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Short communication

# A new HPLC micromethod to measure total plasma homocysteine in newborn

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**Abstract**

Total plasma homocysteine (tHcy) in children may be an useful biochemical marker for genetic risk of premature cardiovascular disease. We reported a rapid, isocratic HPLC method able to process very small amount of newborn plasma samples. A blood sample from heel capillary circulation was collected, using a heparinized capillary glass tube. Plasma sample from 1 to 10 μl was derivatized with ammonium-7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate after reduction with tri-*n*-butylphosphine and analyzed on Discovery C18 column, with a solution of acetonitrile–dihydrogenphosphate 0.1 M (8:92 v/v pH*2.1). This assay ensures a good recovery (95%), precision (CV 4.5%) and linearity ($y = 2.41x + 0.31$, $r = 1$). Due to its simplicity and reliability, our method is suitable for routine analysis of tHcy and other aminothiols (Cys, Cys-Gly, GSH) assessed for clinical and research purposes. With this HPLC method we have assayed tHcy levels in 1400 apparently healthy newborn babies (tHcy mean value = $4.9 \pm 2.7$ μM). In conclusion, this accurate and linear HPLC method allows measurement of tHcy in newborn during the routinary capillary blood collection in the fourth living day without any other invasive procedure. 

*Keywords:* Total plasma homocysteine; Newborn; High-performance liquid chromatography

## 1. Introduction

Homocysteine (tHcy) is a thiol-containing amino acid produced during the transulfuration pathway of methionine. Intracellularly, tHcy is metabolized either to cystathionine or to methionine. Cystathionine is further transformed to cysteine (Cys), a precursor of glutathione (GSH) that is catabolyzed by γ-glutamyl transpeptidase in cysteinylglycine (Cys–Gly). These thiols (tHcy, Cys, GSH, Cys–Gly) are metabolically related in the blood [1]; tHcy metabolism is regulated by the cellular content of vitamins $B_6$, $B_{12}$ and folic acid.

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Therefore, deficiencies of these vitamins may significantly influence tHcy levels and their effects on the development of atherosclerosis. In the last decade several methods to assay tHcy have been reported, including radioenzymatic assays [2], gas chromatography–mass spectrometry [3] and above all high-performance liquid chromatography (HPLC). A great number of these methods are used to determine tHcy in an adult population, but unfortunately they are not suitable to assay hyperomocysteinemia in a neonatal population because of too large amount of plasma sample. Concerning the pediatric tHcy values, only few studies regarding little cohorts have been realized [4–7].

Newborn tHcy levels may be a useful biochemical marker both for cobalamin–folate deficiencies and for a genetic risk of premature atherosclerosis. The measurement of this aminothiol might be important to identify, in a neonatal population, subjects at risk of vascular disease. Aim of this study was to perform a HPLC method able to process very small amount of newborn plasma sample. Blood sample was drawn during the normal routine controls in hospital by heel without any further trauma for baby. Moreover this study looks to evaluate tHcy levels in newborns in order to identify hyperomocysteinemia or vitamin deficiencies already in neonatal age.

## 2. Experimental

### 2.1. Chemicals

Cysteine, Cys–Gly, Hcy, GSH, tri-*n*-butylphosphine (TBF), dimethylformamide, ammonium-7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate (SBD-F), potassium dihydrogenphosphate, acetonitrile ($CH_3CN$), sodium hydroxide (NaOH), boric acid, ethylenediaminetetraacetic acid sodium salt (EDTA), trichloro acetic acid (TCA), physiological solution (NaCl 0.14 M), were purchased from Fluka (Sigma-Aldrich, Milan, Italy).

### 2.2. Apparatus

A Pro Star 240 HPLC Solvent Delivery Modules coupled to a Pro Star 410 HPLC Autosampler (Varian, Walnut Creek, CA) were fitted with TSK-GEL TOSOHAAS ODS-80Ts analytical column (4.6 mm ID × 250 mm), TOSOH Corporation, Japan. Fluorescence intensities were measured using a Jasco FP-1520 Fluorimetric Detector coupled to a Star Chromatography Workstation (Varian).

### 2.3. Sample collection

The study involved 1400 newborns (727 males and 673 females) apparently healthy (by history and analytical data), between March 1998 and April 2000 in the Neonatology Department of FBF Hospital, Erba, Italy.

From each newborn, about 20 μl of whole blood from heel were collected in heparinized capillary glass tubes. Plasma was obtained within 30 min by immediate centrifugation of the capillary at 2000*g* for 5 min and stored at 20°C until analysis.

### 2.4. Reagents and solutions

- TBF (50 μl) was dissolved in dimethylformamide (450 μl) and stored in a glass vial at 4°C. The solution was prepared freshly every 3 days.
- Precipiting solution was made by dissolving 10 g of TCA and 0.0372 g of EDTA in 100 ml of distilled water, this solution was stored at room temperature.
- NaOH (1.55 M): 6.2 g of NaOH was solved in 100 ml of distilled water; the solution was stored at room temperature.
- Borate buffer (1 M) was prepared as follows: in 100 ml of boric acid (1 M), 0.15 mg EDTA was added and then the solution was adjusted to pH 11 with NaOH (5 M).
- Derivatizing solution: 10 mg of SBD-F was solved in 1 ml of borate buffer 0.125 M (pH 9.5); the solution was stored at 4°C in darkness.

### 2.5. Standard solution

A Hcy standard (Hcy std) stock solution (600 μM) was prepared solving 4 mg of homocysteine standard in 5 ml of methanol and adding 45 ml of distilled water later.

Stock solution of Cys (150 μM), Cys–Gly (30 μM) and GSH (10 μM) were prepared solving each standard in HCl (0.1 M). To assay the linearity of the method the Cys, Cys–Gly, Hcy and GSH stock solutions were diluted to yield appropriate concentration in the following ranges: Cys (9.375–150 μM), Hcy (1.25–20 μM), Cys–Gly (1.875–30 μM), GSH (1.25–10 μM). The sample volume used to perform the calibration curves was 10 μl.

### 2.6. Reduction and derivatization

Plasma thiols were reduced and derivatized according to a method used to test tHcy in an adult population [8]. One to ten microliters of plasma or standard were added to water (90–99 μl) and filled up to a final volume of 100 μl, then 10 μl of TBF was added. The mixture was incubated at 4°C for 30 min to reduce and cleave protein-bound thiols (Cys, Cys–Gly, Hcy and GSH). After incubation, proteins are removed by precipitation adding 100 μl of TCA (10%), mixed and centrifuged at 10 000*g* for 5 min. Subsequently 100 μl of clear supernatant were added to 10 μl of NaOH (1.55 M), 100 μl of borate buffer (1 M) and 10 μl of derivatizing solution. This final reaction mixture is heated at 60°C for 60 min, then cooled at 4°C for 15 min before HPLC analysis. From 5 to 50 μl are injected. Because all fluorescent adducts are light sensitive and this behavior is different among different thiols, it is important to keep samples in the darkness after derivatization.

### 2.7. HPLC analysis

The determination of thiols was performed by an isocratic run, using acetonitrile–potassium dihydrogenphosphate buffer 0.1 M (8:92, v/v) (pH* 2.1). The buffer was filtered through a 0.22 μm filter.

The analytical column is conditioned with mobile phase at flow rate of 1 ml/min. The optimal response of derivatives is observed using a fluorimetric detector when the excitation (Ex) and emission (Em) wavelengths are set at 385 and 515 nm, respectively.

### 2.8. Statistical analysis

Cys, Cys–Gly, Hcy and GSH sample concentrations are obtained from peak area (or height) and automatically calculated by software.

## 3. Results and discussion

The present method requires only a capillary blood sampling that may be performed without further trauma for newborns; it allows the measurement of low-molecular-mass thiols in plasma simultaneously. A typical chromatogram of newborn plasma thiols is shown in Fig. 1. The retention time for Cys, Cys–Gly, Hcy and GSH were 4.39, 5.67, 7.1 and 9.41 min, respectively. All peaks were symmetrical, distinctly separated and have good resolution. Detection condition was optimized for all thiols even if GSH levels were relatively low.

We were able to confirm the excellent features of SBD-F as analytical reagent for biological thiols, because SBD-F itself and its hydrolysis products do not provide peaks in the chromatographs and it is not reactive towards amines [9]. This constitutes a major advantage with regards to monobromobimane, which has been reported to form fluorescence degradation products [10,11] and 7-fluoro-2,1,3-benzoxadiazole-4-sulfonamide (ABD-F) that is weakly reactive towards amines too.

Some authors have observed that addition of thiol standards to buffer or water or physiological solution, during preparation of the calibration curves, yielded 20% less fluorescence than when the same amounts were added to plasma [12–14]. They attributed this behavior to the presence of plasma proteins, ionic strength or other species affecting the derivatization step. Because the method reported here shows no differences in the

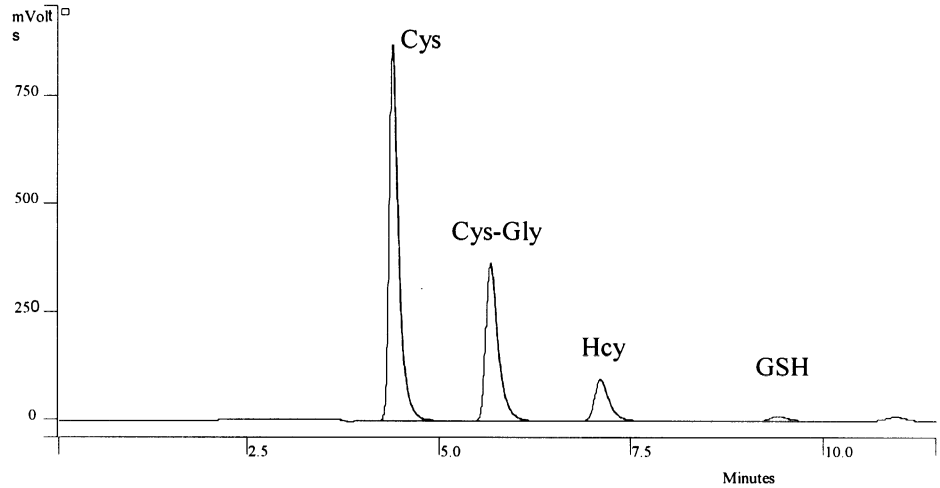


Fig. 1. HPLC determination of newborn's total plasma thiols: cysteine (Cys), cysteinylglycine (Cys–Gly), homocysteine (Hcy) and glutathione (GSH). Mobile phase: acetonitrile–potassium dihydrogenphosphate buffer (0.1 M) (8:92, v/v) (pH* 2.1). The analysis was carried out on TSK-GEL ODS-80Ts TOSOHAAS column (4.6 mm ID × 250 mm). Flow rate (1 ml/min); Ex = 385 nm and Em = 515 nm.

calibration slopes performed in water, physiological solution or plasma, it is simpler and equally effective to dilute Hcy std (calibrators) in water. This might be due to high ionic strength of borate buffer (1 M) used; in fact, when the molarity of borate buffer decreases, the difference between two calibration curve slopes increases (data not shown). The same phenomenon occurs, when the proteins in the plasma sample are precipitated by a less concentrated TCA 1%, instead of TCA 10%; therefore the calibration slopes for Hcy diluted in two different matrices are indistinguishable if borate buffer (1 M) and TCA 10% are used (Fig. 2). In this condition thiol calibration curves were linear over the range described above. The linear regression analysis were: $y = 0.023x + 4.355$, $r = 0.999$; $y = 0.005x + 0.799$, $r = 0.999$; $y = 2.41x + 0.31$, $r = 1$; $y = 0.014x - 0.66$, $r = 0.997$ for Cys, Cys–Gly, Hcy and GSH, respectively, where $y$ is the peak area (mV) and $x$, the thiol concentration (μM).

In our laboratory, the absolute detection limit, determined as three times the baseline noise level, was 0.2 pmol for tHcy, but however it is related to the fluorimetric detector performance.

To determine the within-run precision, 10 different extractions of pooled plasma samples (tHcy = 10 μM) were made on the same day. The coefficient of variation (CV%) was 2.5. Between-assay precision was calculated using the result from 10 separate determinations obtained within 1-month period; the CV% was 4.5 for Hcy. The recovery test has been performed adding known

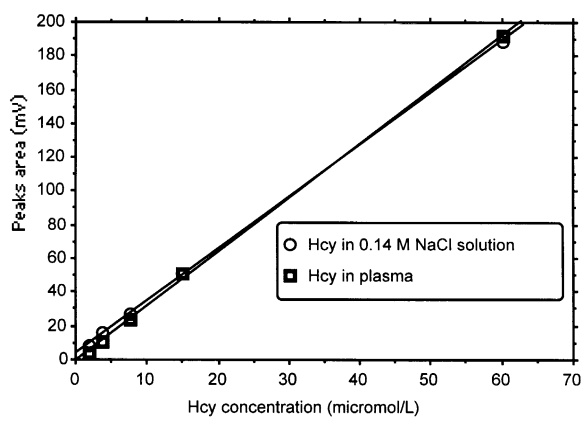


Fig. 2. Linear regression analysis performed by addition of homocysteine standard (Hcy), increasing concentration (1–60 μM) to a 0.14 M NaCl solution pH 7.2 (○) or to a plasma (□).

Hcy concentrations ranged from 1 to 30 μM to the same plasma sample ($n = 10$); analytical recovery was 98% (ranged from 95% to 102%) for Hcy. No differences have been experimentally identified when different sample volumes were injected in HPLC, using the same calibration curves (data not shown).

Moreover, this method shows a good linearity from 1 to 10 μl of plasma samples. If plasma volume is less than 10 μl, it is possible to use the same calibration curve by making up the volume with water; sample is processed as described above and injected using volumes inversely related to the starting plasma volumes. For instance if the fluorimetric detector sensitivity allows injection of 5 μl of SBD-adduct in normal condition (10 μl plasma), 50 μl will be injected if 1 μl of plasma is the starting sample volume.

In order to define the physiological plasma thiol levels, we determine Cys, Cys–Gly, tHcy and GSH in plasma samples by heparinized capillary glass tubes from 1400 healthy newborn in four living days. The mean tHcy value was $4.9 \pm 2.7$ μM.

## 4. Conclusion

This procedure represents an improvement of different HPLC methods. Due to its easiness and reliability, it is suitable for routine analysis of tHcy and other aminothiols (Cys, Cys–Gly, GSH) in biological samples. This method requires only a capillary blood sampling without further trauma for newborn.

We believe that this study might give important contributions to the knowledge of the atherosclerotic process from the neonatal age and its prevention already in a pediatric population.

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