


Plasma Homocysteine Determined by Capillary Electrophoresis with Laser-induced Fluorescence Detection, Elizabeth Caussè1*, Nathalie Siri,1 Hélène Bellet,2 Sandrine Champagne,1 Christophe Bayle,3 Pierre Valdiguié,1 Robert Salvayre,1 and Françoise Couderc4 (1 Laboratoire de Biochimie/Institut National de la Santé et de la Recherche Médicale, U466, Centre Hospitalier Rangueil, 1 avenue chimie/Institut National de la Sante ´ et de la Recherche

**Homocysteine (Hcy) has emerged as another risk factor for the development of coronary heart disease (1). Genetic abnormalities of the enzymes cystathionine-β-synthase and methylene tetrahydrofolate reductase (folic acid and vitamin B6 and B12 cofactors) can cause raised plasma Hcy concentrations. Deficiencies in folic acid and vitamins B6 and B12 may also contribute to this increased concentration (1–3). Consequently, patients with coronary heart disease have been treated successfully with folic acid, vitamin B12, or vitamin B6 (1), but the benefit of such treatment in reducing morbid cardiovascular endpoints is presently unknown (3).

A widely used technique for measuring total plasma Hcy is reversed-phase HPLC with fluorescence detection after derivatization of plasma thiols (4–6). Some studies use gas chromatography–mass spectrometry techniques (7) with various derivatization protocols (8).

The increasing demand for the determination of plasma total Hcy prompted us to develop a rapid, automated method based on capillary electrophoresis (CE) and laser-induced fluorescence detection (LIF), in which all specific thiols are detected selectively.

Blood was collected by venipuncture into evacuated tubes containing EDTA from patients with episodes of deep-vein thrombosis or vascular disease. The plasma was separated by centrifugation and was stored at −20 °C until analysis.

Plasma samples (100 μL) were treated for 30 min at room temperature with 10 μL of a novel thiol-reducing agent [50 mmol/L tris(2-carboxyethyl)phosphine; Molec-

ular Probes], as reported recently by Gilfix et al. (9). Compared with the widely employed tributyl phosphate, this new phosphine is nonvolatile, stable, soluble in water, and without disagreeable odor (5,6). When we used this procedure, total Hcy (mixed and symmetric disulfides, including protein-bound Hcy) was analyzed in its reduced form.

All patient samples and plasma-based calibrators were incubated with tris(2-carboxyethyl)phosphine under the same conditions. The internal standard [5 μL of 10 mmol/L β-penicillamine (DP)] was mixed with plasma. The solution was deproteinized with 60 μL of 0.8 mol/L 5-sulfosalicylic acid with vortex-mixing, followed by centrifugation at 10 000g for 15 min.

We mixed 100 μL of the supernatant with 50 μL of 0.3 mol/L carbonate buffer, pH 9.5, and 5 μL of 5 mol/L sodium hydroxide. We then added 50 μL of a 1 g/L 6-iodoacetamidofluorescein solution (6-IAF) in dimethyl sulfoxide (Sigma Chemical Co.), and the mixture was incubated overnight in the dark at room temperature to allow analysis after the reaction has plateaued (>2 h). IAF-labeled samples were stored at −20 °C until analysis; they could be preserved for up to 3 months. Each derivatized plasma was analyzed directly after being diluted 5000-fold before the CE injection. The analyses were performed on a Zeta CE instrument (Zeta Technology) equipped with a modular LIF detector and an argon ion laser (wavelength, 488 nm). The different thiols were separated on a 75 cm × 50 μm (i.d.) fused-silica capillary (Polymicro Technology) with an effective length of 43 cm and a total length of 75 cm. The separation buffer consisted of 10 mmol/L sodium dodecyl sulfate, 50 mmol/L boric acid, and 20 mmol/L 3-(cyclohexylamino)-1-propanesulfonic acid adjusted to pH 9.5 by addition of a sodium hydroxide solution (10 mol/L). The separation voltage was +25 kV, producing an electrophoretic current of 25 μA.

All samples were assayed in triplicate. Peaks were identified by use of solutions of Hcy, Cys, or glutathione, and the internal standards cysteamine or DP. Fig. 1A shows the thiol solutions prepared in carbonate 0.3 mol/L buffer, pH 9.5, and then diluted in deionized water. Under the electrophoretic conditions used, the Hcy derivative is well separated with a migration time of 7.2 min.

Electropherograms of a human plasma sample with added DP as internal standard produced several peaks (Fig. 1, B and C). Known quantities of Hcy, Cys, or DP solutions were added to the plasma sample to identify Hcy, Cys, and DP peaks.

Hcy calibrators were prepared from a plasma pool (30, 15, 10, 5, and 2 μmol/L), and the concentrations were calculated according to peak heights. The calibration curves for Hcy quantification were obtained by plotting the peak height ratio of Hcy/DP and Hcy/Cys. The equations, as a function of the Hcy concentration x (expressed in μmol/L) were, respectively, as follows: y = 0.033x + 0.013 (r = 0.9998) for Hcy/DP and y = 0.011x + 0.003 (r = 0.9992) for Hcy/Cys.

The detection response was linear over the concentration range between 2 and 200 μmol/L for all the plasma
thiols. The detection limit for Hcy, defined as a signal-to-noise ratio $>3$, was $\sim 0.25 \mu mol/L$ in plasma and was quite identical for the other thiols. The detection limit of the diluted sample injected (after the 5000 fold-dilution) was 50 pmol/L, corresponding to a quantity of 1.1 atto-moles.

The within-day reproducibility of the assay for Hcy was determined on aliquots ($n = 10$) prepared independently from the same plasma sample, and the between-day reproducibility was calculated from the analysis of the same plasma sample that was derivatized each day during 10 consecutive days. The coefficients of variation (CVs) which take into account every step (i.e., reduction, precipitation, centrifugation, derivatization, and injection into the CE-LIF) were 4.9% and 7.8%, respectively, whereas the CVs for injection only were 1.8% and 2.6%.

To determine the recovery of the method, we added 10 $\mu L$ of different concentrations of Hcy ($2, 5, 10, 15, 20, 25, 30, 35, 40, 45$, and $50 \mu mol/L$) to 90 $\mu L$ of the same pooled plasma. The average recovery was $100.5\% \pm 4.87\%$ (mean $\pm$ SD). The recovery (mean $\pm$ SD) of Hcy added to six plasma samples analyzed in triplicate (Table 1) was $101.1\% \pm 6.99\%$ ($n = 18$).

We used this CE-LIF technique to analyze serum samples from 55 fasting subjects with episodes of deep-vein thrombosis or arterial occlusive disease, and we compared the results with those obtained from an ion-exchange amino acid analyzer (Beckman 6300 analyzer). The mean control values ($n = 27$) for the amino acid analyzer method ($y$) were $8.89 \pm 2.85 \mu mol/L$ vs $11.73 \pm 4.45 \mu mol/L$ for CE-LIF ($x$). The regression equation was as follows: $y = 0.91x + 2.26; n = 55; S_{yx} = 7.49; r = 0.87$, which is comparable to previous observations ($5, 8, 10$).

The same calibrator, i.e., homocystine (Sigma), was used in both methods: in the ion-exchange chromatography method, homocystine was diluted in lithium citrate buffer (0.2 mol/L, pH 7.0), and the calibrator curve in aqueous solution was determined using the ninhydrin reaction (specific for amine groups); in the CE-LIF method, homocystine was diluted in carbonate buffer (pH 9.5), and the calibrator curve in plasma solution was determined using IAF, which reacts specifically with thiol groups.

The major advantage of this IAF method is its ability to quantify Hcy, Cys, and glutathione simultaneously in a single analysis. Our derivatized thiols were stable for several months. The method is thiol-specific and the detection limit (0.25 $\mu mol/L$ Hcy in plasma) is similar to other HPLC or amino acid analyzer procedures ($4, 5$). The IAF method saves time compared with the HPLC method, and we encountered no problems with interference from the reducing agent. When compared with the fluorescein isothiocyanate method, described previously ($11$), which quantifies Hcy and all amino acids through the reaction with amine groups, the IAF-based procedure allows a more selective detection of thiol-containing amino acids. In addition, in the IAF method, the migration times are shorter, the peak resolution is better, and co-injection of a calibrator is not required. The use of an internal standard [DP or homocysteic acid ($11$)] also improves the accuracy and the standardization ($12$) of the method.

The IAF method is simple, low in reagent cost ($11$), sensible, reproducible, and suitable for routine determi-

![Electropherograms of fluorescence intensity.](image)

**Fig. 1.** Electropherograms of fluorescence intensity.

Fluorescence intensity measured in relative fluorescence units (RFU). (A), solutions of thiol amino acids; **peak 1**, 0.5 nmol/L cysteamine; **peak 2**, unreacted 6-IAF; **peak 3**, 0.5 nmol/L Hcy; **peak 4**, 0.5 nmol/L DP (internal standard); **peak 5**, 2.5 nmol/L cysteine; **peak 6**, 2.5 nmol/L glutathione. (B), patient plasma containing 14 $\mu mol/L$ Hcy; (C), patient plasma containing 57.7 $\mu mol/L$ Hcy. The thiol amino acids are labeled with 6-IAF at 1 nmol/L and diluted 5000-fold. The migration conditions are described in the text.

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nation of serum/plasma Hcy concentrations in a clinical laboratory. This method will allow us to investigate and evaluate the potential atherogenic properties of Hcy.

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References

Validation of a Point-of-Care Assay for the Urinary Albumin:Creatinine Ratio, Marie P. Parsons,1 David J. Neuman,1,3 Ronald G. Nevall,2 and Christopher P. Price1* (1 St. Bartholomew’s and the Royal London School of Medicine and Dentistry, Turner Street, London E1 2AD, UK; 2 Bayer plc, Diagnostics Division, Bayer House, Strawberry Hill, Newbury, Berks RG14 1JA, UK; 3 SW Thames Institute for Renal Research, St. Heller NHS Trust, Wrythe Lane, Carshalton, Surrey SM5 1AA, UK; * address correspondence to this author at: Department of Clinical Biochemistry, St. Bartholomew’s and the Royal London School of Medicine and Dentistry, Turner Street, London E1 2AD, UK; fax 44 171 377 1544, e-mail c.p.price@mds.qmw.ac.uk)

Microalbuminuria is a risk factor for the development of overt nephropathy in type 1 and type 2 diabetic patients (1, 2). Importantly, improvement of glycemic control and early intervention with antihypertensive drugs can retard the development of microalbuminuria and possibly its progression towards overt nephropathy (3, 4). Microalbuminuria is defined as an increased excretion of albumin above the reference range for healthy nondiabetic subjects, but which is undetectable by the Albustix dipstick test (5). It has also been defined as a urinary albumin excretion rate between 20 and 200 μg/min in an overnight or 24-h sample on at least two of three occasions within a period of 6 months (6). This is equivalent to 30–300 mg/24 h or 3–30 g/mol creatinine.

Although there are no clinical data to define the analytical requirements for the detection and monitoring of microalbuminuria, it has been suggested that a method should have an interassay precision of <12% over the concentration range 5–200 mg/L and be sensitive enough to reliably detect changes of 10 mg/L in the concentration range 5–35 mg/L (5). The method must also be reproducible over long periods because patients may be screened only two or three times throughout the year.

Albumin may be measured in urine by radial immunodiffusion methods, which include a rapid, gold sol labeled variant of the technique capable of semiquantitative measurement (7), and by direct immunoturbidimetric and nephelometric methods that enable automation and rapid turnaround of results. The latter methods are prone to a “hook effect” at high antigen concentrations (8). Latex-enhanced methods, in addition to providing improved sensitivity, also allow the use of an inhibition format, thereby avoiding a hook effect (9).

The DCA 2000TM desktop microalbumin system (Bayer plc, Newbury, UK) is a disposable cartridge device encapsulating an immunoturbidimetric assay for albumin and a colorimetric assay for creatinine, together with a programmable photometer with an incubation chamber. The cassette includes separate reservoirs that contain buffer and antibody reagent and reagents for a creatinine assay. Each batch of reagents is calibrated, and the algorithm is encoded in a bar code strip on the cartridge. The analyzer prints out the albumin:creatinine ratio as well as the concentrations of albumin and creatinine. The analyzer can also be used for the quantitation of HbAIC (10). We have evaluated the performance of this system and compared it with a quantitative particle enhanced immunoinhibition method (9) (Dade Behring aca IV® analyzer, Dade Behring Inc., Wilmington, DE).

In the DCA method, albumin-specific goat anti-human polyclonal antibody binds to albumin in the presence of polyethylene glycol. Formation of albumin-antibody complexes increases turbidity, which is measured by the absorbance at 531 nm. The preprogrammed calibration curve uses calibrator values assigned by the use of dilutions of the Reference Preparation for Proteins in Human Serum (CRM470) (11).

In the kinetic creatinine assay, creatinine complexes with 3,5-dinitrobenzoic acid at an alkaline pH to form a