CREATINE METABOLISM: DETECTION OF CREATINE AND GUANIDINOACETATE IN SALIVA OF HEALTHY SUBJECTS

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ABSTRACT

Creatine (Cr) plays an important role in storage and transmission of phosphate-bound energy. Cerebral creatine deficiency syndromes comprise three inherited defects in Cr biosynthesis and transport. The aim of this study was to investigate whether Cr and Guanidinoacetate (GAA) can be detected in saliva of healthy subjects and to establish the relationship between salivary and plasma levels of these molecules. An adapted gas chromatography (GC) method is described for the quantification of Cr and GAA biomarkers in saliva. Reference values were established for GAA and Cr in saliva. These values were age dependent (p= 0.001). No difference between genders was observed. We detected a difference between GAA and Cr concentrations in saliva and in plasma. The GC method for simultaneous determination of GAA and Cr in human saliva is fast, reliable, sensitive, non-invasive and precise to use as a biochemical approach in early detection of cerebral creatine deficiency syndromes.

Key words: Creatine deficiency, biomarkers, saliva, brain, gas chromatography.

METABOLISMO DE CREATINA: DETECCIÓN DE CREATINA Y GUANIDINOACETATO EN SALIVA DE SUJETOS SANOS

RESUMEN

La creatina (Cr) juega un importante rol en el almacenamiento y el transporte de energía unida al fosfato. Los síndromes de deficiencia de creatina cerebral comprenden tres defectos genéticos en la biosíntesis y transporte de creatina. Es propósito de este estudio investigar si el guanidinoacetato (GAA) y la Cr pueden ser detectados en saliva de sujetos sanos e investigar la relación entre los valores de GAA y Cr en saliva con los niveles en plasma de estas moléculas. Se describe un método modificado de cromatografía gaseosa para la cuantificación de los biomarca-dores, Cr y GAA en este biofluído. Se establecieron valores de referencia para GAA y Cr. Estos valores dependen de la edad (P=0.001). No se observaron diferencias entre género. Se detectó una diferencia entre la concentración de GAA y Cr en saliva con respecto al plasma. El método adaptado de cromatografía gaseosa para la determinación simultánea de GAA y Cr en saliva humana es fácil, seguro, sensible, no invasivo y preciso para utilizar como aproximación bioquímica en la detección temprana de los síndromes de deficiencia de creatina cerebral.

Palabras clave: deficiencia de creatina, biomarcadores, saliva, cerebro, cromatografía gaseosa.

INTRODUCTION

The creatine (Cr)/phosphocreatine (PCr) creatine kinase (CK) system plays essential roles, maintaining the high energy levels necessary for the development and functions of the central nervous system by regenerating ATP and buffering ATP levels[1,2]. In humans, the Cr/PCr pool is maintained by endogenous biosynthesis and nutritional intake of Cr. Cr biosynthesis is a two-step process occurring mainly in kidney, pancreas and liver[3]. In the first step, L-arginine: glycine amidinotransferase synthesizes guanidinoacetate (GAA) from arginine and glycine. In the second step, guanidinoacetate methyltransferase methylates GAA to form Cr. The methyl group donor is S-adenosylmethionine, which is subsequently converted to S-adenosyl homocysteine. Finally, Cr is distributed and actively taken up by different via the Cr transporter (CRT, encoded by *SLC6A8*)[4] (Fig.1).

Of the three cerebral creatine deficiency syndromes (CCDS), L-arginine: glycine amidinotransferase (AGAT) deficiency and guanidinoacetate N-methyltransferase (GAMT) deficiency are caused by defects in the enzymes, whereas CRT deficiency results from the defect in CRT caused by *SLC6A8* defect. Cr is found in blood and cerebrospinal fluid, but cannot enter brain cells, as the cell membranes are an effective barrier to Cr transport[5]. The clinical
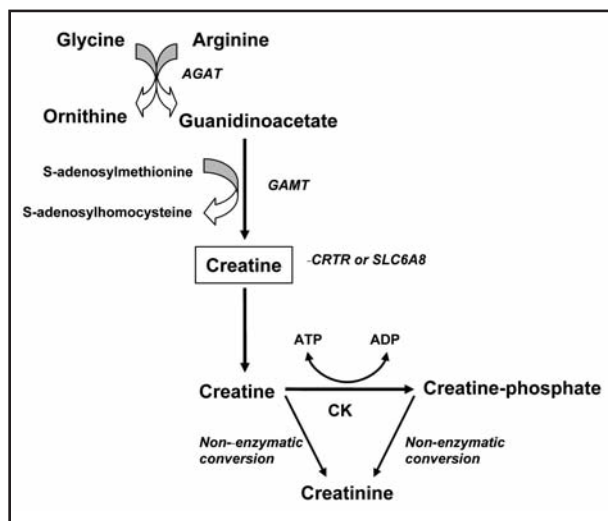
Fig. 1: Metabolic pathway of creatine/creatine-phosphate. AGAT: arginine:glycine amidinotransferase; GAMT: guanidinoacetate methyltransferase; CRTR: creatine transporter; CK: creatine kinase.

presentation of all CCDS patients is characterized by developmental delay/arrest, mental retardation, language delay and autism spectrum disorders[6]. Proton magnetic resonance spectroscopy (MRS) of affected patients shows absence or decrease of the Cr peak[7, 8].

Saliva is a biofluid that is useful for novel approaches to prognosis, laboratory or clinical diagnosis, and monitoring and management of patients with oral disorders such as caries and periodontal disorders, and systemic diseases of different etiologies, including AIDS, systemic lupus erythematosus, Sjogren´s syndrome, hepatitis A, B and C, rheumatoid arthritis, Type II diabetes, cystic fibrosis, hormonal dysfunctions, oral cancer, neurological and cardiovascular disorders[9] and in some lysosomal storage disorders diagnosed at the Center for the Study of Inherited of Metabolic Diseases, Córdoba, Argentina (Cemeco) [10]. It is easily collected and stored and ideal for early detection of disease because it contains specific soluble biomarkers. Saliva contains multiple biomarkers which make it useful for multiplexed assays that are being developed such as point-of-care devices, rapid tests, or in more standardized formats for centralized clinical laboratory operations. Recent studies indicate coexistence of total salivary proteins and their mRNAs, which can be used as proteomic and genomic biomarkers of specific disorders, such as oral cancer[11]. Progress in the use of saliva as a diagnostic biofluid has been remarkably influenced by biotechnological progress, the analysis of small samples (microfluidic), its high sensitivity and specificity, ease of use and low cost[12]. In addition, the values of serum components can be measured and compared through saliva[13]. The aim of this study was to determine whether Cr and GAA can be detected in saliva of healthy subjects, and to investigate the relationship between salivary and plasma levels of these molecules compared to our reference values. We found that saliva can be used as a biological indicator to facilitate the recognition of CCDS in our environment, with the advantages of using a non-invasive, readily available method.

MATERIALS AND METHODS
Sample collection
Control values were determined in saliva samples from individuals (age range: male: 2 - 48, female: 3 - 67) with no metabolic, renal or neurologic disorder. A sample of unstimulated whole saliva (+/- 0.3 ml) was collected from the mouth under the tongue with a disposable Pasteur pipette. The saliva was obtained 60 minutes after mouth cleansing and before breakfast from 97 subjects (48 male and 49 female). Subjects were recruited from healthy volunteers at Hospital de Niños Córdoba, Argentina. Following collection, the saliva samples were immediately placed on ice and stored at -20°C. Blood samples were collected by venipuncture into heparin tubes and immediately centrifuged to separate blood components. Plasma supernatants were transferred by cryotubes for storage and frozen at -20°C.

This study was approved by the Institutional Committee of Health Research Ethics, CIES, of Polo Hospitalario (Hospital de Niños de la Santísima Trinidad, Nuevo Hospital San Roque, Hospital Rawson), Argentina. Informed consents were signed by the study population.

Sample preparation
The method mainly follows Struy et al.[14], with modification. Fifty µl saturated aqueous sodium bicarbonate, 50 µl hexafluoroacetylacetone and 500 µl toluene were added to 100 µl saliva or plasma. The mixture was heated to 80°C for 12h. From the upper toluene phase, 400 µl were transferred to a clean vial and dried under nitrogen flow at room

temperature. Pentafluorobenzyl (PFB) derivates were formed by treating the residue with 10 µl triethylamine and 100 µl 7% pentafluorobenzyl bromide in acetonitrile (v/v) at room temperature for 30 min. After adding 200 µl 0.5 N HCl, the derivatives formed were extracted with 0.5 ml hexane and then analyzed by GC.

Standard curves
Calibration curves were established with different concentrations of Cr (2.5- 180 µmol /L) and GAA (0- 35 µmol/L) and a constant amount of internal standard β-guanidinopropionic acid (254 µmol/L). The linear regression equations derived from calibration curves were used for calculating Cr and GAA concentrations in saliva and plasma samples.

Gas chromatography
The samples were analyzed in a Hewlett Packard gas chromatograph 5890 series II. Chromatographic separation was achieved on a DB-35 MS capillary column, 30 mm in length, 0.25 mm in internal diameter and 0.25 µm in film thickness. One microliter of each sample was injected in the splitless mode. Injector temperature was 250 °C. Initial oven temperature was 110 °C, followed by a ramp up to 152ºC at 3°C/min, then by a ramp up to 280ºC at 70°C/min. Helium was used as the carrier gas at an initial pressure of 20 psi, constant flow.

Statistical analysis
The population was initially subdivided into two age-related subgroups (*GAA: 0-15 years (y); >15 y; Cr: 0-10 y; > 10 y*) because the concentrations of Cr and GAA in plasma are age-dependent[15]. The value followed a non-Gaussian distribution. The data for Cr and GAA concentration in saliva were analyzed with t-Student for one parameter in order to compare the average values in saliva vs. standard average values in plasma, obtained from our population; plasma value: *(GAA, 0-15 y: 0.01 to 2.8 µM; >15 y: 0.3 to 3 µM; Cr, 0-10 y: 7 to 142 µM; > 10 y: 20 to 96 µM)*. The Wilcoxon Test for independent samples was performed to compare salivary values between age ranges. Statistical significance was set at $p \leq 0.05$.

RESULTS
Salivary GAA and Cr concentrations were analyzed in the 97 samples. The method was selective and no interfering peaks were observed. Cr and GAA were expressed and detected in all saliva samples (Fig. 2 A, B).

In saliva, it was observed that the GAA value ranges for control subjects were for 0-15 years (n=48)**:** 0.1 to 13.4 µM, for > 15 years (n= 49)**:** 0.08 to 6.1 µM (Table 1). Average GAA concentration in saliva was found to be significantly higher (*p*-value= 0.000) in individuals aged 0-15 (3.94 µM) in comparison with subjects over 15 years old (2.55 µM) with regard to the upper limit value in blood per age range studied (2.8 µM in 0 to 15, and 3 µM in > 15). On the other hand, a remarkable difference was observed between age ranges in males (*p*-value = 0.0057) for concentrations of GAA in saliva within the age ranges.
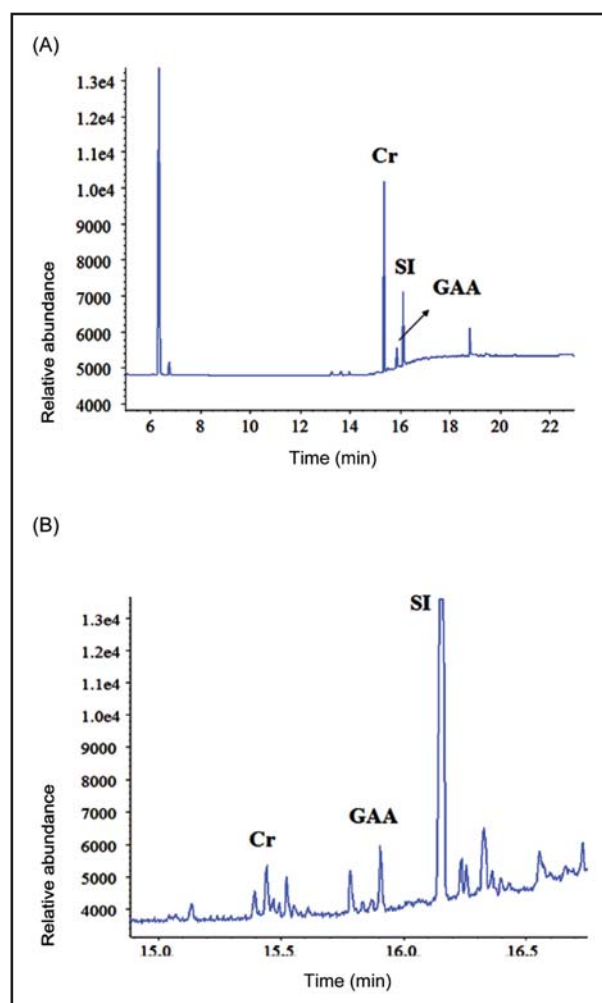

Fig. 2: Representative GC chromatograms. (A) Creatine (Cr), guanidinoacetate (GAA) standards and β-guanidinopropionic acid Internal standard (SI); (B) saliva sample from a healthy individual.

Table 1: Concentration of GAA (A) and Cr (B) in saliva of healthy adult subjects (n= 97), according to two age ranges and gender.

	Gender	Agerange (years)	n	Average	SD	Minimum	Maximum	P(25)	P(50)	P(75)
GAA µM	Female (n=48)	0-15	22	3.94	2.92	0.11	13.40	1.60	3.70	5.40
		>15	26	2.69	1.60	0.16	6.15	1.60	2.40	3.60
	Male (n=49)	0-15	26	4.02	2.16	0.67	8.70	3.10	3.40	5.20
		>15	23	2.38	1.44	0.08	5.40	1.40	2.10	3.20
Cr µM	Female (n=48)	0-10	21	8.07	2.98	1.40	14.30	6.30	8.22	9.50
		>10	27	10.67	5.07	2.44	22.60	7.60	9.40	10.70
	Male (n=49)	0-10	23	7.65	3.12	0.29	12.20	5.90	8.40	9.30
		>10	26	9.24	2.76	5.20	15.20	7.50	8.60	10.60

The range of Cr for control subjects was: for 0-10 (n=44): 0.2 to 14.3 µM for > 10 (n= 54): 2.4 to 22.6 µM (Table 1). Cr concentrations in saliva were significantly lower than standard blood parameters (p-value = 0.000) in subjects aged 0-10 (7.85 µM) and over 10 (9.98 µM) and with regard to the highest values in plasma for each age range studied (0-10: 142 µM and 96 µMin > 10). On the other hand, there was a remarkable difference between age ranges in females (p-value= 0.0001) for concentrations of Cr within the age ranges.

Normal GAA and Cr ranges are very wide in different age groups, which are explained by the fact that reference values in the population do not follow normal distribution.

In saliva, GAA and Cr were found to differ according to age (p=0.001). No gender-related difference was observed for either compound (p (GAA)= 0.6874; p(Cr)= 0.2620).

DISCUSSION

So far, several methods have been developed to measure Cr and GAA in plasma, urine and CSF [16-18]. We have described a modified method to measure GAA and Cr in saliva by gas chromatography[14]. According to our results, this technique may be suitable for analyzing GAA and Cr in saliva, and could therefore be used to study Cr biosynthesis and its relation to diseases associated with altered Cr metabolism. These findings suggest that the detection of Cr and GAA in saliva may be used as a noninvasive, safe, inexpensive tool for diagnosing Cr genetic disorders. We have not found any recently published data on GAA and Cr concentration or total GAA and Cr content in saliva. In AGAT deficiency, low Cr and GAA levels are found in plasma, urine and CSF whereas in GAMT deficiency, low Cr and high GAA levels are diagnosis hallmarks[19, 20]. In CRTR deficiency, Cr and GAA are normal in plasma but elevated in urine in male patients[21].

The clinical spectrum of CCDS has widened in recent years: epilepsy, mental retardation and autism have remained the principal neurological signs reported to date[2, 3]. In addition, AGAT and GAMT deficiencies are easily treatable[22]. Consequently, early diagnosis of these inherited conditions is very important.

In conclusion, paper provides reference values for metabolites in Cr metabolism (GAA, Cr) in saliva for different age groups. We also show that salivary GAA and Cr concentration is age-dependent but not under the influence of gender. In our opinion, this biochemical approach should be offered for patients with unexplained mental retardation, dysphasia, epilepsy, and /or autistic behavior. Follow-up studies by molecular analysis of the gene and/or functional tests (AGAT, GAMT activities or Cr uptake) are warranted for definition diagnosis.

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