

INTRODUCTION

Glutathione (GSH) is a tripeptide. It contains an unusual peptide linkage between the amine group of cysteine and the carboxyl group of the glutamate side chain. Glutathione, an antioxidant, protects cells from toxins such as free radicals. Glutathione is found almost exclusively in its reduced form, since the enzyme which reverts it from its oxidized form (GSSG), glutathione reductase, is constitutively active and inducible upon oxidative stress. In fact, the ratio of reduced to oxidized glutathione within cells is often used scientifically as a measure of cellular toxicity. Glutathione is not an essential nutrient since it can be synthesized from the amino acids L-cysteine, L-glutamate and glycine. It is synthesized in two adenosine triphosphate-dependent steps: first, gamma-glutamylcysteine is synthesized from L-glutamate and cysteine via the enzyme gamma-glutamylcysteine synthetase (a.k.a. glutamate cysteine ligase, GCL). This reaction is the rate limiting step in glutathione synthesis; second, glycine is added to the C-terminal of gamma-glutamylcysteine via the enzyme glutathione synthetase.

Glutamate cysteine ligase (GCL) is a heterodimeric enzyme composed of a catalytic (GCLC) and modulatory (GCLM) subunits. GCLC constitutes all the enzymatic activity, while GCLM increases the catalytic efficiency of GCLC. In healthy tissue, more than 90% of the total glutathione pool is in the reduced form and less than 10% exists in the disulfide form (GSSG). An increased GSSG/GSH ratio is considered indicative of oxidative stress.

Glutathione participates in leukotriene synthesis and is a cofactor for the enzyme glutathione peroxidase. It is also important as a hydrophilic molecule that is added to lipophilic toxins and waste in the liver during biotransformation before they can become part of the bile. Glutathione is also needed for the detoxification of methylglyoxal, a toxin produced as a by-product of metabolism.



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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.

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TECHNICAL FEATURES

Principle of the Method:

The Total Glutathione (oxidate + reduced) is analysed treating the whole blood with a suitable reductant and derivatizing the reduced Glutathione with a specific reagent at 55 °C for 15 minutes. After deproteinization the solution is directly injected into HPLC system.

The Reduced endogenous Glutathione is analysed derivatizing the whole blood directly (without reductant) with a specific reagent at 55 °C for 15 minutes.

After deproteinization the solution is directly injected into HPLC system.

The Oxidate Glutathione is calculated by difference from **Total Glutathione** and **Reduced Glutathione endogenous**.

Sensitivity :

5,0 mg/l

Dynamic Range of the Method :

5,0 – 1000 mg/l

Accuracy intra serie (relative error %) :

Ci	Cs
250 mg/l	750 mg/l
3,94%	6,08%

Accuracy inter serie (relative error %) :

Ci	Cs
250 mg/l	750 mg/l
7,44%	9,49%

Reproducibility intra serie (coefficient of variation %):

C LLOQ	Cm	Cs
5 mg/l	500 mg/l	1.000 mg/l
4,65%	1,65%	3,59%

Reproducibility inter serie (coefficient of variation %):

C LLOQ	Cm	Cs
5 mg/l	500 mg/l	1.000 mg/l
7,44%	10,76%	7,64%

Coefficient of Correlation + DS :

0,9913 ± 0,0045

Normal Range of Reduced Endogenous Glutathione in whole blood:

307 – 450 mg/l

Normal Range of Total Endogenous Glutathione in whole blood:

not available

<u>Components of the kit :</u>	All the components are ready-to-use, except for Reagent A and B , and stable 3 years at 2-8°C.
Reagent A – Reducing Solution, 1 x 20 mg	(To reconstitute with 3 ml of Reagent D – Buffer Sol.)
Reagent B – Derivatization Solution, 2 x 0,5 ml	(Each Reagent B must be diluted with 1 ml of Reagent D – Buffer Sol. before using)
Reagent C – Deproteinization Solution, 1 x 5 ml	
Reagent D – Buffer Solution, 1 x 25 ml	
Reagent E – Internal Standard Solution, 1 x 5 ml	
Reagent F – Test Solution, 1 x 5 ml	
Reagent G – Whole Blood Calibrator lyophil., 1 x 1 ml	<u>Code Z30016</u>
Reagent L – Lysantis Solution, 1 x 200 ml	<u>Packed separately – See data sheets</u>
Reagent M – Mobile Phase, 2 x 500 ml	

Minimum Instrumental equipment required:

Isocratic HPLC System with loop of 50 µl
 Fluorimetric Detector $\lambda_{EX}=385\text{ nm}$ $\lambda_{EM}=515\text{ nm}$
 Chromatograms Recorder
 Autosampler
 Automatic Samples Preparation
 Operational Computer

Optional Equipment:

Whole Blood Collection Procedure :

Pipette 3 ml of whole blood in a tube with anticoagulant **citrate**.
 Stable 3 weeks at 2–8 °C or 4 months at – 20 °C.

PREANALYTICAL PROCEDURE – TOTAL GLUTATHIONE in WHOLE BLOOD

STEP 1 : Reconstitution of **Reagent A** and **Reagent B**

- Add at **Reagent A – Reducing Sol.** 3 ml of **Reagent D – Buffer Sol.**
- Add at **Reagent B – Derivatization Sol.** 1 ml of **Reagent D – Buffer Sol.** (Stable 6 weeks at 2–8 °C)

STEP 2 : Test Solution at about 37,1 mg/l Preparation

Pipette in a tube :

- 900 µl of H₂O HPLC grade
- 100 µl of **Reagent F – Test Sol.**

This is a Test Solution at about 37,1 mg/l

Pipette in a 1,5 ml tube with cap:

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

Vortex for 10 sec.

- Incubate at 55 °C for 15 min.

Cooling

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

Vortex for 10 sec.

- Centrifuge at 5000 rpm for 5 min.
- Take 200 µl of clear surnatant

INJECTION :

- Inject 10 µl of this solution in the chromatographic system.

Verify that the Test Solution has retention time similar to fig. 1. 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 – TOTAL GLUTATHIONE in WHOLE BLOOD

STEP 1 : Samples Preparation

Dilute the Calibrator and the samples in this way:

- 1950 µl of **Reagent L – Lysantia Sol.** + 50 µl of Whole Blood

STEP 2 : Pipette in a tube :

	Calibrator	Samples
Reagent D – Buffer Sol.	200 µl	200 µl
Diluted Calibrator	50 µl	
Diluted Samples		50 µl
Reagent A – Reducing 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 3 : Incubate at 55 °C for 15 min.

Cooling

STEP 4 : Add 50 µl of **Reagent C – Deproteinization Sol.**

Vortex for 10 sec.

STEP 5 : Centrifuge at 5000 rpm for 5 min.

STEP 6 : Take 200 µl of clear supernatant

N.B.: at this step, the sample is stable 3 days at 2-8 °C

INJECTION :

- Inject 10 µl of this solution in the chromatographic system.

PREANALYTICAL PROCEDURE – REDUCED GLUTATHIONE in WHOLE BLOOD

STEP 1 : Reconstitution of **Reagent A** and **Reagent B**

- Add at **Reagent A – Reducing Sol.** 3 ml of **Reagent D – Buffer Sol.**
- Add at **Reagent B – Derivatization Sol.** 1 ml of **Reagent D – Buffer Sol.** (**Stable 6 weeks at 2–8 °C**)

STEP 2 : Test Solution at about 37,1 mg/l Preparation

Pipette in a tube :

- 900 µl of H₂O HPLC grade
- 100 µl of **Reagent F – Test Sol.**

This is a Test Solution at about 37,1 mg/l

Pipette in a 1,5 ml tube with cap:

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

Vortex for 10 sec.

- Incubate at 55 °C for 15 min.

Cooling

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

Vortex for 10 sec.

- Centrifuge at 5000 rpm for 5 min.
- Take 200 µl of clear supernatant

INJECTION :

- Inject 10 µl of this solution in the chromatographic system.

Verify that the Test Solution has retention time similar to fig. 1. 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 – REDUCED GLUTATHIONE in WHOLE BLOOD

STEP 1 : Samples Preparation

Dilute the Calibrator and the samples in this way:

- 1950 µl of **Reagent L – Lysantia Sol.** + 50 µl of Whole Blood

STEP 2 : Pipette in a tube :

	Calibrator	Samples
Reagent D – Buffer Sol.	200 µl	200 µl
Diluted Calibrator	50 µl	
Diluted Samples		50 µl
Water	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 3 : Incubate at 55 °C for 15 min.

Cooling

STEP 4 : Add 50 µl of **Reagent C – Deproteinization Sol.**

Vortex for 10 sec.

STEP 5 : Centrifuge at 5000 rpm for 5 min.**STEP 6** : Take 200 µl of clear supernatant

N.B.: at this step, the sample is stable 3 days at 2-8 °C

INJECTION :

- Inject 10 µl of this solution in the chromatographic system.

GLUTATHIONE in WHOLE BLOOD - Warnings

REAGENT F : TEST SOLUTION

GLUTATHIONE	371 mg/l
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SPECTROFLUORIMETRIC DETECTOR PARAMETERS

λ_{EX}	385 nm
λ_{EM}	515 nm
GAIN	1 x 100
INTEGRATION TIME	10 sec.

HPLC COLUMN PROTECTION

To save the analytical column Reverse Phase Poroshell EC C18 (4,6 x 50 mm, 2,7 μ , the use of Javelin Colfilters (1 x 10 pcs.) cod. S90199511 is obligatory.
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HPLC COLUMN CONDITIONING

Install a <u>new</u> analytical C ₁₈ Reversed-Phase column (Poroshell EC C18 (4,6 x 50 mm, 2,7 μ). Disconnect the detector and filter 30 ml of H ₂ O: Methanol (20 : 80 v/v) solution and subsequently 30 ml of H ₂ O per HPLC, set flow at 1,2 ml / min. Don't recycle the washing solutions. Filter the mobile phase with a vacuum system and a suitable filter of 0,22 μ . Condition the column with the mobile phase at a flow of 1,2 ml / min. and discharge the first 30 ml. Condition further on the column for 30 min. also at recycling phase. Finally inject the 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.
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COLUMN CLEANING

Disconnect the detector. Flux 30 ml of H ₂ O and discharge. Flux a solution made of H ₂ O : Methanol (20 : 80 v/v) for 30 min and discharge. When you re-use the column, flux 30 ml of H ₂ O before a new conditioning with the Mobile Phase.
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INJECTION NEEDLE WASHING

Wash with a solution of H ₂ O / Ethanol (75 : 25 v/v).

HPLC PARAMETERS

LOOP	50 μ l
FLOW	1,2 ml/min.
PRESSURE	About 150 bar

ACCESSORIES AND CONSUMABLES

CODE	DESCRIPTION	PACKAGING
Z30016	Calibrator for Glutathione in whole blood	4 x 1 ml
Z30019	Control for Glutathione in whole blood - Levels 1 and 2	2 x 5 x 1 ml
Z699675902	Poroshell 120 EC C18 (50 x 4,6mm -2,7 μ m) Analytical Column	1 Pc
S90199511	Javelin Colfilters	1 x 10 Pcs
S51843550	Clear glass vials with reduced volume from 1,5 ml to 15 μ l	1 x 100 Pcs
S51820717	Caps for Clear glass vials with reduced volume from 1,5 ml to 15 μ l	1 x 100 Pcs



GLUTATHIONE IN WHOLE BLOOD (Reference Chromatograms)

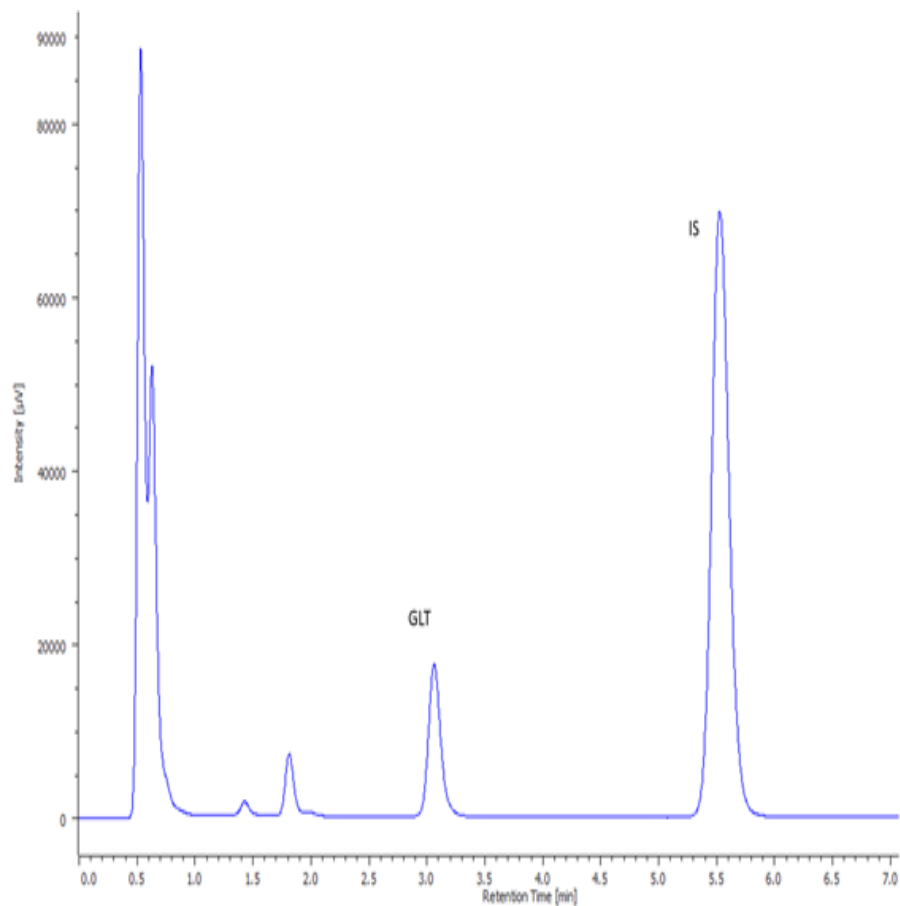


Fig. 1 :	Calibrator in whole blood
	R.T. 3.1 Total Glutathione 250 mg/l
	R.T. 5.6 Internal Standard

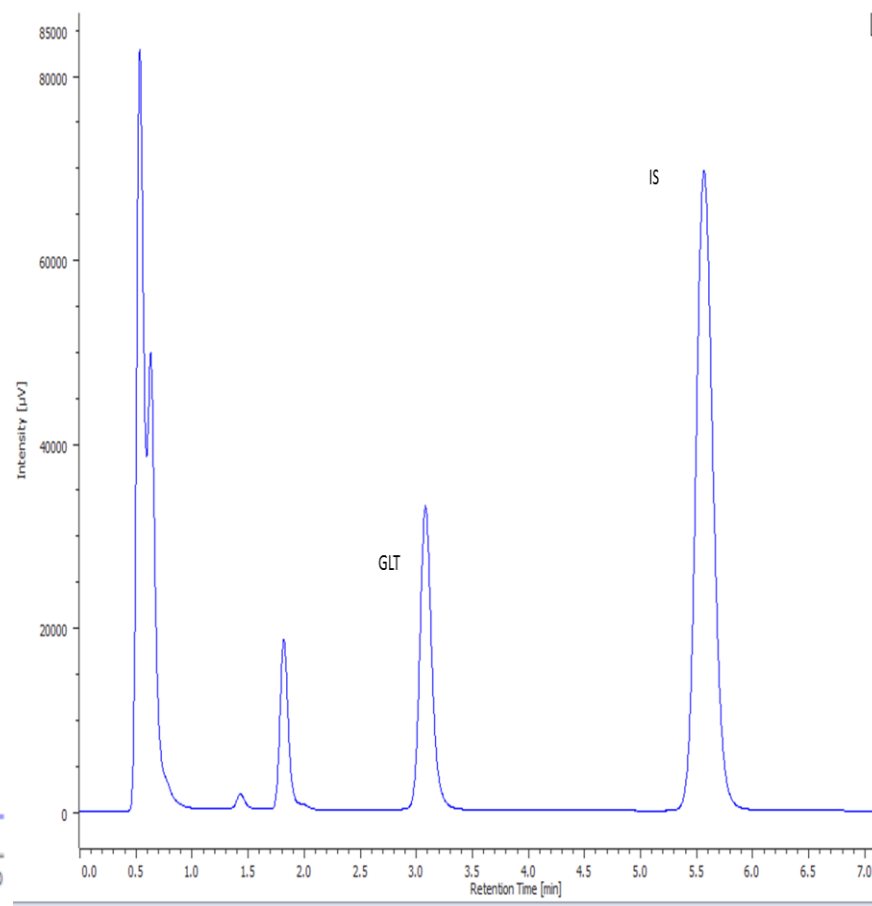


Fig. 2 :	Calibrator in whole blood
	R.T. 3.1 Total Glutathione 500 mg/l
	R.T. 5.6 Internal Standard

GLUTATHIONE IN WHOLE BLOOD (Reference Chromatograms)

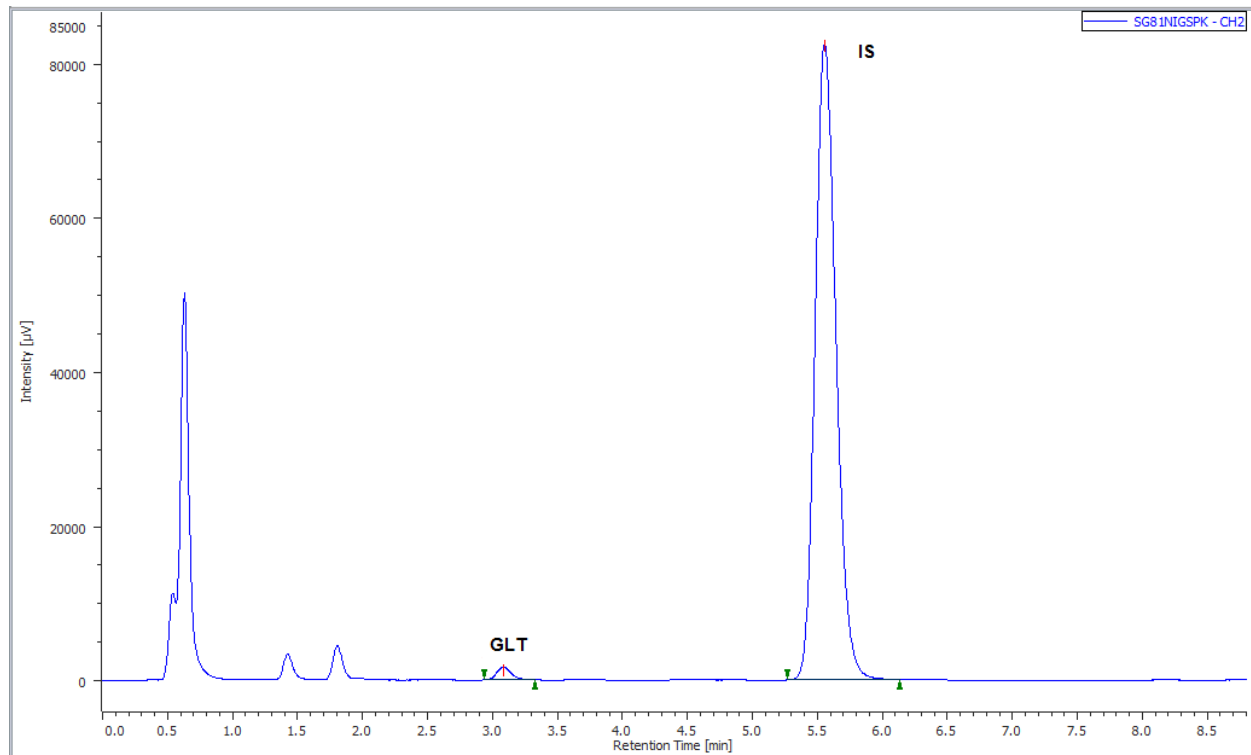


Fig. 3 :	Calibrator in whole blood
	R.T. 3.1 Total Glutathione
	R.T. 5.6 Internal Standard