Short communication

Automated high-performance liquid chromatographic method for the determination of guanidinoacetic acid in dried blood spots: a tool for early diagnosis of guanidinoacetate methyltransferase deficiency

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Abstract

A new automated method for the assay of guanidinoacetic acid (GAA) in dried blood spot (DBS) on filter paper is reported. The method, based on reversed-phase (RP)-HPLC, precolumn derivatisation with benzoin and fluorescence detection, has shown good precision and sensitivity and requires only minimal sample handling. The validity of the method was demonstrated by analysing the neonatal blood spot of a patient affected by guanidinoacetate methyltransferase (GAMT) deficiency. GAA concentration was found to be nearly 12-fold higher than the mean control value. We propose this method as an inexpensive and widely applicable tool for the diagnosis of GAMT deficiency.   © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Guanidinoacetate methyltransferase (GAMT, EC 2.1.1.2) catalyses the biosynthesis of creatine by the transfer of a methyl group from *S*-adenosylmethionine to guanidinoacetic acid (GAA) [1]. GAMT deficiency is a recently discovered inborn error of creatine metabolism [2]. The first case was described in 1994 and so far only a few patients have been reported in the literature [2–6]. The disease is characterised by creatine depletion and GAA accumulation in the nervous system, which can be detected by in vivo proton magnetic resonance spectroscopy (^{1}H-MRS) [2–6]. Since the clinical symptoms of the disease (early onset epilepsy, neurological deterioration, movement disorder and mental retardation) [6] are quite common in paediatric neurology, a large number of patients could be considered as candidates for the diagnosis of this rare disorder. The clinical improvement seen after creatine monohydrate replacement therapy [7] suggests that early diagnosis and treatment can

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prevent the irreversible neurological damage of this metabolic disease, improving the prognosis. Therefore a fast, specific and inexpensive diagnostic tool is required to assess a large number of patients. The determination of the concentration of GAA in biological fluids, whose increase is a marker of the disease [8], could meet these criteria.

Our aim was therefore to develop a new automated method for the assay of GAA in dried blood spot (DBS) on filter paper. DBS, a universally used type of sample for neonatal screening, has the advantage of being easily storable and transportable.

Several methods for the determination of GAA (alone or in association with other guanidino compounds) in biological fluids have been previously described. These methods are based on liquid chromatography with post-column derivatisation with ninhydrin [8–12], thin layer chromatography followed by a Sakaguchi colorimetric detection [13], liquid chromatography–mass spectrometry [14], gas chromatography–mass spectrometry [15,16] and liquid chromatography with pre-column derivatisation with benzoin [17,18]. Only a few of these methods were tested for their application in the diagnosis of GAMT deficiency [8,13,15,16]. Here we describe, for the first time, a method for the determination of GAA in DBS. This method, easily applicable to routine analysis, is based on automated pre-column derivatisation with benzoin, RP-HPLC and fluorimetric detection.

2. Experimental

2.1. Materials

GAA and sodium sulfite salts were obtained from Sigma (St. Louis, MO, USA), benzoin and 2-mercapoethanol (2-MCE) were obtained from Fluka (Buchs, Switzerland), 2-methoxyethanol was obtained from Aldrich (Sigma–Aldrich, Steinheim, Germany) and glacial acetic acid, sodium acetate, potassium hydroxide, methanol (HPLC gradient grade) were from Merck (Darmstadt, Germany). Ultrafree-MC 5000 mass cut-off filters were purchased from Millipore (Bedford, MA, USA). Phosphate buffer solution (PBS) 1× was purchased from HyClone (Cramlington, NE, USA). All aqueous solutions were prepared using highly purified water, produced by a Millipore Milli-RO/Milli-Q system (Millipore, Bedford, MA, USA). The analytical system was composed of two pumps model 2248 and a controller model 2252 (Pharmacia Biotech, Brussels, Belgium), a Croco-Cil column oven (Cil-Cluseau, Paris, France) and a FP1520 fluorescence detector (Jasco, Tokyo, Japan). The derivatisation procedure was accomplished by an ASPEC XL sample processor (Gilson, Villiers-le-Bel, France) equipped with racks maintained at different temperatures: the first rack, in which samples were loaded, was at room temperature, the second rack, in which the derivatisation reaction occurs, was electrically heated at 100°C and the third rack, used to quickly cool samples before the injection into HPLC system, was maintained at 1°C using a F20CR05E refrigerated circulator (Techne, Duxford, Cambridge). The column was a TSK gel Super-ODS 100×4.6 mm I.D., 2 μm (TosoHaas, Montgomeryville, PA, USA), in conjunction with an ODS Hypersil 20×4 mm I.D. guard column (Hewlett-Packard, Waldbronn, Germany). Data were stored and processed using HP ChemStation Chromatographic software. Filter paper used for dried blood spots was Schleicher & Schull (Dassel,Germany) grade 2992.

2.2. Methods

GAA was eluted in 200 μl of PBS 1×, pH 7.4, from 6-mm diameter DBS by sonication for 30 min at room temperature. The volume of whole blood in a 6-mm diameter DBS was calculated from the data of Hill et al. [19] and Adam et al. [20] to be 7.8±0.8 μl. The eluate was then ultrafiltered to remove the proteins using Ultrafree-MC filters at 5000 g for 20 min. The filtrate obtained was subjected to a complete automatic procedure of derivatisation: 100 μl of sample, 50 μl of 30 mM benzoin in 2-methoxyethanol, 50 μl of an aqueous solution containing 0.1 M 2-MCE and 0.2 M sodium sulfite and 100 μl of 2 M potassium hydroxide, was dispensed into a vial maintained at 1°C; after a mixing step (draw and eject 300 μl twice) the mixture was dispensed into a vial maintained at 100°C and heated for 15 min; the mixture was then dispensed into a vial maintained at 1°C and cooled for 1 min; then 40 μl of 5 M acetic acid were added and mixed (draw and eject 340 μl

twice) and 20 μl of the final mixture were injected onto HPLC.

The separation of GAA was obtained in 23 min using a flow-rate of 0.8 ml/min and the following gradient conditions: the initial mobile phase consisted of 40% B and 60% A, buffer B was increased to 50% in 5 min, maintained constant at 50% for 15 min, increased to 100% in 1 min, maintained constant at 100% for 3 min and decreased to 40% in 1 min. Equilibration time between injections was 4 min. Mobile phase A was 20 mM sodium acetate buffer, pH 7.35, and mobile phase B was 20% (v/v) 100 mM sodium acetate buffer, pH 7.35, and 80% v/v methanol. Column temperature was 38°C. The excitation and emission wavelengths were, respectively, 325 and 435 nm.

3. Results

A good separation of GAA from other guanidino compounds and interfering arginyl-containing oligopeptides was achieved in 23 min (25 min including column regeneration). A chromatogram obtained from a standard solution containing the most important biogenic guanidino compounds (guanidinosuccinic acid (GSA), creatine (CT), creatinine (CTN), guanidinoacetic acid (GAA), β-guanidinopropionic acid, argininic acid, α-N-acetylarginine, γ-guanidinobuturic acid, arginine, homoarginine, guanidine, methylguanidine) at a concentration of 0.8 μM each, except for CT and CTN at 40 μM, is shown in Fig. 1A. Whereas the peaks of GSA, CT+CTN and GAA are shown in the chromatogram, the other guanidino compounds elute later (therefore they are not shown in Fig. 1A) and do not interfere with the GAA analysis. Fig. 1B shows a chromatogram obtained from a DBS of a normal neonate.

The linear correlation between peak areas and concentrations was assessed in the range 0.02–2 μM using six different concentrations (0.02, 0.04, 0.08, 0.4, 0.8, 2 μM) in triplicate. The correlation coefficient for GAA was 0.999 and the equation was rsp$=1.27\times10^{4}$ (amt)-167 (intercept standard error: 98.5, slope standard error: 244.9). The detection limit was 46 fmol with a signal-to-noise ratio of 3.

The precision of the derivatisation and chromatog-

raphy analysis (without considering DBS extraction) was assessed using ten consecutive analyses of a standard solution at a concentration of 0.08 μM and the area relative standard deviation (RSD) obtained was 3.9%. The within-run precision was evaluated by performing ten analyses of a DBS sample (mean absolute value was 0.26 μM) on the same day and the RSD obtained was 6.7%. The between-run precision was assessed by performing ten analyses of the same DBS sample stored at 5°C on different days within 2 weeks and the RSD obtained was 9.6% (Table 1).

Recovery was evaluated by comparing spiked and unspiked blood spot controls prepared from the same whole blood pool. Human blood containing heparin and intact red cells was divided into four portions for enrichment with GAA to obtain added concentrations of 20, 10, 5 and 2 μmol/l of blood. The whole-blood pools were dispensed in 30-μl portions (spot size similar to those of the newborn-screening samples) onto filter paper from which 6-mm spots were punched. The samples obtained were analysed in triplicate and the mean recovery obtained was 94±9%. In order to determine the reference values, 32 DBS samples from neonatal screening (2–4 days old, mean birth weight 3391±462 g, 12 subjects breast-fed) were analysed. Whole blood from finger or heel puncture was spotted on filter paper, air dried at room temperature and stored at 5°C. The mean value of GAA was 1.2±0.3 μmol/l of blood.

The validity of the method was demonstrated by analysing the blood spot of a patient affected by GAMT deficiency, diagnosed at 4 years old and already described by our group [6] (Fig. 1C). The sample was collected though a neonatal screening program and stored at room temperature for 4 years and 6 months. At the time of the sampling, the patient was 3 days old and was breast-fed. The concentration of GAA was found to be extremely high (14.0 μmol/l of blood, reference values 1.2±0.3 μmol/l of blood).

4. Discussion

The determination of GAA has been performed by several analytical methods [8–18], among which cation-exchange chromatography with post-column
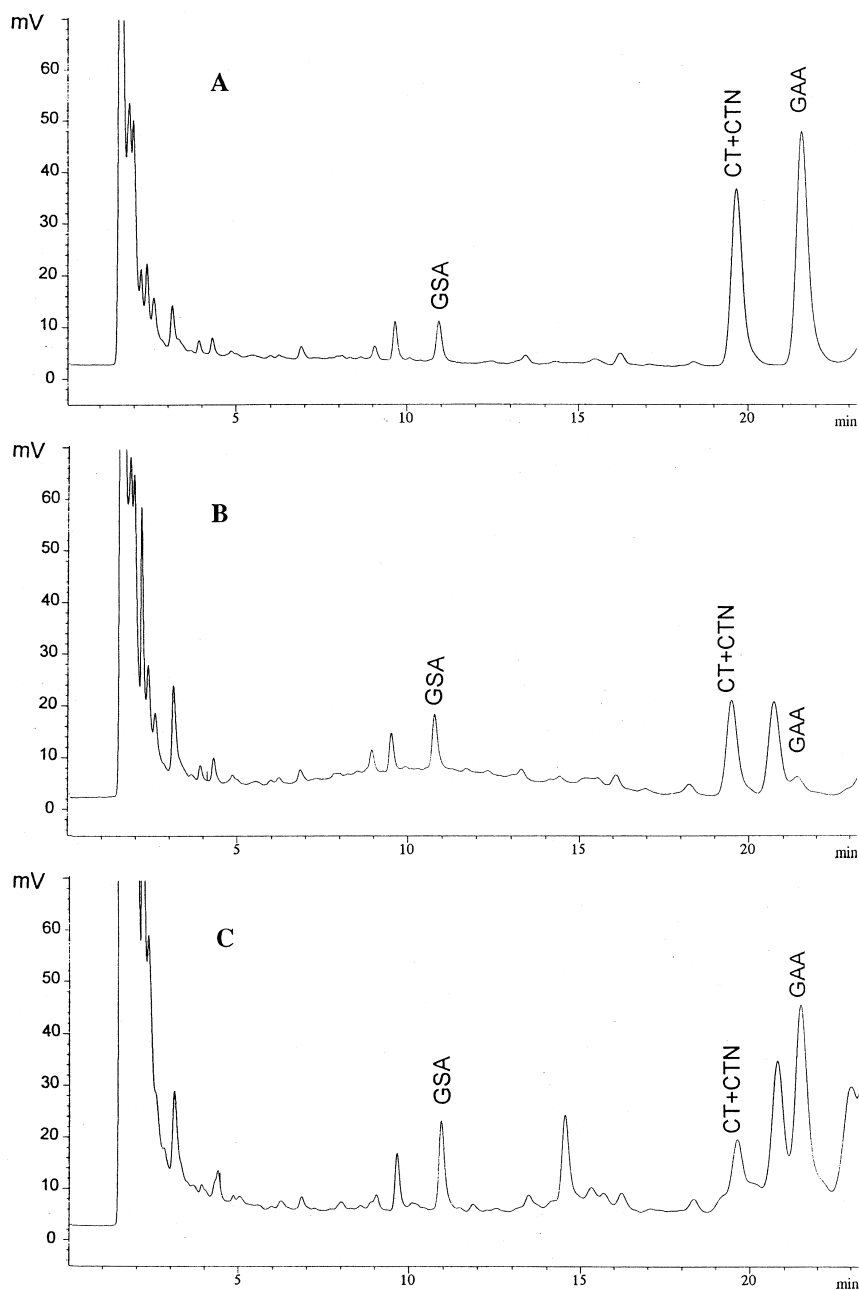
Fig. 1. (A) Chromatogram of a standard solution containing 12 guanidino compounds including GAA, CT and CTN at a concentration of 0.8 μM each, except for CT and CTN at 40 μM. (B) Chromatogram of a dried blood spot of a normal neonate (GAA 1.01 μmol/l of blood). (C) Chromatogram of a dried blood spot of a patient affected by GAMT deficiency obtained in neonatal period (GAA 14.0 μmol/l of blood). The GAA concentration is nearly 12-fold higher than mean control value.

Table 1
Precision of the derivatisation and chromatography analysis[a]

	Repeatability of peak areas	Within-run (μM)	Between-run (μM)
	1053	0.25	0.25
	986	0.26	0.26
	1032	0.27	0.27
	1008	0.23	0.23
	972	0.28	0.30
	1105	0.27	0.28
	1024	0.29	0.31
	998	0.28	0.29
	1047	0.25	0.24
	982	0.27	0.28
Mean	1020.7	0.27	0.27
SD	40.45	0.018	0.026
RSD (%)	3.9	6.7	9.6

[a] The precision of the method was evaluated by analysing a 0.8 μM standard solution. Areas of GAA peak are reported. The within-run precision was assessed by performing ten analyses of a DBS sample in the same day. The between-run precision was assessed by performing ten analyses of the same DBS sample stored at 5°C on different days over 2 weeks.

derivatisation with ninhydrin has been widely used. Since the sensitivity of the on-line post-column derivatisation was limited by the dilution of HPLC column eluate with the reagent solution, we decided to use benzoin, the unique reagent reported for pre-column derivatisation [17–21], to achieve the maximum sensitivity (fmol level). The fluorescent derivatives of guanidino compounds by benzoin reaction in an aqueous 2-methoxyethanol/potassium hydroxide solution, correspond to the chemical structures of 2-substituted amino-4,5-diphenylimidazoles [22] and can be separated using RP-HPLC. Different concentrations of reagent solutions and different conditions of reaction were tested to establish the optimum conditions and to set up a completely automated procedure. Although the automation allows a greater throughput, the procedure can theoretically be manually performed.

The diagnosis of GAMT deficiency is based on the detection of brain creatine depletion and GAA increase by in vivo ^{1}H-MRS [2–6]. This is a non-invasive but expensive procedure, requiring sedation in non-cooperative patients. The determination of GAA in DBS, described here, provides clinicians with a fast, simple, inexpensive alternative for the diagnosis of GAMT deficiency. Furthermore our

method allows the determination of CT+CTN. GAMT deficiency causes a depletion of CT in biological fluids and in tissues but, even if CTN excretion measured in affected patients' 24-h urine is lower than the controls, the CTN concentration in randomly collected urine and plasma is not always different from control values [5]. The quantitation of creatine and creatinine, even if it cannot be considered a reliable tool for the diagnosis of GAMT deficiency, increases the diagnostic impact of the method and is useful for monitoring the therapy.

The method has shown good precision and sensitivity and requires only minimal sample handling. Furthermore the samples can be mailed to the laboratory, thus allowing the paediatrician easy access to the test. It enabled us to show that blood GAA increases from the first days of life in GAMT deficiency (Fig. 1C).

In conclusion the method we have set up allows systematic screening of symptomatic subjects, whose clinical condition suggests GAMT deficiency. Finally, it promises to be a candidate method for pre-symptomatic neonatal diagnosis, provided the frequency of this treatable disease can be proved to warrant a neonatal screening program.

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