HOMOCYSTEINE IN PLASMA BY FLUORIMETRY FAST - CODE Z09710

INTRODUCTION

Homocysteine (HCY) is a sulfurated aminoacid that derives from the demethylation of **Methionine**. (Fig. 1)

HOMOCYSTEINE

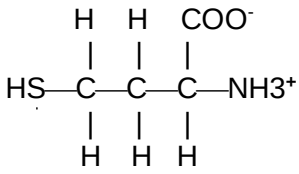


Fig. 1 : 2-Amino-4-Mercaptobutyric acid

It does not take part in the proteins synthesis. It is present in the bloodstream above all as disulfuric compounds such as **HCY-HCY (homocystine)** and **HCY-Cysteine**. These molecules can be free or bound to proteins.

BIOCHEMISTRY

It derives from **Methionine**, which is an essential aminoacid. Methionine is converted to **S-adenosylhomocysteine**, and than hydrolysed in homocysteine.

Homocysteine can be involved in two different metabolic pathways:

1) Homocysteine can be methylated again to **Methionine** by the **5-10 methylene tetrahydrofolate reductase enzyme** (5-10-Metil-THF) This reaction needs **B_{12} Vitamin** as cofactor and **N-methyl tetrahydrofolate** as donor of -CH_3 groups.

2) **Homocysteine** can be linked to **Serine** and than can be converted first in **Cystathionine** and then cysteine by the **Cystathionine β-synthethase** enzyme. This reaction needs **B_6 Vitamin** as cofactor.

Hyperhomocysteinemia can be caused by enzymatic impairment of one of these pathways. The impairment can be:

- **total**, when the enzymatic dysfunction has an **homozygote** heritage
- **partial**, when the enzymatic dysfunction has an **heterozygote** heritage

The incidence of homozygote **hyperhomocysteinemia** is estimated to be around 1:200.000. These patients have markedly increased **HCY** levels markedly increased (sometimes over 400 µM/l). Atherosclerotic cerebrovascular diseases and venous thrombosis are often present in this syndrome.

EUREKA S.R.L. LAB DIVISION
Via M. D'Antona 28
60033 Chiaravalle (AN) - Italy
Tel. +39 071 7450790
eureka-support@sentinel.it
www.eurekakit.com

CE

This product fulfills all the requirements of Directive 98/79/EC on in vitro diagnostic medical devices (IVD). The declaration of conformity is available upon request.

Mudd et al (1985) published the results of an international survey on clinic manifestation and natural evolution of 629 patients affected by deficit of cystathionine β-synthetase enzyme. Thromboembolic complications were present in 158 patients (25%). These patients had 253 thrombotic events as following:

- 51% deep venous thrombosis
- 32% acute cerebrovascular events
- 11% arterial peripheral obstructions
- 4% myocardial infarctions.

Vascular thrombotic diseases are the most common causes of premature death in patients with high HCY. It has been demonstrated that high HCY is an independent factor **of early coronary illness in man. Boushey et al. has recently demonstrated that the increase of** 5 µM/l of HCY **in plasma levels or the increase of** 20 mg/dl of cholesterol **has the same effect on cardiovascular risk.**

Moderate hyperhomocysteinemia **(heterozygote heritage) can determine only a slight increase of the fasting level of HCY. However, this condition can be better identified by an oral methionine load that can evidences a strong increase of homocysteine plasma levels respect to healthy subjects.**

Intermediate hyperomocysteinemia effects 5-7% of the Caucasian population and it is defined as an independent cardiovascular risk factor. It is potentially dangerous as few symptoms are present until the thrombotic event.
Folates, B_{12} and B_6 Vitamins intake can be successfully used in different forms of hyperhomocysteinemia. For this reason an early diagnosis is fundamental to avoid the risk of cardiac and cerebrovascular thrombotic diseases.

TECHINCAL FEATURES

Principle of the Method:

Homocysteine is detected in plasma after treatment of sample with a reducing reagent and a specific derivatization reagent at room temperature for 15 min. After deproteinization the sample is directly injected into the HPLC.

Recovery :	100%
Sensitivity :	0,2 µmol/l
Dynamic range of the method :	0,2 – 500 µmol/l
Normal values in Plasma :	5 – 15 µmol/l

Components of the kit :	All the components are ready-to-use except for **Reagents A and B** and stable 3 years at 2 – 8°C.
Reagent A – Reducing Solution, **1 x 100 mg**	(to be reconstituted with 15 ml of Reagent D)
Reagent B – Derivatization Solution, **2 x 2,5 ml**	(each vial has to be diluted with 5 ml of Reagent D)
Reagent C – Deproteinization Solution, **1 x 25 ml**	
Reagent D – Buffer Solution, **1 x 50 ml**	
Reagent E – Internal Standard, **10 x 2 mg**	To reconstitute with **10 ml of H_2O** HPLC grade and store at **2 – 8 °C**
Reagent F – Test Solution, **1 x 10 ml**	
Plasma Calibrator lyophil., 2 x 2 ml	**Code Z09016 (packed separately – see data sheets)**
Reagent M – Mobile Phase, **10 x 500 ml**	

Minimum Instrumental equipment required:	Isocratic HPLC System with loop of 20 µl Fluorimetric Detector **λ_{EX}=385 nm λ_{EM}=515 nm** Chromatograms Recorder
Optional Equipment:	Autosampler Operational Computer
Plasma Collection Procedure:	Collect 3 ml of blood with sodium citrate. Centrifuge **immediately** at 4.000 rpm for 5 min. Plasma samples should be stored at **–20 °C** for no longer than **1 month**.

PREANALYTICAL PROCEDURE

Reconstitution of **Reagent A**, **Reagent B** and **Reagent E**

- Add 15 ml of **Reagent D –** Buffer Solution to **Reagent A –** Reducing Solution
- Add 5 ml of **Reagent D –** Buffer Solution to **Reagent B –** Derivatization Sol (**Stable 6 weeks at 2–8 °C**)
- Add at **Reagent E** – Internal Standard 10 ml of H_2O HPLC grade (**Stable 3 months at 2–8 °C**)

Test Solution at about 80 µmol/l Preparation

Dispense in a tube :
- 900 µl of H_2O HPLC grade
- 100 µl of **Reagent F** – Test Solution

This is a Solution Test at about 80 µmol/l

Dilute the **Reagent E** – Internal Standard Solution 1 : 20 with H_2O HPLC grade before the analytical session.

Pipette in a vial of 1,5 ml with cap:

- 50 µl of Test Sol. 100 µmol/l
- 30 µl of **Reagent A** – Reducing Sol.
- 50 µl of **Reagent D** – Buffer Sol.
- 50 µl of **Reagent E** – Internal Standard Sol.
- 30 µl of **Reagent B** – Derivatization Sol.

Vortex for 10 seconds

- Incubate at room temperature for 15 min.

Cooling at Room Temperature

- Add 50 µl of **Reagent C –** Deproteinization Sol.

Warning: deproteinizate directly on Vortex for 10 sec.!!!

- Centrifuge at 5000 rpm for 5 min.

- Take 200 µl of clear surnatant

INJECTION :

- Inject 5-10 µl of the solution into the chromatographic system.

Verify that the Test Solution has retention time similar to fig. 2. If the Test is all right you can start with the analytical procedure; if not, check the functionality of the analytical system.

Important : Don't use this solution to calibrate!

ANALYTICAL PROCEDURE

STEP 1 : Samples Preparation

Dilute the **Reagent E** – Internal Standard 1: 20 with HPLC grade H_2O before the analytical session. ***(if the internal standard signal changes from session to session, apply less stringent dilution)***

Dispense in a tube :

	Calibrator	**Sample**
Calibrator	50 µl	
Sample		50 µl
Reagent A – Reducing Sol.	30 µl	30 µl
Reagent D – Buffer Sol.	50 µl	50 µl
Reagent E – Internal Standard Sol.	50 µl	50 µl
Reagent B – Derivatization Sol.	30 µl	30 µl

Vortex for 10 sec.

STEP 2 : Incubate at room temperature for 15 minutes

Cooling at Room Temperature

STEP 3 : Add 50 µl of **Reagent C –** Deproteinization Sol.

Warning: deproteinizate directly on Vortex for 10 sec.!!!

STEP 4 : Centrifuge at 5000 rpm for 5 min.

STEP 5 : Take 200 µl of clear surnatant

N.B.: at this step, the sample is stable 7 days at 2-8 °C

INJECTION :

- Inject 5-10 µl of the solution into the chromatographic system.

HOMOCYSTEINE IN PLASMA FAST - Warnings

REAGENT F : TEST SOLUTION

HOMOCYSTEINE	about 800 µmol/l

PARAMETERS FOR THE FLUORIMETRIC DETECTOR

λ_{EX}	385 nm
λ_{EM}	515 nm
GAIN	1 x 100
INTEGRATION TIME	5 sec.

HPLC COLUMN PROTECTION

To save the analytical column Reverse Phase POROSHELL 120 EC-C18 (50 x 4,6 mm, 2,7 µm), the use of Javelin Prefilters (1 x 10 pcs), cod. S90199511 is obligatory.

HPLC COLUMN CONDITIONING

Disconnect the detector and flux 30 ml of H_2O : Acetonitrile (20 : 80 v/v) at pH > 3 solution and subsequently 30 ml of **H_2O : Acetonitrile (80 : 20 v / v)**, set flow at 1,5 ml/min. **Don't recycle the washing solutions**. Condition the column with the mobile phase at a flow of 1,5 ml/min. and discharge the first 15 ml. Condition further on the column for 15 min. also at recycling phase. Finally inject the derivatized Chemical Standard and verify the quality of the HPLC run.
It is possible to make analysis at recycling phase, providing that you filter the same mobile phase with a filter of 0,22 µ before any analytical run. If room temperature is > 20 °C store the Mobile Phase at 2-8 °C between an analytical session and another.

COLUMN CLEANING

Disconnect the detector. Flux 30 ml of H_2O : Acetonitrile (80 : 20 v / v) and discharge. Flux a solution of H_2O HPLC grade : Acetonitrile (30 : 70 v/v) for 30 min. Discharge. A new use of the column needs a flow of 15 ml of H_2O : Methanol (20 : 80 v / v) before a new conditioning with the mobile phase. **It is recommended to run the washing of the analytical column at the end of each run.**

HPLC PARAMETERS

LOOP	20 µl
Recommended Flow rate	1,2 ml/min.
Pressure	About 120 bar

ACCESSORIES AND CONSUMABLES

CODE	DESCRIPTION	PACKAGING
Z09016	Plasma Calibrator lyophilized for Homocysteine	4 x 2 ml
Z09019	Plasma Control lyophilized for Homocysteine, Levels 1 and 2	2 x 5 x 2 ml
Z699975902	Poroshell 120, EC-C18 (50 x 4,6 mm - 2,7 um) Analytical Column	1 Pc
S90199511	Javelin Prefilters	1 x 10 Pcs
S51843550	Clear glass vials with reduced volume from 1,5 ml to 15 ul	1 x 100 Pcs
S51820717	Caps for Clear glass vials with reduced volume from 1,5 ml to 15 ul	1 x 100 Pcs

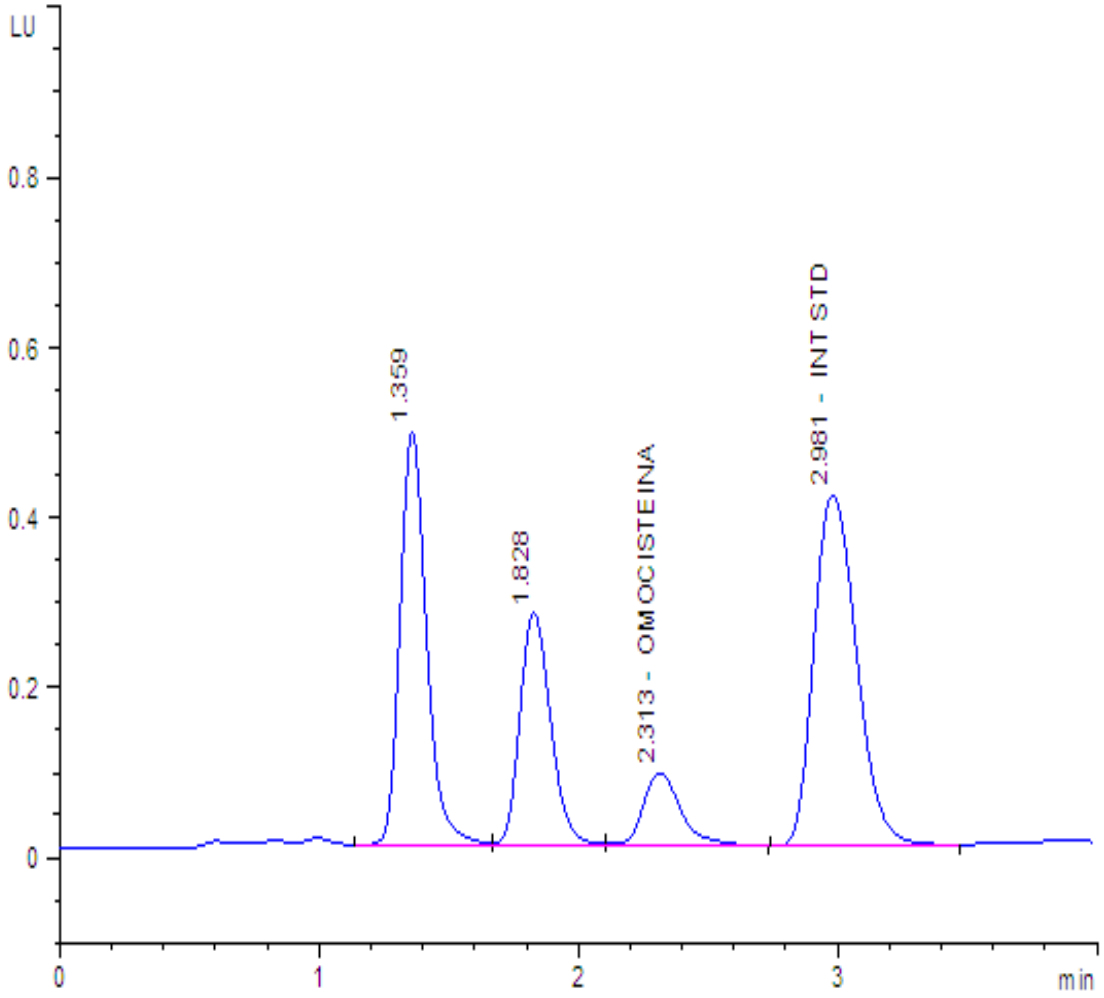
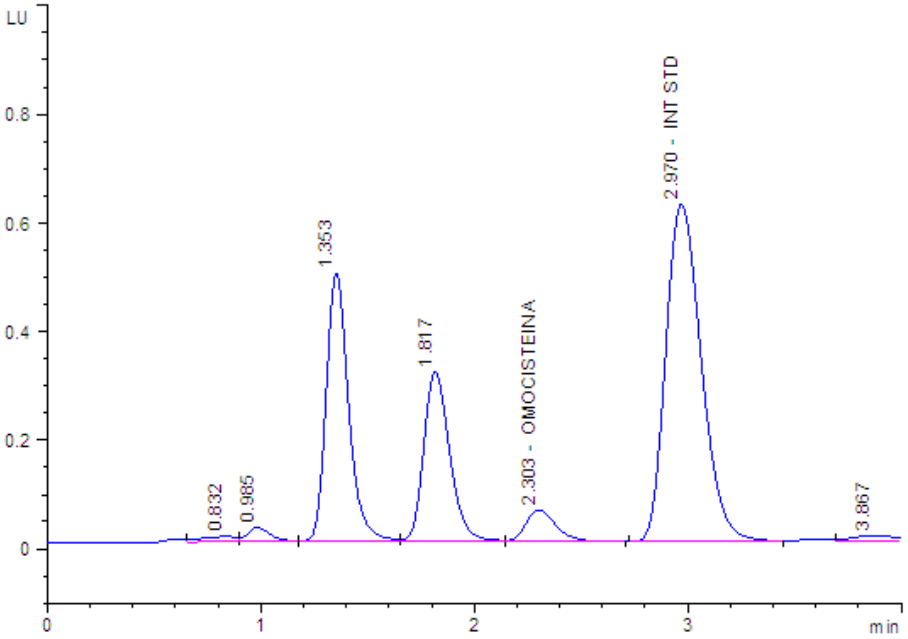


Fig. 2:	***Plasma Calibrator***		
	R.T. 2.31	Homocysteine	20 µmol/l
	R.T. 2.98	Internal Standard	

HOMOCYSTEINE IN PLASMA FAST

(Reference Chromatograms)



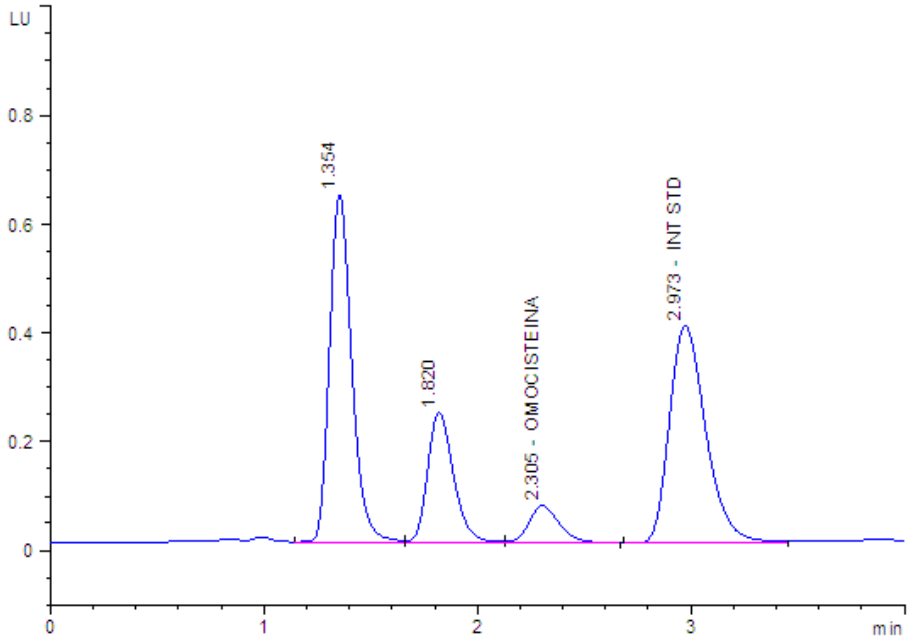


Fig. 3:	***Plasma Control Level 1***		
	R.T. 2.30	Homocysteine	8,1 µmol/l
	R.T. 2.97	Internal Standard	

Fig. 4:	***Plasma Sample***		
	R.T. 2.30	Homocysteine	15,4 µmol/l
	R.T. 2.97	Internal Standard	