^{13}C-NMR (D_2O, δ 45.5, d, J_{CC} 53 Hz, CH_2; δ 176.6, d, J_{CC} 53 Hz, COOH) and high performance liquid chromatography (HPLC), and found to be $>98\%$. Isotopic purity of the bis(trifluoromethyl)pyrimidine pen-

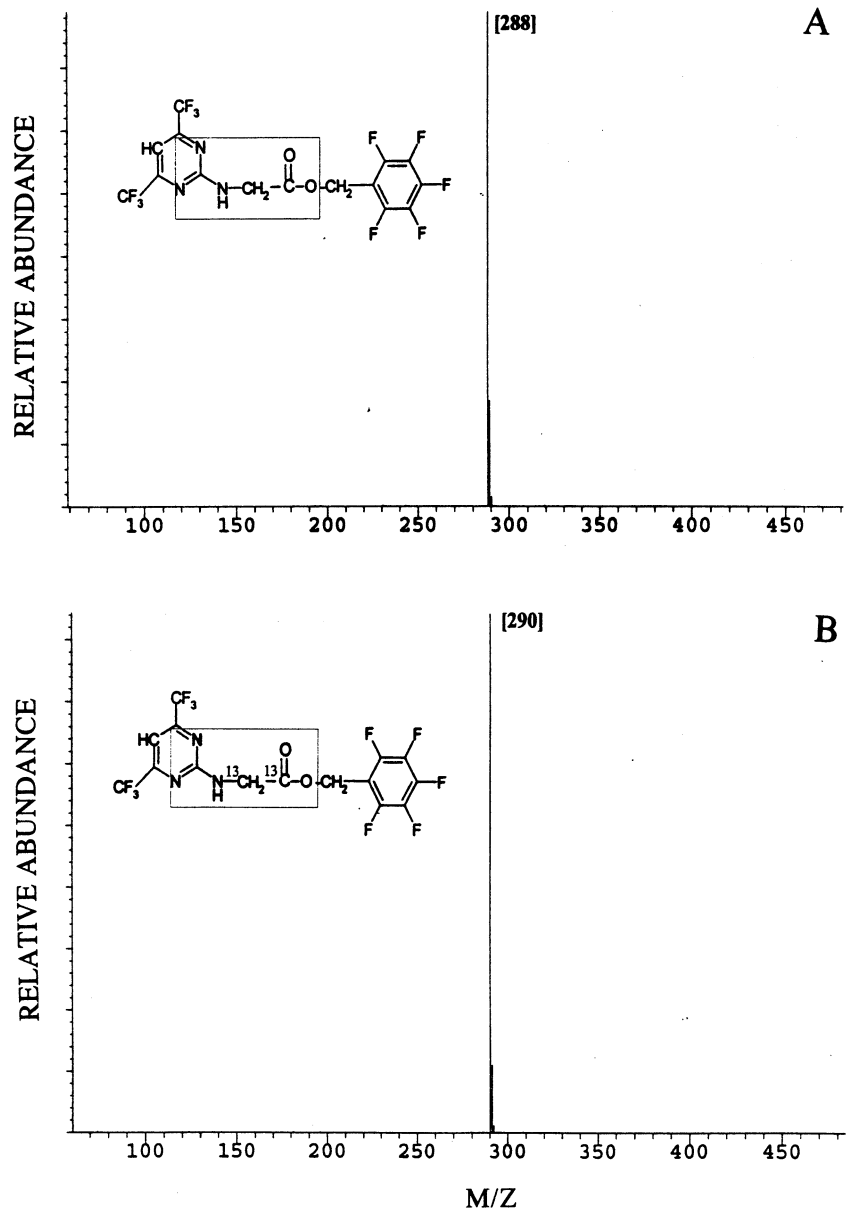

Fig. 1. NCI mass spectra of guanidinoacetate (A) and $^{13}C_2$-guanidinoacetate (B).

tafluorobenzyl derivative, determined by GC–MS was $>98\%$ $^{13}C_2$. NMR spectra were measured at 25°C on a Bruker AC 200 spectrometer (^{1}H NMR: 200.0 MHz; ^{13}C NMR: 50.3 MHz). HPLC used for quality definition of the synthesized $^{13}C_2$-guanidinoacetate was performed on a Supelcosil LC-18S (150×4.6 mm I.D.; 5 μm) analytical column. Isocratic elution was performed at ambient temperature using a flow-rate of 1 ml min^{-1}. The mobile phase consisted of a mixture of water and tri-ethylamine 999.5/0.5 (v/v). The UV-absorbance detector was set at 195 and 210 nm.

Table 1
Intra- and inter-assay variability of the described NCI GC–MS method[a]

	Urine	Plasma	CSF	Amniotic fluid
Intra-assay ($n=10$)	40.5 ± 0.7	2.51 ± 0.05	0.217 ± 0.010	3.40 ± 0.04
	$V=1.7\%$	$V=2.0\%$	$V=4.8\%$	$V=1.1\%$
Inter-assay ($n=5$)	41.6 ± 1.2	4.25 ± 0.10	0.212 ± 0.009	3.51 ± 0.05
	$V=2.9\%$	$V=2.3\%$	$V=4.4\%$	$V=1.4\%$

[a] Urine concentrations in mmol mol^{-1} creatinine; plasma, CSF and amniotic fluids concentrations in μmol l^{-1}.

2.5. Sample preparation

To a 100 μl sample, 50 μl of saturated aqueous sodium bicarbonate, 50 μl hexafluoroacetylacetone and 500 μl toluene were added. The amount of labelled internal standard added to urine samples was 12.5 nmol, to plasma and amniotic fluid samples 0.25 nmol and to CSF samples 0.1 nmol. The mixture was heated to 80°C for 2 h under continuous stirring and allowed to cool. From the upper toluene phase, 100 μl was transferred to another test tube and blown to dryness with nitrogen. Pentafluorobenzyl (PFB) derivatives were formed by treating the residue with 10 μl triethylamine and 100 μl 7% pentafluorobenzyl bromide in acetonitrile (v/v) at room temperature for 15 min. After adding 200 μl 0.5 N HCl, the formed derivatives were extracted with 1 ml hexane. From this hexane extract 1 μl was injected into the GC–MS. Calibration curves were established by carrying various amounts of guanidinoacetate and constant amounts of internal standard through the entire procedure. The observed ratios of standards towards internal standard were used for linear regression analysis. The concentration of guanidinoacetate in a sample was obtained by interpolation of the observed ratio.

2.6. Gas chromatography and mass spectrometry conditions

GC–MS analyses were performed using a Hewlett Packard 5890 GC connected to a Hewlett Packard mass spectrometer type Engine. Chromatographic separation was achieved on a SGE BPX-70 (25 m × 0.32 μm I.D., film thickness 0.25 μm) capillary column, coated with a very polar phase. Samples were injected splitless at a temperature of 300°C. The initial oven temperature was maintained at 100°C for 1 min, followed by a ramp of 15°C min^{-1} to 260°C. The temperature of the transfer line to the mass spectrometer was set at 300°C. The column was inserted directly into the ion source which was set at 250°C. The quadrupole temperature was 150°C. Ammonia was used as moderating gas at an optimized gas pressure. The mass spectrometer was operated under electron capture negative chemical ionization in the single ion monitoring mode. The negative ions measured for guanidinoacetate were: m/z −288.13 (endogenic guanidinoacetate) and m/z −290.13 ($^{13}C_2$-guanidinoacetate).

3. Results

3.1. Negative chemical ionization mass spectra of guanidinoacetate and $^{13}C_2$-guanidinoacetate

The use of PFB-derivatives under NCI-conditions results in the release of the PFB-group yielding a predominant $[M\text{-}PFB]^-$ fragment. As can be seen in Fig. 1, the mass spectrum of guanidinoacetate only shows a mass fragment m/z −288.13, which arises from (M-PFB). The mass spectrum of $^{13}C_2$-guanidinoacetate only reveals a mass fragment m/z −290.13, which differs, because of the two ^{13}C-carbons, two mass units from the non-labelled compound.

3.2. Standard curves of guanidinoacetate

For urine samples, the linearity was determined over a range from 5 to 100 nmol absolute amount

Table 2
Control ($n = 10$) and patient ($n = 1$) values of guanidinoacetate in different body fluids (average $\pm$ standard deviation (range))[a]

	Urine	Plasma	CSF	Amniotic fluid
Controls	53.9 $\pm$ 25.9 (10.3–98.8)	1.08 $\pm$ 0.31 (0.65–1.44)	0.114 $\pm$ 0.068 (0.045–0.262)	3.44 $\pm$ 0.64 (2.72–4.40)
Patient	3597	38.4	13.7	–

[a] Urine concentrations in mmol mol^{-1} creatinine; plasma, CSF and amniotic fluid concentrations in μmol l^{-1}.

of added standard. The equation of linear regression of the analytical data was $y = (0.069 \pm 0.0004)x + (0.07 \pm 0.01)$, where y is the observed ratio and x is the absolute amount in nmol of the standard. The correlation coefficient (r) found was 0.9999. For plasma, CSF and amniotic fluid samples, the linearity was determined over a range from 0.01 to 1 nmol absolute amount of added standard. This resulted in the following linear regression of the analytical data: $y = (4.04 \pm 0.05)x + (0.03 \pm 0.01)$; $r = 0.9998$. In case the found ratio in an unknown sample was more than two times higher than the highest ratio in the calibration curve, the sample preparation was repeated whereby the sample was properly diluted.

3.3. Inter- and intra-assay analyses of the methodology

Table 1 shows the results of the inter- and intra-assay variability for the different matrices. Intra-assay variability was established by the analyses of ten identical samples in one sample preparation. For the establishment of the inter-assay variability, one sample was processed in five independent sample preparations on 5 different days.

3.4. Limit of detection and limit of quantification

The lower limit of detection (LOD) ($S/N = 5$) of guanidinoacetate in a 100 μl sample volume is 0.01 μmol l^{-1}, whereas the lower limit of quantitation (LOQ) ($S/N = 10$) is 0.02 μmol l^{-1}. Both LOD and LOQ were estimated by verifying the observed noise in a CSF sample.

3.5. Control and patient values of guanidinoacetate

Control values of guanidinoacetate in urine ranged from 10.3 to 98.8 mmol mol^{-1} of creatinine with an average of 53.9 mmol mol^{-1} of creatinine. In plasma and CSF, the average control values were much lower; 1.08 μmol l^{-1} (0.65–1.44 μmol l^{-1}) and 0.114 μmol l^{-1} (0.045–0.262 μmol l^{-1}), respectively. In CSF, no gradient following sample taking was observed. Control values of guanidinoacetate in amniotic fluid ranged from 2.72 to 4.40 μmol l^{-1} with an average of 3.44 μmol l^{-1}. Table 2 shows the concentrations of guanidinoacetate in the different body fluids of our patient. The highly elevated levels of guanidinoacetate in the body fluids from the patient led to the diagnosis of GAMT-deficiency in this patient. The absence of a creatine signal using ^{1}H-MRS of the brain and the non-detectable GAMT activity confirmed the diagnosis. Fig. 2 shows the mass fragmentogram of the guanidinoacetate-derivative in a urine sample of our patient. We had to dilute the urine sample 20-fold prior to processing the sample. As the labelled internal standard consists of two ^{13}C-carbons, no retention shift is observed; therefore, both the endogenic guanidinoacetate and the labelled internal standard have exactly the same retention time.

4. Discussion

In patients suffering from GAMT-deficiency, the biosynthesis of creatine and creatinine is disturbed, and as a consequence guanidinoacetate, as

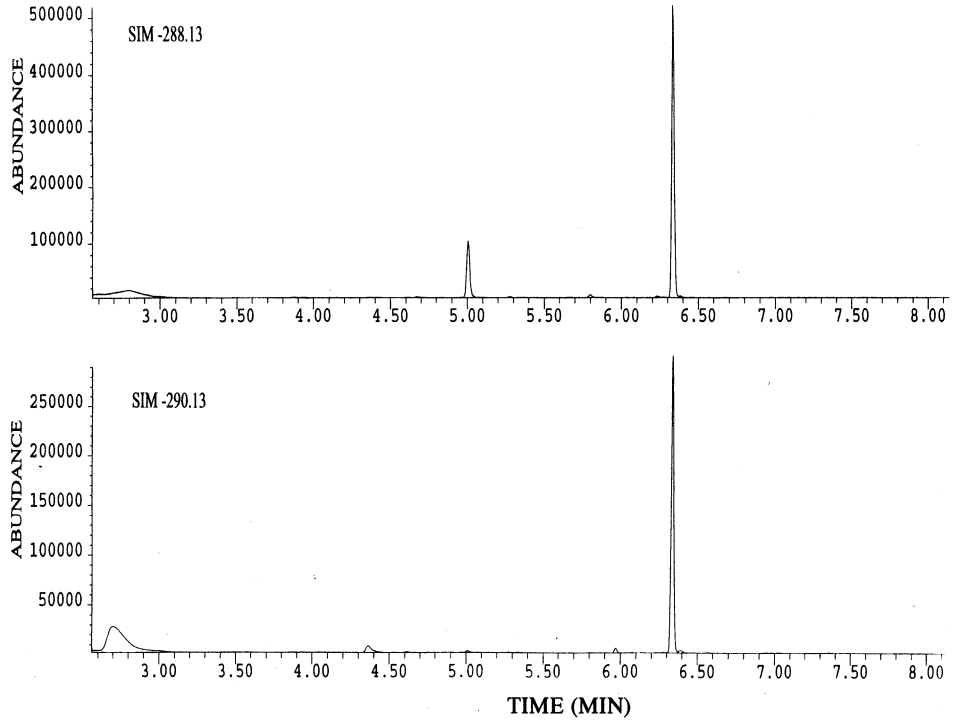

Fig. 2. NCI mass fragmentogram of guanidinoacetate in a 20-fold diluted urine sample of our patient. Upper trace shows the derivative of endogenic guanidinoacetate; lower trace shows the derivative of $^{13}C_2$-guanidinoacetate (both eluting at a retention time of 6.35 min).

a precursor, accumulates. Levels of amino acids and organic acids in urine are usually related to creatinine. Thus, a general elevation of these levels in urine samples of patients suffering from GAMT-deficiency will be found, pointing in the direction of a lowered creatinine excretion caused by GAMT-deficiency. The suspicion of GAMT-deficiency can be confirmed by the quantification of guanidinoacetate in urine, plasma and CSF. We have established a new GC–MS method for the quantification of guanidinoacetate in different body fluids. The method is a step forward with respect to simplicity and precision due to the use of a PFB-derivative which enables selective and sensitive measurements, and the use of a labelled internal standard. The PFB-derivative introduces no second isotope in contrast with the trimethylsilyl derivative used by others [4]. Due to the selective derivatisation of the cyanamide-group of guanidinoacetate with hexafluoroacetylacetone, the mass fragmentograms are relatively free of other components, particularly in the case of urine samples. The use of a labelled internal standard has the advantage that the reaction of guanidinoacetate with hexafluoroacetylacetone does not necessarily has to be complete as both standard and internal standard behave in the same way during derivatization. This allows shorter derivatization time: 2 h versus 24 h without a loss of accuracy. Both intra- and inter-assay variability for guanidinoacetate in the different body fluids indicate that the presented method has a good accuracy and sensitivity. The sensitivity of this method allows the use of only a 100 μl sample for all of the examined body fluids, which is important in the case of plasma and CSF samples due to their limited availability. Control values obtained using our NCI GC–MS method correlate well with control values described by others as can be seen in Table 3. In many inherited metabolic disorders, the measurement of a characteristic metabolite can be used for prenatal diag-

Table 3
Comparison of guanidinoacetate control values determined by NCI GC–MS with control values described by others[a]

Reference	Analytical method	Urine	Plasma	CSF	Amniotic fluid
[4]	GC–MS	17–116	–	–	–
[6]	LC-fluorescence	25–100	0.83 ± 0.31	0.055 ± 0.032	–
[7]	LC-fluorescence	28–98	0.77 ± 0.14	–	2.56 ± 0.40
	NCI GC–MS	10–99	1.08 ± 0.31	0.114 ± 0.068	3.44 ± 0.64

[a] Urine concentrations in mmol mol^{-1} creatinine; plasma, CSF and amniotic fluid concentrations in μmol l^{-1}.

nosis [8]. The here presented method might potentially be used for the prenatal diagnosis of GAMT-deficiency by the quantification of guanidinoacetate in amniotic fluid.

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