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Improved Sample Processing to Optimize Stability for Total Glutathione Determination in Human Plasma using a Fit-For-Purpose Approach

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PURPOSE

Glutathione determination is highly impacted by its short half-life in human plasma, which is caused by oxidation and enzymatic degradation. Therefore, the collection procedure and the storage temperature of the human plasma samples are of high importance. The purpose of this study was to develop and validate a biomarker assay for the quantitative determination of total glutathione in human plasma using a fit-for-purpose approach.

OBJECTIVE(S)

- Improve the stability of total glutathione in human plasma
- Optimize the collection procedure and
- Validate a fit-for purpose method to support a biomarker category I assay

METHOD(S)

Glutathione colorimetric assay kit from Biovision (K261-100) was used for the validation of total glutathione in human plasma, based on the glutathione recycling system.

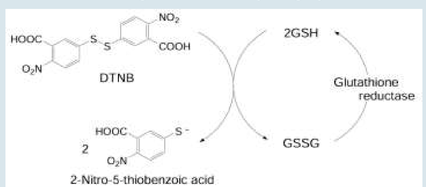

Fig. 1. Glutathione recycling system by DTNB (glutathione substrate) and glutathione reductase.

- Treatment of human plasma samples with 5% SSA prior analysis
  - Remove proteins
  - Prevent oxidation of GSH
- Addition of samples to a microplate
- Addition of Glutathione reductase, Glutathione Reaction Buffer and NADPH
- Addition of Substrate solution (DTNB)
- Reaction of DTNB and glutathione (GSH), which generate 2-nitro-5-thiobenzoic acid (yellow color).
- Measurement of intensity of the color at 405 nm
- Concentrations are calculated against a no-weighting 4-Parameter Logistic curve fit with a theoretical concentration range of 1.003 µM to 13.002 µM.

RESULT(S)

Impact of 5% SSA addition on GSH Stability

When added to samples, 5% SSA is reported to:

- Reduce the auto-oxidation
- Reduce the enzymatic degradation by γ-glutamyl transpeptidase

The impact of adding 5% SSA into freshly collected human plasma, maintained on ice following the sample processing was investigated in two different donors. The data demonstrated:

- A reduction of 13.4% and 22.7 % of total GSH when the SSA is added after 17 minutes as opposed to 7 minutes
- Its importance for the GSH stability

Freshly Collected Human Plasma GHS Concentration at Day 0								
Time of addition of 5% SSA (minutes)	7		17		37		85	
Donor ID	1	2	1	2	1	2	1	2
Observed Concentration (µM)	2.39	3.97	2.07	3.07	1.72	NA	1.53	1.60
Recovery (%)	**NA**		**86.6**	**77.3**	**72.0**	**NA**	**64.1**	**40.3**

* Reference Value

Fig. 2. Recovery (%) of GSH following the addition of SSA at different time points on freshly collected human plasma samples maintained on ice

Impact of Process of Collection on GSH Concentration

Fig. 3. Concentration of freshly collected plasma from 2 donors using K_2 EDTA and ADC Vacutainers at different conditions: Immediately frozen on dry ice or kept on ice

RESULT(S)

Due to the difficulty of reproducing this collection procedure at all the clinical sites within the required timeframe of 7 minutes, the following was performed to enable a consistent approach for collecting samples and stabilizing their total GSH concentration in a uniform way:

- Rapid freezing (-80°C nominal) of the freshly collected plasma samples into single use aliquots at the clinical sites.
- Immediate addition of the 5% SSA to samples upon thawing at the bioanalytical lab.

Stability in different collection tubes

Stabilities for Human Plasma Collected in ACD Vacutainer Tubes				
	Short-Term in ice/water bath (60 min)	Short-Term in ice/water bath (257 min)	Freeze-Thaw (2 cycles)	Long-Term at -80°C nominal (327 days)
Nominal Conc. (µM)	2.005	2.505	2.505	2.005
Observed Conc. (µM)	1.901	2.091	2.697	1.913
%C.V.	4.4	5.4	4.2	3.2
% Nominal	**94.8**	**83.5**	**107.6**	**95.4**

Stabilities for Human Plasma Collected in K2 EDTA Vacutainer Tubes				
	Short-Term in ice/water bath (60 min)	Short-Term in ice/water bath (257 min.)	Freeze-Thaw (2 cycles)	Long-Term at -80°C nominal (327 days)
Nominal Conc. (µM)	2.187	3.115	3.115	2.187
Observed Conc. (µM)	2.288	1.230	3.055	2.324
%C.V.	6.0	15.3	4.9	3.1
% Nominal	**104.6**	**39.5**	**98.1**	**106.2**

Fig.4. Stability in ACD and K_2 EDTA collection tubes

The resulting stability evaluations demonstrated acceptable stability for both collection tubes, with an improved performance for the ACD solution A collections tubes for short-term stability at 4°C nominal.

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VALIDATION

Glutathione Validation Summary*			
Proxy QCs Between-run	Accuracy 96.5% to 99.1%	Precision 7.9% to 9.9%	Total Error 9.4% to 13.4%
Proxy QCs Within-run	Accuracy 87.7% to 89.2%	Precision 0.8% to 2.2%	Total Error 12.4% to 14.0%
Matrix QCs in K_2 EDTA Vacutainer Between-run	Accuracy 100.5% to 101.0%	Precision 4.8% to 6.9%	Total Error 5.8% to 7.5%
Matrix QCs in K_2 EDTA Vacutainer Within-run	Accuracy 99.3% to 105.5%	Precision 1.7% to 2.3%	Total Error 3.0% to 7.2%
Matrix QCs in ACD Vacutainer Between-run	Accuracy 99.9% to 101.7%	Precision 4.0% to 5.9%	Total Error 4.1% to 7.6%
Matrix QCs in ACD Vacutainer Within-run	Accuracy 104.7% to 107.0%	Precision 0.6%	Total Error 5.3% to 7.6%
Whole Blood Stability	K_2 EDTA Vacutainer QCs: up to 1.0 hour in an ice/water bath		ACD Vacutainer QCs: up to 0.5 hours in an ice/water bath
Freeze and Thaw Stability	K_2 EDTA Vacutainer QCs: 2 cycles		ACD Vacutainer QCs: 2 cycles
Short-term Stability in human plasma	K2 EDTA Vacutainer QCs: up to 1.0 hour in an ice/water bath		ACD Vacutainer QCs: up to 4.2 hours in an ice/water bath
Long-term Stability in human plasma	K_2 EDTA Vacutainer QCs: up to 327 days at -80°C nominal		ACD Vacutainer QCs: up to 327 days at -80°C nominal

*Hook effect, parallelism, selectivity were not evaluated due to short-half of Glutathione.

CONCLUSION(S)

The validation of the Total Glutathione biomarker assay with acceptable stability is very challenging due to its short half-life in plasma. We have demonstrated that improving the sample processing steps during the collection of the samples at the clinical sites, as well as the addition of 5% SSA into the plasma samples immediately upon thawing to prevent GSH degradation, both significantly enhanced the determination of Total Glutathione in human plasma using an enzymatic colorimetric method and allowed its successful validation as a biomarker category I.