GLUTATHIONE AND OTHER DISULFIDES

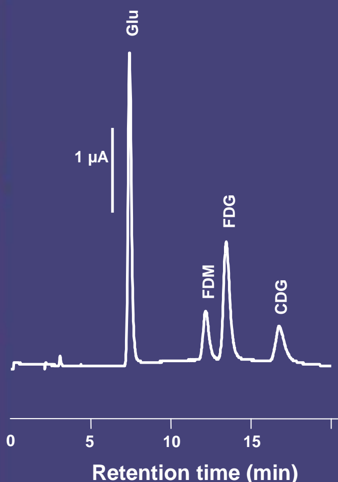
THE MOST RELIABLE LC-EC APPLICATIONS FOR PHARMACEUTICAL & BIOTECH ANALYSIS EVER FORMULATED

Aminoglycosides
- *Amikacin*
- *Framycetin Sulphate*
- *Gentamicin Sulphate*
- *Kanamycin Sulphate*
- *Lincomycin*
- *Neomycin*
- *Spectinomycin*
- *Tobramycin*

PET imaging tracer
- *FDG*

Macrolide antibiotics
- *Azithromycin*
- *Azaerythromycin*
- *Clarithromycin*
- *Erythromycin*
- *Roxithromycin*

Bioanalysis of pharmaceutics
- *Artemisinin*
- *Dihydro-artemisinin*
- *Artemether*
- *Etoposide*
- *8-OH-DPAT*
- *mesna BNP7787*
- *Vincristine*



INTRODUCTION

Glutathione (GSH) is a tripeptide composed of glutamate, cysteine and glycine that has numerous important functions within cells. Glutathione is involved in detoxification, it binds to toxins, such as heavy metals, solvents, and pesticides, and transforms them into a form that can be excreted in urine or bile. Glutathione is conjugated to drugs to make them more water soluble, is involved in amino acid transport across cell membranes (the γ-glutamyl cycle), is a part of the peptidoleukotrienes, serves as a cofactor for some enzymatic reactions and as an aid in the rearrangement of protein disulfide bonds.

- In compliance with the United States Pharmacopeia
- Flexcell with exchangeable gold electrode
- Analysis of main substituent and impurities
- Reproducible & Robust

Summary

In this publication the analysis of GSH, GSSG and several other (di-)thiols is described using a DECADE II electrochemical detector with Dual Cell Control (DCC). The DCC option makes it possible to control two electrochemical cells and use them for data acquisition.

Fig. 1. ALEXYS Disulfides analyzer.

The role of GSH as a reductant (antioxidant) is extremely important particularly in the highly oxidizing environment of the erythrocyte. The sulfhydryl of GSH can be used to reduce peroxides formed during oxygen transport. The resulting oxidized form of GSH consists of two molecules disulfide bonded together (GSSG). The enzyme glutathione reductase utilizes NADPH as a cofactor to reduce GSSG back to two moles of GSH. Hence, the pentose phosphate pathway is an extremely important pathway of erythrocytes for the continuing production of the NADPH needed by glutathione reductase. In fact as much as 10% of glucose consumption, by erythrocytes, may be mediated by the pentose phosphate pathway.

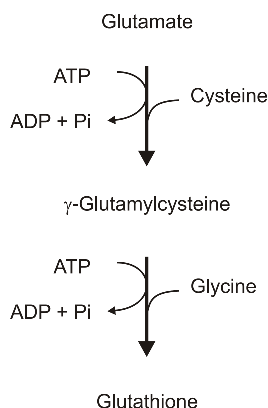
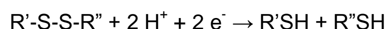


Fig. 2. Synthesis of Glutathione.

The ratio of GSH/GSSG can be used to indicate the oxidative stress in vivo. The measurement of GSH, GSSG, Cysteine, Cystine and other thiol and disulfides levels in biological samples is essential for the redox and detoxification status of cells and tissue.

Method

An ALEXYS 100 micro LC-EC system has been used with a reactor cell for *reduction* of disulfides to thiols.



$$R'\text{-}S\text{-}S\text{-}R'' + 2\,H^{+} + 2\,e^{-} \rightarrow R'SH + R''SH$$

In the second cell thiols are detected by *oxidative* amperometric detection. Thiols as well as the oxidised free disulfides can be quantified this way, which is not possible when a pre-column reduction of disulfides is applied.
The HPLC system consisted of an ALEXYS 100 Disulfides I system.

Experimental conditions are summarised in Table 1 unless specified otherwise.

Table 1

Conditions	
HPLC system	ALEXYS 100 Disulfides I (p.n. 180.0068)
Flow rate	50 µL/min
Toven	35 °C (separation and detection)
Reactor cell	GC electrode vs. Hy-REF reference elec-
Second cell	FLEXCELL with gold WE and Hy-REF
Range	100 nA/V
ADF	0.5 Hz

Results

In this work we have chosen for microbore HPLC with a 1 mm ID column. The electrochemical conversion of a reactor cell is higher at low (microbore) flow rates which is a pre-requisite for serial red-ox or ox-red configurations.
First, detection settings are optimised for the analysis of glutathione, cystine, and homocystine, followed by a linearity and reproducibility study. To improve reproducibility a working electrode cleaning pulse was applied. Optimisation of mobile phase pH, and ion pairing agent has been investigated.

Optimisation of working potential

Optimum electrode potential for oxidative analysis of glutathione, cysteine and homocysteine was 600 mV vs. ISAAC. This corresponds to about 550 mV vs. HyREF.

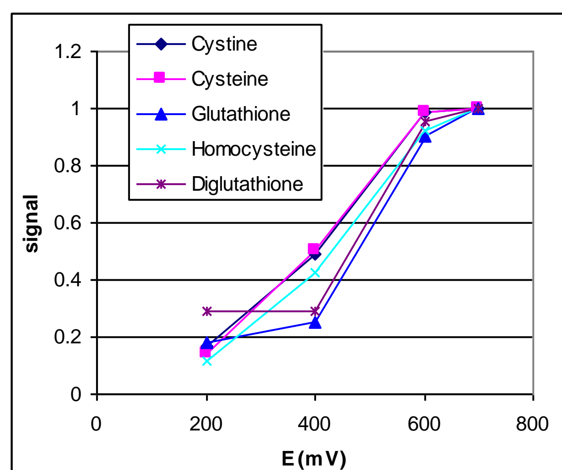


Fig. 3. Normalised IE-curves for oxidative detection of thiols.

The optimum working potential for the reactor cell was found to be -1.4 to -1.6 V.

Cleaning step for the gold electrode

For detection of thiols a FLEXCELL with gold electrode is applied. Electrode contamination and oxidation processes compromise reproducibility, therefore the gold electrode surface has to be regenerated regularly. At the start of each run a short cleaning pulse is applied of +1 V and -1 V for 3 s (Fig. 4). This potential step is programmed in the DC mode events table of the ALEXYS software. In about 10 minutes the baseline is stabilised again.

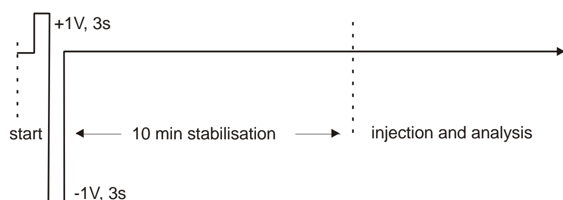


Fig. 4. Schematic representation of a cleaning pulse.

Chromatography

Separation of amino acids is affected by pH, concentration ion pairing reagent and percentage modifier. Decreasing the mobile phase pH below 4 which is the pKa of carboxyl group, will result in an increase of retention times (Fig. 5 upper two traces). Amino acids with a free amine group will always protonated at acid pH. Retention times of these substances will increase with the concentration of ion pairing reagents (Fig. 5). However, when more functional groups are involved the retention behaviour becomes less predictable. For example, at higher OSA concentration retention of di-glutathione decreases (Fig. 5, top and third trace).

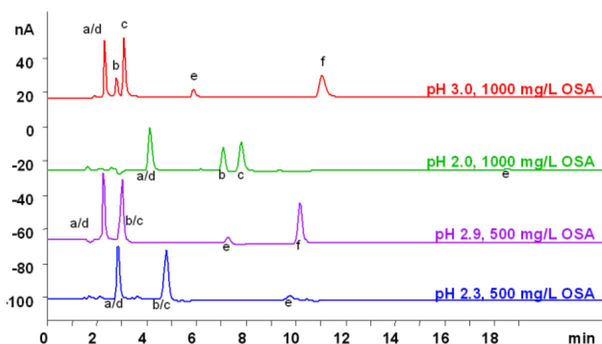


Fig. 5. Effect of pH on retention times of CSH (a), GSH (b), HCSH (c), CSSC (d), GSSG (e) and HCSSCH (f) analysed at two different mobile phase OSA concentrations and further conditions according to Table 1.

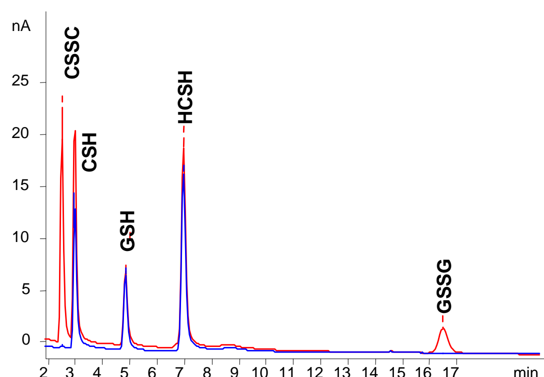


Fig. 6. Fig. 4. Analysis of 2 µL 1 µM cystine (CSSC), cysteine (CSH), GSH, homocysteine (HCSH) and GSSG in mobile phase with reactor on (red) or off (blue).

Reproducibility

Reproducibility has been investigated for 10 injections (2 µL) of 1 µmol/L CSSC, GSH and HCSH. Before each run a cleaning pulse was applied as described above. The RSD was between 2 and 7 % for peak heights and between 2 – 6 % for areas. In case of strongly tailing peaks reproducibility of peak area's was influenced by integration, and manual integration is advised.

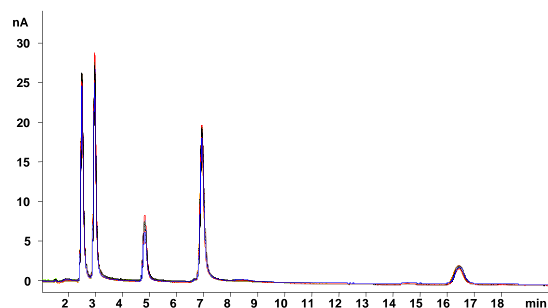


Fig. 7.Overlay of 10 chromatograms of 1 uM CSSC, CSH, GSH, HCSH and GSSG (peaks from left to right) dissolved in mobile phase. Injection volume 2uL (partial loop fill).

Table 2

Reproducibility

	Retention		Height		Area	
	tr (min)	RSD (%)	h (nA)	RSD (%)	A (nA.s)	RSD (%)
CSSC	2.48	0.2	22.8	3.2	168.9	2.7
CSH	2.95	0.2	24.7	3.1	195.4	3.6
GSH	4.8	0.1	6.8	6.5	69.6	6.0
hCSH	6.92	0.1	18.8	2.7	233.4	2.8
GSSG	16.42	0.1	2.2	5.2	55.5	5.0
CSSC	2.48	0.2	22.8	3.2	168.9	2.7

Linearity and LOD

The linearity was investigated in the range of 0.1 - 1 µmol/L (0.1, 0.25, 0.5, 0.75 and 1 µmol/L) for CSSC, CSH, GSH, HCSH and GSSG. Correlation coefficients of 0.998 – 0.9994 were found for peak heights. Linear regression data is given in Table 3.

Table 3

Linearity Y = a + bX

	R	Slope b	Intercept a
CSSC	0.99949	22.8 ± 0.4	0.23 ± 0.3
CSH	0.9998	25.1 ± 0.3	-0.67 ± 0.2
GSH	0.99836	6.88 ± 0.2	-0.27 ± 0.1
hCSH	0.99943	19.5 ± 0.4	-0.47 ± 0.2
GSSG	0.99661	2.16 ± 0.1	-0.03 ± 0.06

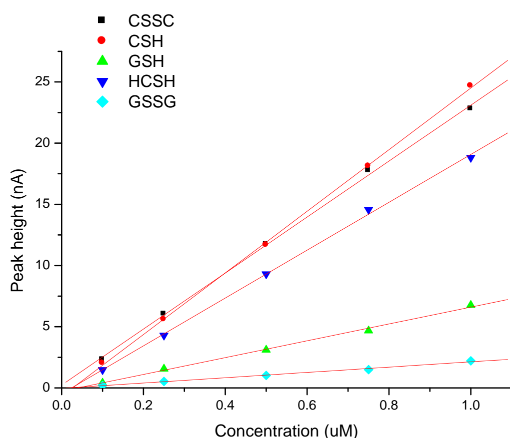


Fig. 8. Calibration lines for CSSC, GSH and HCSH in the range of 0.1 - 1 µmol/L.

Detection limits were calculated as the concentration resulting in a signal that is 3 times the peak-to-peak noise of the baseline. For CSSC, GSH and HCSH a detection limit of 10 nmol/L was found.

Conclusion

Thiols as well as the oxidised free disulfides can be quantified using the 'ALEXYS 100 Disulfides I'. A cleaning step for the gold working electrode improves the reproducibility. A detection limit down to 10 nmole/L has been achieved.

PART NUMBERS	
180.0068B	ALEXYS Disulfides Analyzer
250.1114	ALF-115, 150 x 1 mm (ID), particle size 3 µm

Antec Leyden B.V. - Industrieweg 12 - 2382 NV - Zoeterwoude - The Netherlands - info@myantec.com - www.myantec.com
Antec (USA) - P.O. Box 111418 - Palm Bay FL 32911 - USA - info.usa@myantec.com - www.myantec.com