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Short communication

# Quantitation of homocysteine in human plasma by capillary electrophoresis and laser-induced fluorescence detection

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**Abstract**

Homocysteine (Hcy) represents a branching point between the transsulfuration and transmethylation pathway of methionine. A large increase of plasma concentration of Hcy is observed in patients with inherited hyperhomocysteinemia. A moderated increase (above 10 $\mu M$) is also observed in various pathological conditions, such as arterial occlusion, hypertension, hyperlipidemia and chronic renal failure. While amino acids were largely studied using capillary electrophoresis with UV or laser-induced fluorescence detection (LIF), thiol-amino acids were not. In this work we present a new approach for testing homocysteine in human plasma using CE–LIF and fluorescein isothiocyanate. The low fluorescence yield of the fluorescein thiocarbamyl (FTC) thiol-amino acids limits, probably, the sensitivity of the detection to $8 \cdot 10^{-10}$ $M$ (instead of $10^{-12}$ $M$ for FTC–arginine). 

*Keywords:* Homocysteine; Amino acids; Fluorescein isothiocyanate; Fluorescence

## 1. Introduction

Capillary electrophoresis (CE) associated with laser-induced fluorescence (LIF) detection is largely used to detect and quantify amines and amino acids in various media such as wine [1], urine [2], cerebrospinal fluid [3,4], microdialysate of rat brain [5,6], unique cell [7], cheese [8,9]. To detect low amounts of amines and amino acids, various tags have been considered such as fluorescein isothiocyanate (FITC) [1,4,8,10–14], naphtalene dicarboxaldehyde [5,6], 3-(4-carboxybenzoyl)-2-quinoline carboxaldehyde (CBQCA) [3], dansyl chloride [2], and carbocyanide [9]. In our laboratory we derivatized amino acids with FITC because of (i) its efficient derivation of primary and secondary amines, (ii) its compatibility with the 488 nm argon ion laser wavelength and (iii) its very high fluorescence quantum yield and molecular extinction coefficient which allows sensitive detection. The main disadvantage of FITC is the large number of FITC–buffer by-products which interfere and disturb the interpretation of electropherograms.

Only a few studies deal with sulfur amino acids such as cysteine or homocysteine using CE–LIF [10,15]. To our knowledge, no study has been done on the quantitation using CE. Usually, homocysteine (Hcy) is determined by either high-performance liquid chromatography (HPLC) according to Refsum et al. [16] and Fermo et al. [17], radioenzymatic assay [18], gas chromatography–mass spectrometry (GC–MS) [19], automated fluorescence polarization

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immunoassay [20], or ion-exchange chromatography [21].

Homocysteine represents the branching point between the transsulfuration and transmethylation pathway of methionine. A large increase of plasma concentration of Hcy is observed in homozygous patients with inherited hyperhomocysteinemia disease [20]. A moderate increase is observed in heterozygous patients suffering from the same disease and also in various pathological conditions, such as arterial occlusion, hypertension, hyperlipidemia or chronic renal failure.

We present here the separation of sulfur amino acids and the quantitation of plasma homocysteine.

## 2. Experimental

### 2.1. Sample preparation

All chemicals where purchased from Sigma (St. Quentin Fallavier, France).

Blood was collected by venipuncture into vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA) from patients with episodes of deep-vein thrombosis or vascular disease. Plasma was separated by centrifugation and was stored at −20°C until analysis. The plasma (100 μl) was treated with 10 μl of reducing agent (dithiothreitol, DTT 8 m*M*) and Hcy was assayed in its reduced form. A 2-μl volume of 10 m*M* L-homocysteic acid (internal standard) was added to the plasma and incubated for 60 min at 37°C. The solution was deproteinized with 60 μl of 10% perchloric acid under vortex mixing, followed by a 10-min centrifugation at 13 000 *g*.

### 2.2. Standard solutions

L-Homocystine, other amino acids and the internal standard (homocysteic acid) were dissolved in a carbonate buffer (0.3 *M*, pH 9.8) to give a concentration of 10 m*M* and stored at −20°C until analysis. These stock solutions were further diluted with water to various concentrations. A standard calibration curve was prepared by adding known concentrations (3–100 μ*M*) of homocysteine to plasma.

### 2.3. Derivatization procedure

A 100-μl aliquot of the clear supernatant was mixed with 50 μl carbonate buffer (0.3 *M*, pH 9.8) and alkalinized with 3 ml of sodium hydroxide (5 *M*). After vortex mixing, 50 μl of FITC solution was added. This solution of FITC isomer I (fluorescein-5-isothiocyanate) was prepared by dissolving 1 mg of FITC in 2 ml acetone. The reaction was performed in the dark for one night at ambient temperature. At the same time, blank and standard solutions were mixed under the same conditions but alkalinisation was not necessary.

### 2.4. Identification and quantification

Each derivatized solution was either 1000- or 10 000-fold diluted prior to injection. The separation buffer consisted of 10 m*M* sodium dodecyl sulfate (SDS, Aldrich) and 70 m*M* boric acid, adjusted to pH 9.8 using 5 *M* NaOH. The peak of homocysteine was identified by spiking the diluted plasma with known quantities of this fluorescein thiocarbamyl (FTC) amino acid. It was quantified using the linear calibration curve based on the ratio of the homocysteine/homocysteic acid peak heights. The linearity of the calibration curve was assessed using standard least-squares analysis of the logarithm of peak height versus logarithm of concentration plots. Injection of known quantities of homocysteine and homocysteic acid showed that experimental error on quantitation remains below 3%.

### 2.5. Instrumentation and separation conditions

We used a modular injector and a high-voltage power supply Prime Vision (Europhor Instrument, now Zeta Technology, Ramonville, France) and a modular LC–CE–LIF detector (Zeta Technology) equipped with a 488 nm argon ion laser (Uniphase, 2014, San Jose, CA, USA). The capillary was rinsed with 1 *M* NaOH for 2 min, 0.1 *M* NaOH for 2 min, water for 2 min and then with separation buffer for 3 min. Samples (15 nl) were injected by hydrodynamic injection for 1 s. Separation conditions were 10 m*M* SDS, 50 m*M* boric acid at pH 9.8, 25 kV for plasma samples, and 10 m*M* SDS, 50 m*M* boric acid, 20

m*M* 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) at pH 9.8, and 15 kV for standards.

Data issued from this method for total homocysteine in plasma were compared with the ones given by an amino acid analyser [21].

## 3. Results and discussion

Fig. 1 shows the electropherogram of the FITC-derivatized standard amino acids involved in the metabolic pathway of methionine. The amino acids were derivatized at a concentration within the micromolar range, which is the mean concentration recorded in plasma. The quantities injected in our CE–LIF system were around 30–190 amol. Surprisingly FTC–cysteine (FTC–Cys) and FTC–homocysteine (FTC–Hcy) seemed to give less sensitive results than FTC–serine or FTC–methionine.

The FTC–amino acids are negatively charged. Basic amino acids (small migration time) migrate first, then neutral amino acids and finally acidic amino acids (higher migration time). Homocysteine migrates slower than cysteine. This is explained by the fact that homocysteine is more hydrophobic than cysteine, thus possessing one more methylene group than cysteine and that it has a higher affinity for micelles, thus slowing down this amino acid migration, prior to cysteine.

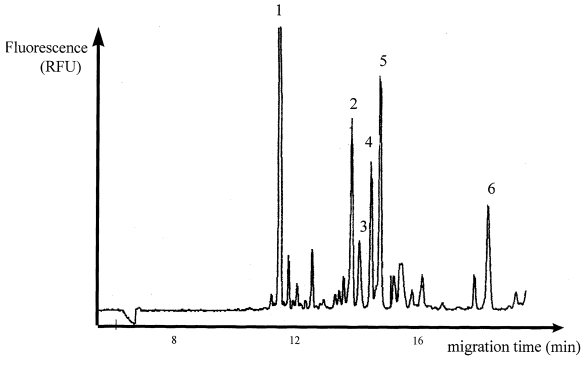


Fig. 1. Electropherogram of standard amino acids involved in the metabolic pathway of methionine. The amino acids are labelled with FITC at $0.5 \cdot 10^{-4}$ *M* and diluted 1000×. Migration conditions: 10 m*M* SDS, 50 m*M* boric acid, 20 m*M* CAPS at pH=9.8, +15 kV (14 μA). (1) Unreacted FITC, (2) Met, $18.75 \cdot 10^{-10}$ *M*; (3) homocysteine (Hcy), $93.75 \cdot 10^{-10}$ *M*; (4) Ser, $18.75 \cdot 10^{-10}$ *M*; (5) Cys, $125 \cdot 10^{-10}$ *M*; (6) *S*-carboxymethylcysteine, $93.75 \cdot 10^{-10}$ *M*.

*S*-Carboxymethyl cysteine standard allows us to record the difference of migration time with cysteine. It migrates slower than cysteine because of the net negative charge of the added carboxylic group, compared to the partially charged sulfhydryl group [22] at the pH of our electrolyte buffer (pH 9.8).

The inter-plasma reproducibility of the migration times is sometimes affected. FTC–Hcy or FTC–Cys can be difficult to identify using solely the retention times as recognition criteria. It is necessary to spike the sample with FTC–Cys or FTC–Hcy standard solutions. The variation of migration time could be due to small pH changes in the different samples. The $pK_a$ values of FTC–Cys and FTC–Hcy are unknown but the pH of underivatized species where the concentrations of charged ([$-S^-$]) and uncharged ([–SH]) sulfhydryl groups are equal, are around 9.6 and 10, respectively [22]. A very strong dependence of the ratio [$-S^-$]/[–SH] was observed following small variations of pH [22]. The amount of these differently charged species affects the electrophoretic mobility if the sample pH is not perfectly constant.

To obtain good and efficient separations, we diluted our derivatized samples in water, and, in order to reach the best LIF sensitivity, we had to use a pH higher than 9.5. Consequently, to minimize the electrophoretic mobility variations of partially charged sulfhydryl group, we derivatised cysteine and homocysteine, prior to FITC derivatization, with iodoacetic acid to obtain *S*-carboxymethyl thiol amino acids. This additional step in the amino acid extraction protocol allowed us to obtain very reproducible migration times, independent of matrix effects. However, the reaction was not completed over the studied concentration range (3–100 μ*M*). Moreover, this derivatization step was time consuming and induced some large and unwanted peaks in the electropherograms e.g., labelling of DTT with iodoacetic acid and FITC. Therefore we have stopped using iodoacetic acid derivatization. Using CAPS in the electrolyte buffer minimized the peak displacement of cysteine and homocysteine, but some loss of electrophoretic efficiency was observed due to an increase of the current in the capillary resulting in higher migration times.

To quantitate homocysteine, standards were prepared from plasma samples, without detectable Hcy, in a range of 3–100 μ*M* and derivatised using a

FITC solution of $0.5 \cdot 10^{-4}$ *M*. To improve the reproducibility of the assay, we used homocysteic acid (HA) as an internal standard (I.S.) at a fixed concentration of 20 μ*M*. Four separate standard calibrations were analysed by this method, each standard curve was prepared on a different day, and the variability, expressed in terms of the relative standard deviation (R.S.D.), is therefore likely to accurately reflect the day-to-day reproducibility of the standard curve. The following equation was used to evaluate the linearity of the method:

$$\ln R = \ln A + a \ln C \quad (1)$$

where ln *C* is the logarithm of the analyte concentration, ln *R* refers to the logarithm of the ratio of relative fluorescent intensity of homocysteine to that of homocysteic acid, and *a* is the slope of the curve ln *R* = f(ln *C*). The regression equation of the curve gives ln *A* = −4.55 (R.S.D. = 2.2%) (for *C* expressed in μ*M*) and *a* = 0.98 (R.S.D. = 0.6%), very close to 1.00. This is indicative of a good linearity response of the detector. A linear regression coefficient *r* = 0.9914 is also obtained, which refers to the relatively good linearity of the calibration curve.

At high concentration (above 50 μ*M*), there are two peaks representing cysteine and homocysteine. These peaks were attributed to the two different chemical sites (amine or thiol) which can be labeled by the isothiocyanate function of the FITC [23]. The relative height of these two peaks of each sulfur amino acid changes with the time. No peak doubling was observed at concentrations lower than 50 μ*M* of cysteine or homocysteine.

DTT is labelled by the excess of FITC in the plasma, and affects the separation giving a broad peak with a high migration time (14 min).

Fig. 2 shows an electropherogram of two human plasmas with high and low homocysteine concentrations, respectively. Quantitative data are reported in Table 1. The within-day R.S.D. is lower than 3.0% (*n* = 8). The results were compared to ion-exchange chromatography quantitation by the use of non-parametric Spearman's test [24]. The difference between the two methods is not significant (*r* = 0.9). The limit of Hcy detection in plasma is 1 μ*M*, and the limit of quantitation is 3 μ*M* by this method. The detection limit of diluted standard FTC–Hcy and FTC–Cys is $8 \cdot 10^{-10}$ *M*. The R.S.D. of Hcy migration times in inter-plasma samples is 8.5% (*n* = 4) using the SDS–boric acid buffer and 2.5% (*n* = 4) in the SDS–boric acid–CAPS buffer. It is 1.7% (*n* = 3) for the same sample analysis in the CAPS buffer. These results show the importance of matrix effects.

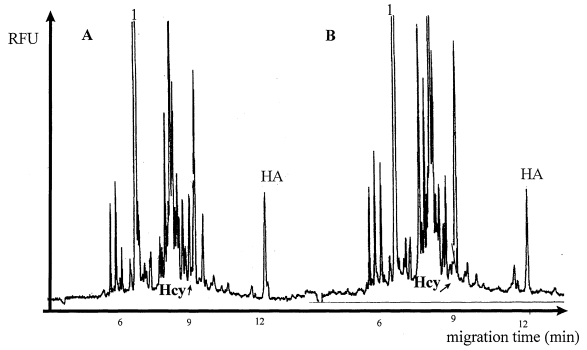


Fig. 2. Electropherograms of two plasmas containing low and high concentration of homocysteine, using homocysteic acid as an internal standard. Electrophoretic conditions: 10 m*M* SDS, 50 m*M* boric acid, pH 9.8, 25 kV (45 μA). Plasmas were diluted 1000× in water prior to analysis.

The CE migration of plasmatic homocysteine and homocysteic acid is recorded in less than 12 min, compared to 100 min for amino acid analyser (ninhydrin derivatives), and 12 min for HPLC and monobromobimane derivatives [25]. This methodology is probably one of the quickest and the cheapest of all methods commonly used. Its cost is below US$ 0.2 per analysis. Derivatization of plasma using FITC also allows to quantitate other amino acids in plasma, as described in several previous studies [4,8,26]. Moreover, one of the most important advantages of the use of FITC is to label all the amines or amino acids (i.e., primary or secondary amines). The several peaks of unreacted FITC and the very high sensitivity of FITC to the light,

Table 1
Comparison of CE–LIF data and ion-exchange chromatography for five patients

| Plasma sample No. | CE–LIF (μmol/l) | Ion-exchange chromatography (μmol/l) |
|---|---|---|
| 1 | 13 | 8 |
| 2 | 15 | 10 |
| 3 | 63 | 51 |
| 4 | 8 | 8 |
| 5 | 61 | 48 |

however may induce some difficulties in using such a tag.

In conclusion, we have separated and determined homocysteine in plasma using FITC derivatives and CE–LIF. The results were available in less than 12 min and the R.S.D.s are lower than 3%. This method, which can be easily automated is a rapid, non-sample consuming, low cost analytical methodology which can be used in the clinical laboratory for diagnosis of hyperhomocysteinemia, atherosclerosis and thromboembolic disease.

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