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High-throughput capillary electrophoresis method for plasma cysteinylglycine measurement: evidences for a clinical application

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Summary. Increased levels in plasma homocysteine and cysteine, and more recently, decreased levels in cysteinylglycine have been indicated as a risk factor for vascular diseases. Most assays focused their attention only on homocysteine determination and when also other thiols were measured, analytical times drastically increased. By modifying our previous method for thiols detection, we set up a rapid capillary electrophoresis method for the selective quantification of plasma cysteinylglycine, cutting the analysis time of about 50%. Samples were treated with tri-n-butylphosphine as reducing agent, proteins were precipitated with trichloroacetic acid and released thiols were successively derivatized by the selective thiol laser-induced fluorescence-labeling agent 5-iodoacetamidofluorescein and separated by capillary electrophoresis. A baseline separation between peaks was obtained in about 2 min using 3 mmol/L sodium phosphate/2.5 mmol/L boric acid as electrolyte solution with 75 mmol/L N-methyl-D-glucamine at pH 11.25 in a 47 cm long capillary with a cartridge temperature of 45 °C. The method application was checked by measuring plasma Cys-Gly levels in a group of patients affected by retinal vein occlusion (RVO), an important cause of visual loss in the elderly. The low levels of Cys-Gly found in the RVO patients suggest that these small thiols may have importance in the disease development.

Keywords: Cysteinylglycine – Cysteine – Homocysteine – Capillary electrophoresis – Retinal vein occlusion

Abbreviations: CE, capillary electrophoresis; Cys, cysteine; Cys-Gly, cysteinylglycine; Hcy, homocysteine; 5-IAF, 5-iodoacetamidofluorescein; LIF, laser induced fluorescence; RVO, retinal vein occlusion; TBP, tri-n-butylphosphine

Introduction

Cysteine (Cys), cysteinylglycine (Cys-Gly), homocysteine (Hcy) and glutathione (GSH) are the most common plasma low-molecular-mass aminothiols involved in numerous important functions in metabolism and homeostasis. Their metabolic pathway is strongly linked to each other and although the metabolic correlations among these thiols are well-known, there are still few information on the dynamic relationships in either health or disease (Kleinman and Richie, 2000). Several studies have reported that a moderate elevation of total plasma homocysteine is associated with an increased risk of atherosclerotic vascular disease (Gerhard and Duell, 1999; Nygard et al., 1999; Aguilar et al., 2004; Kalra, 2004). The atherogenicity of Hcy may involve a variety of mechanisms in which the redox property of the sulfhydryl group is believed to play an important role (Heinecke et al., 1987; Heinecke et al., 1993; Jacobsen, 2000). Structurally similar and metabolically linked to Hcy, Cys shows some of the chemical properties of Hcy for the presence of the sulfhydryl group in the molecule (Stamler and Slivka, 1996). An increase in the total amount of this compound should be noxious, but only few studies have shown its possible and still uncertain relationship to the risk of cardiovascular disease (Jacob et al., 1999; El-Khairy et al., 2001; van den Brandhof et al., 2001; Ozkan et al., 2002). Recently it has been besides reported that also cysteinylglycine may have important funcions in the atherosclerosis pathogenesis by modulating the interaction between apoprotein B-100 and homocysteine (Zinellu et al., 2006). In particular, elevated Cys-Gly levels may act inhibiting the S-homocysteinilated LDLs formation that has been reported to be more atherogenic than native LDLs (McCully and Wilson, 1975; Ferretti et al., 2004). Moreover, it is well known that Cys-Gly is an

important precursor for glutathione biosyntesis with which it shares antioxidative and likely anti-atherogenic properties (Kugiyama et al., 1998; Lapenna et al., 1998; Morrison et al., 1999). There is a large number of methods for plasma thiols determination, such as amino acid analyzers (Gupta and Wilcken, 1978), radioenzymatic methods (Refsum et al., 1985), and several chromatographic methods by GC-MS (Kataoka et al., 1995; Sass and Endres, 1997), by HPLC with fluorimetrics (Svardal et al., 1990; Cornwell et al., 1993; Fermo et al., 1998), or with electrochemical detectors (D'Eramo et al., 1998; Martin et al., 1999) and CE methods (Causse et al., 2000; Zinellu et al., 2003). Among these methods HPLC and ELISA are the most used ones; on one hand HPLC allows simultaneous thiols measurement but it is a long procedure in term of sample preparation and separation time, on the other hand ELISA is a faster procedure but up to now only the determination of homocysteine is possible. At this moment, literature lacks of specific methods for a rapid evaluation of plasma Cys-Gly. Recently a very rapid HPLC assay that focused on Hcy, Cys and Cys-Gly measurement has been developed by Frick et al. (2003). Starting from other previous methods, these authors developed a new procedure where the analytes separation needs about 2 min and half and the entire run time is of about 6 min. Speed has become a crucial factor in research and clinical laboratories: cutting the analysis time even of half can greatly reduce costs when dealing with hundreds of samples daily. As already largely reported, methods employing capillary electrophoresis (CE) technology allow to shorten analysis time and to reduce costs as well. Therefore, starting from our previous work on the measurement of total plasma thiols (Zinellu et al., 2003), we have set up a new rapid assay by CE with laser induced fluorescence detection for the quantification of plasma cysteinylglycine. The method was tested by measuring Cys-Gly levels in a group of patients affected from retinal venous occlusive disease. The RVO is an important cause of moderate to severe visual loss in older people and its pathogenesis remains still uncertain, but is likely multifactorial. Two different mechanisms have been proposed to explain RVO pathogenesis, that are: thrombosis in the vein resulting from compression by atherosclerotic changes in the adjacent artery and a local alteration of the blood flow resulting from unfavourable physiologic factors. The most common risk factors involved in RVO disease are age, arterial hypertension, and primary open-angle glaucoma (Mitchell et al., 1996; Klein et al., 2000; Cugati et al., 2006).

Materials and methods

Chemicals

Homocystine, cystine, $Na_3PO_4 * 12H_2O$, H_3BO_3, KBr, NaCl, Na_2EDTA, N-methyl-D-glucamine, DMSO (dimethylsulfoxid), DMF (N,N-dimethylformamide), TBP (tri-n-butylphosphine), NaOH, 5-IAF (5-iodoacetamidofluorescein), TCA (trichloroacetic acid), were obtained from Sigma (St. Louis, USA). Membrane filters (0.45 μm), obtained from Millipore (Bedford, USA), were used to filter all buffer solutions before CE analysis.

Patients recruitment

The present study used a case-control design, recruiting 73 consecutive patients with RVO (CRVO or BRVO) admitted to our Institute between April 2003 and November 2005. The duration of visual symptoms, ocular medication, and ocular history were noted. A full ophthalmic evaluation of both eyes was carried out, including best corrected visual acuity (BCVA), slit-lamp examination, applanation tomometry, fundus biomicroscopy, and fluorescein angiography. Medical conditions, including diabetes, systemic hypertension, cardiovascular status, decreased renal function, relevant drug history, and presence of blood dyscrasias were also recorded. Exclusion criteria were age <18 years, renal failure, and current medication with vitamin B6, B12, or folic acid.

Similarly to other studies analyzing the relationship between plasma homocysteine and cysteine levels and vascular occlusive disease, we chose a control group of apparently healthy subjects. The control group included 71 subjects, recruited from accompanying relatives or friends of patients or hospital personnel. Exclusion criteria for controls were a history of diabetes, systemic hypertension, cardiovascular or cerebrovascular disease, renal failure, blood dyscrasias, tumors, retinal vascular disorders, age <18 years, and current medication with vitamin B6, B12, or folic acid. All controls underwent standard ophthalmic evaluation, including BCVA, slit-lamp examination, applanation tonometry, and fundus examination. Controls were recruited concurrently during the patients' recruitment period.

Sample preparation

Blood samples were collected after an over-night past by venipuncture into EDTA-containing tubes (Becton Dickinson, Rutherford, USA), and immediately processed. Plasma was obtained immediately by centrifugation at $3000 \times g$ for 10 min at 4 °C. Plasma was stored at −80 °C and analyzed within 1 month. The sample obtained was prepared in two different methods for CE and for HPLC analysis. For CE analysis 100 μL of standard or plasma sample with 10 μL of TBP (10%) were mixed, vortexed for 30 sec and subsequently incubated at room temperature for 10 min. At the end of incubation 100 μL of 10% TCA were added, vortexed for 10 sec and then centrifuged for 5 min at $3000 \times g$. 100 μL of supernatant were mixed with 100 μL of 300 mmol/L Na_3PO_4 at pH 12.5 and with 20 μL of 5-IAF (4.1 mmol/L), and subsequently incubated at room temperature for 10 min. The mix was diluted 1/500 before injected in CE-LIF. For HPLC analysis we used the method described by Cornwell (Cornwell et al., 1993).

Capillary electrophoresis

The P/ACE 5510 CE system with 488 nm Ar ion laser was used (Beckman Instruments, Palo Alto, CA, USA). The system was fitted with a 30 kV power supply with a current limit of 250 μA. The dimension of the uncoated fused silica capillary was 75 μm ID and 47 cm length (40 cm to the detection window). Analysis was performed applying 10 nL of sample under nitrogen pressure (0.5 psi) for 1 sec using 3 mmol/L sodium phosphate/2.5 mmol/L boric acid as electrolyte solution with 75 mmol/L N-methyl-D-glucamine at pH 11.25. Since the pH run

buffer mixture was already at the optimum value (pH 11.25), adjustments were not required. The separating conditions (30 kV, 60 μA, normal polarity) were reached in 20 sec and held at a constant voltage for 2.5 min. The separations were carried out at 45 °C. After each run no rinse was necessary; the capillary was equilibrated with run buffer for 0.5 min.

Results

Capillary electrophoresis optimization

We have recently reported an effective and innovative method to measure total plasma thiols by capillary electrophoresis with induced fluorescence detection (Zinellu et al., 2003). Samples were treated with TBP to reduce oxidized thiols, that are successively derivatized by the selective thiol LIF-labeling agent 5-IAF in about 10 min. The adducts 5IAF-thiols (homocysteine, cysteine, glutathione, cysteinylglycine and glutamylcysteine) were baseline resolved in about 5 min in a 57 cm long capillary by using the organic base N-methylglucamine to increase peak resolution. Based on this method we developed a new ultra-rapid assay to rapidly measure cysteinylglycine.

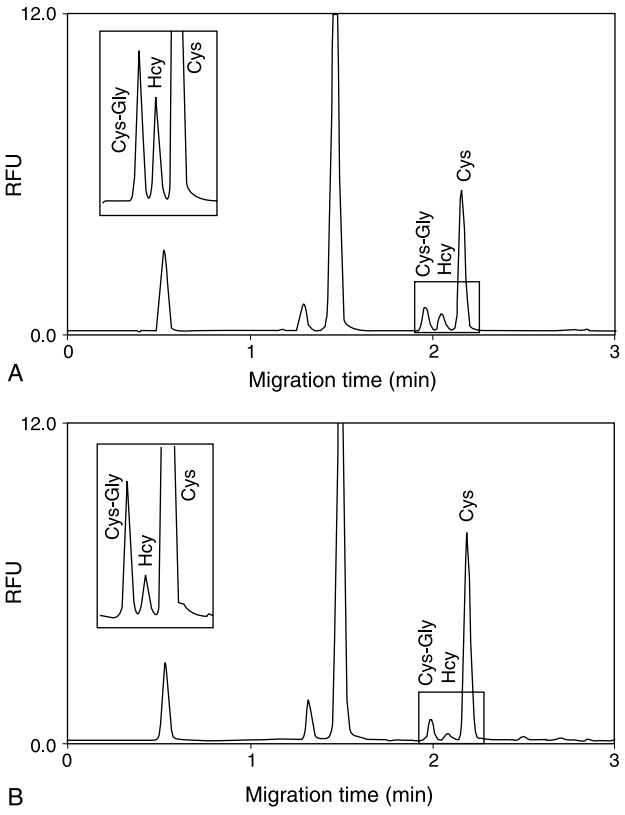

Fig. 1. Typical electropherograms of 5IAF-thiols obtained from a standard mixture (**A**) and from a plasma sample (**B**)

Table 1. Analysis time differences between our CE method and the rapid HPLC assay described by Frick et al. (2003)

	Reduction (min)	Centrifugation (min)	Derivatization (min)	Run time
HPLC	30	10	60	6
CE	10	5	10	3

In particular, shortening the capillary length from 57 to 47 cm, increasing the temperature at 45 °C and reducing the concentration of electrolyte buffer we obtained a baseline separation between Cys-Gly, Hcy and Cys peaks in about 2 min, as reported in Fig. 1. Therefore simultaneous quantification of cysteine and homocysteine together with cysteinylglycine is also possible. The attempt to further reduce the analysis time by cutting the capillary length or removing glucamine from the run buffer failed since overlapping between peaks occurred. However, as reported in Table 1, comparing our CE method with the recent rapid HPLC method described by Frick et al. (2003) to measure Hcy, Cys and Cys-Gly it is plain that the capillary electophoresis method allows to reduce both pre-analytical and analytical times.

Method validation

The linearity of the detector response to different concentrations of water solution standard thiols was determined between 5 and 80 μmol/L for Cys-Gly, 3 and 48 μmol/L for Hcy and 25 and 400 μmol/L for Cys. Calibration curves ($Y = 9.46 + 0.13$ for Cys-Gly, $Y = 8.64X - 7.45$ for Hcy and $Y = 7.84X - 62.74$ for Cys), obtained by five replicate, show good correlation coefficients ($r^2 > 0.999$ for all thiols) ensuring a linear response over the concentrations tested. Injection reproducibility was calculated by injecting ten times consecutively the same standard solution containing Cys, Hcy and Cys-Gly. Within-run precision (intrassay) of the method was evaluated by injecting the same biological sample 10 times consecutively, while between-run (interassay) precision was determined by injecting the same biological sample on 10 consecutive days. As shown in Table 2, precision tests indicate a good repeatability of our method both for migration times (CV < 0.8%) and areas (CV < 1.2%). Moreover a good reproducibility of intrassay and interassay tests was obtained (CV < 3% and CV < 5%, respectively). Thiols recovery was determined by adding authentic standards to plasma samples. The analytical recoveries, evaluated at four different concentrations for every thiol, were between

Table 2. Precision of the assay

	Injection ($n = 10$)		Intrassay ($n = 10$) (μmol/L)	Intrassay ($n = 10$) (μmol/L)
	Migration times (min) Mean (CV%)	Concentration (μmol/L) Mean (CV%)	Mean (CV%)	Mean (CV%)
Cys-Gly	1.967 (0.65)	22.513 (1.17)	22.112 (2.4)	22.313 (4.4)
Hcy	2.040 (0.68)	11.453 (1.13)	11.716 (2.5)	11.601 (4.1)
Cys	2.091 (0.72)	231.6 (1.07)	235.8 (2.7)	233.9 (4.5)

Table 3. Comparison between HPLC and CE methods by Passing and Bablok regression

	Slope	95% CI of slope	Intercept	95% CI of intercept	Correlation coefficient	*P*-value
Cys-Gly	1.002	0.973; 1.037	0.834	−0.114; 1.491	0.972	<0.0001
Hcy	0.915	0.810; 1.021	0.6909	−0.428; 1.965	0.967	<0.0001
Cys	1.036	0.942; 1.131	−12.128	−33.550; 10.069	0.943	<0.0001

96.3 and 102.6%. The limit of detection (LOD), calculated by 10-nL injections of a known solution of standards (after 500 fold water dilution) was 0.5 nmol/L, corresponding to an injected quantity of about 7 amol, with a signal-to-noise ratio of 3. The accuracy of the new CE method was assessed by comparing the plasma thiols levels from 55 healthy subjects obtained by the new method and by a HPLC reference assay (Cornwell et al., 1993). As reported in Table 3, Passing and Bablok regression analysis showed close agreement of both slop and intercept, with the target values of one and zero within the ±95% confidence limits. Moreover the Cusum test for linearity showed no significant deviation from linearity ($p > 0.10$) for all analytes tested.

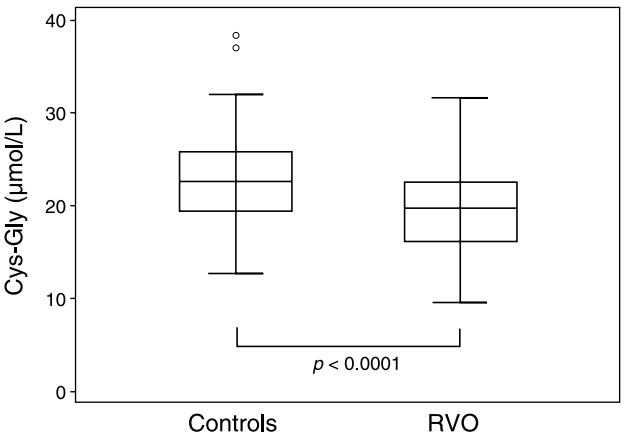

Fig. 2. Box plots of Cys-Gly plasma concentration in controls and RVO patients

Clinical application of method

We measured plasma levels of Cys-Gly in a study cohort consisting of 73 RVO patients (38 men, 35 women; mean age: 64 ± 14 years). The control group consisted of 71 subjects (36 men, 35 women; mean age: 63 ± 8 years); none of them had signs of retinal vascular disorders. We found that RVO patients had significant lower levels of Cys-Gly compared to the control group (19.5 μmol/L vs 22.7 μmol/L, $p < 0.0001$) as reported in Fig. 2. No differences were found on Hcy and Cys levels as also reported in our previous study (Pinna et al., 2006).

Conclusions

Several studies have reported that elevated levels of total plasma homocysteine are related to an increased risk for cardiovascular disease (Gerhard and Duell, 1999; Nygard et al., 1999; Aguilar et al., 2004; Kalra, 2004). Recently some studies have also examined the possible role of cysteine in cardiovascular disease (Jacob et al., 1999; El-Khairy et al., 2001; van den Brandhof et al., 2001; Ozkan et al., 2002). Cys shows some of the chemical properties of homocysteine which make it able to have a part in the process of atherosclerosis, also taking into account that cysteine concentration in plasma is 25–30 times higher than homocysteine. In the last years also Cys-Gly gained importance as metabolite with anti-atherogenic properties (Zinellu et al., 2006). However, among the various methods described in literature RIA and ELISA procedures focused on the measurement of homocysteine alone, while most HPLC, GC and CE assays have been developed to

detect all plasma thiols in the same run with a waste in time and costs. We have set up a rapid CE method for the selective determination of cysteinylglycine. The elevated selectivity of our electrophoretic conditions allows besides to baseline resolve also homocysteine and cysteine in the same run in about 2 min. In comparison with our previous method (Zinellu at al., 2003) and with the most rapid HPLC assay (Frick et al 2003), we are able to double the number of plasma samples that may be processed daily reducing also the analysis costs. The application of the method on a cohort of subjects affected by RVO disease suggest that the decreased levels of Cys-Gly may be involved in the development of pathology. As well known, in fact, Cys-Gly is the precursor of glutathione that is the most important intracelluar antioxidant and is itself a molecule with antioxidant and anti-atherogenic properties. Moreover it has been recently reported that Cys-Gly protect LDL from homocysteinylation, an apoB post-translational modification which makes lipoprotein more atherogenic. In our case-control study the two populations have the same LDL levels (data not shown), but the difference in plasma Cys-Gly concentration may lead to more homocysteynilated LDL in CRVO subjects, thus maybe contributing to vascular damage. In conclusion, the simplicity in sample preparation, the quickness in the analytical times and the low costs of our proposed method make it a reliable tool for clinical laboratories or research groups when an elevate number of samples must be analyzed daily.

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