Establishing an ECD(Electro Chemical Detector)-HPLC System Using a Unique Diamond Electrode
High-precision Quantitative Analysis of SAA (Sulfur Amino Acids)

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Summary

We have established an ECD-HPLC system equipped with a special stabilization-treated conductive diamond electrode, which provides high-precision, stability, selectivity and efficiency compared to the existing ECD detectors.

Existing ECD detectors widely adopt a glassy carbon or graphite electrode as a working electrode, but have the following weaknesses.

· Unsuitable for quantitative analysis as the impurities become adsorbed to the working electrode, resulting in low stability with wide sensitivity variation.

· High-voltage cannot be applied to the electrode, which results in lack of sensitivity.

Conductive diamond electrode has received a lot of attention to overcome the above weaknesses and there has been a report that it produces stable results.

However a sensitivity variation was confirmed on compounds such as SAA, resulting in lack of precision of quantification.

Therefore we have developed a special stabilization-treated conductive diamond electrode and established an ECD-HPLC system as stated earlier.

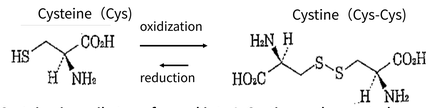
This system was applied to SAA analysis in pharmaceuticals and in biological samples. Existing analytical methods of SAA in infusion solutions cannot quantify an SH-group cysteine and SS-group cystine at once, hence it is analyzed separately which is a time consuming method. Using this system combined with a column switching method, both cysteine and cystine could be analyzed at once within 20 minutes and with high accuracy.

Meanwhile, SAA in biological samples are typically analyzed using ECD equipped with a carbon electrode or fluorescence derivatization method. In the former case, quantitative analysis is unsuitable as has been mentioned. In the latter case, it lacks in stability as there are many complicated sample preparation steps.

By using this system, cysteine, cystine, glutathione and homosysteine could be analyzed simultaneously and in accordance with FDA guidance (2001 May).

1

Relationship between Cysteine and Cystine



Cysteine is easily transformed into L-Cystine under neutral or weakly alkaline conditions.

3

Back Ground

●Signification

Nutrition solutions for infusion contain many kinds of ingredients such as amino acids, glucose and electrolyte. The analysis of L-Cysteine and L-Cystine are important in order to assure the quality of the products, because L-Cysteine is an unstable amino acids and is known to be quickly oxidized into L-Cystine under neutral or weakly alkaline condition (See slide 3). But for the same reason the analysis of these compounds were complex and time-consuming.

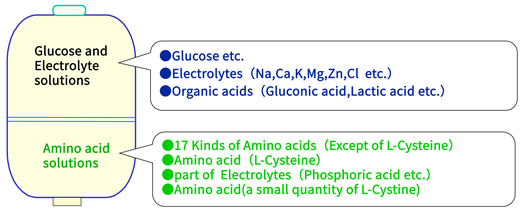
●Original method in nutrition solutions

L-Cysteine: Ultraviolet and Visible Adsorption Spectrophotometry

L-Cystine: Amino Acid Analysis (post-column method : Ninhydrin)

●Characteristic of Original method in nutrition solutions

① Unsimultaneous analysis method
② Long analysis time
③ Complicated pretreatment



●To improve the method of L-Cysteine and L-Cystine

Minimum Requirement

① Simultaneous analysis of L-Cysteine and L-Cystine
② High robustness
③ Short analysis time
④ Easy pretreatment

Our first Choice was ECD-HPLC system. But the Robustness of carbon electrode ECD was not so good.
So we had tried a new ECD equipped with “Diamond electrode” with bio-analysis team and the manufacture of the products.

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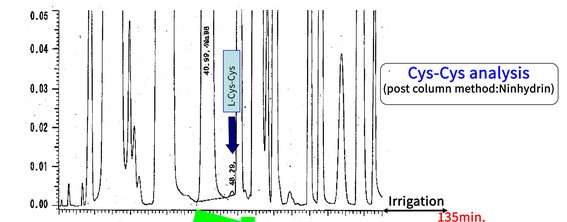
Establishing a New method for Cys and Cys-Cys
Combination of ECD equipped with diamond electrode and column switching system

Original Method

Cys-Cys analysis (Amino Acid Analysis)
Cys analysis (Ultraviolet and Visible Adsorption Spectrophotometry)

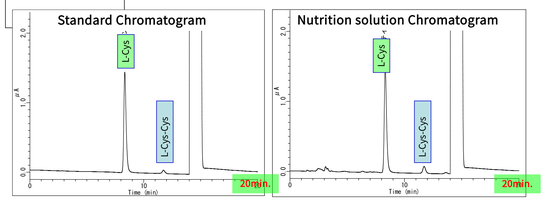


Cys analysis (Unsimultaneous method)

Achieving simultaneous analysis (Short analysis time and simultaneous method)

New method

Stable baseline and Good peak shapes



Analytical Conditions : See Slide 14

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Validation of new method for nutrition injection formulation
According to ICH guideline Q2A and Q2B

<Validation Study>

All items met the criteria according to ICH guideline

Characteristics	L-Cysteine	L-Cystine
Specificity	Good	Good
Linearity (Correlation Coefficient)	0.9999	0.9999
Accuracy (Recovery)	99.8～102.9%	99.3～100.9%
Repeatability	0.9%	0.7%
Intermediate Precision	Good	Good
Quantitation Limit (μg/mL)	0.16	0.0074

<Cross-Validation>

There were no difference between the original and new method !!

Sample	L-Cysteine		L-Cystine	
	Original Method	New Method	Original Method	New Method
	Conc.	Conc.	Conc.	Conc.
Nutrition solutions	100.2 101.0 99.3	98.6 98.3 99.7	2.1 1.2 1.6	1.1 1.1 1.2

Comparison of Original method and New method

Items	Original Method	New Method
Simultaneous method	Compatible	Not Compatible
Analysis time	Cysteine: 360min. Per 20 Samples Cystine : 135min. Per 1 Sample	20min. Per 1 Sample
Number of Analytes per Day	Cysteine: 20 Samples Cystine : 11 Samples	Cysteine and Cysitine: 70 Samples
Working day per Worker	6 days	1.5 days

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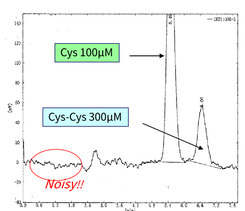
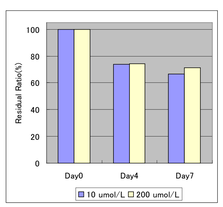
Evaluation of the traditional method
Cys and Cys-Cys analysis in plasma using ECD detector equipped with carbon electrode



Chromatogram of rat plasma
（applied voltage： 900 mV）
Noise level was high and the response of Cys-Cys was low.



Variation of Cys response in rat plasma （applied voltage： 500 mV）
The response decreased about 30% with in just a week !!

Column : Inertsil ODS-3 3 mm i.d.X150mm 3um(GL-Sciences)
Column temp. : 40°C
Solvent :100mM NaH_2PO_4-5mM OSA *Buffer pH2.2 /MeOH = 95/5 (v/v)
Flow rate : 0.8mL/min
Pretreatment : deprotonation using $HClO_4$
*OSA: Octanesulufonic Acid

Weak Point !
1. Need high applied voltage for Cys-Cys Analysis
>>> Low S/N sensitivity
2. Low robustness even at low applied voltage for Cys analysis

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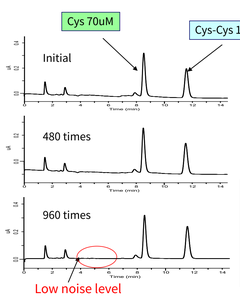
Evaluation of a new method
Cys and Cys-Cys analysis in plasma using ECD detector equipped with diamond electrode

Comparison of baselines and peak areas by continuous analysis (over 13 days) of rat plasma



Cys 70umol/L	0hr (initial)	160hr (480times)	320hr (960times)
Area(uV/sec)	1994126	1942709	1935035
Decreasing Ratio (%)		2.58	2.96

Cys-Cys 15umol/L	0hr (initial)	160hr (480times)	320hr (960times)
Area(uV/sec)	1577049	1524578	1505626
Decreasing Ratio (%)		3.33	4.53

* Analytical conditions : See Slide 14

Remarkable tolerance as an Electro Chemical Detector !!!

- Low Noise level
- Remarkable Tolerance
- High Sensitivity especially for –SS- compounds

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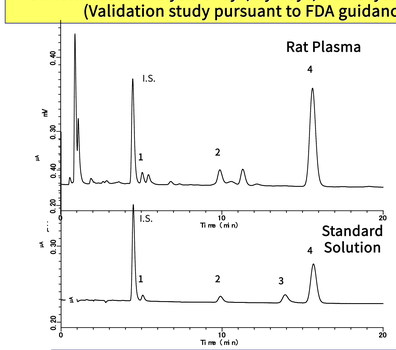
Application for biological samples
Simultaneous analysis of Cys, Cys-Cys, Homosysteine(Hcy)、reduced glutathione (GSH) in rat plasma
(Validation study pursuant to FDA guidance for Bio-Analysis (2001 May))



1 Cystein :Cys (6uM) 2 Reduced Glutation GSH (3uM)
3 Homocystein: Hcy (6uM) 4 Cystine : Cys-Cys (15uM)
I.S. :Internal Standard ( ):Concentration of Standard Solution

Column : Inertsil ODS-3 3.0mm i.d.X100mm 3um(GL Sciences)
Pre-Column : Inertsil ODS-3 3.0mm i.d.X 10mm 3um (GL Sciences)
Column temp. : 45 C
Solvent : 25mM H_3PO_4-20mM Heptanesulufonic Acid/CH_3CN = 98.5/1.5 (v/v)
Flow : 0.75 mL/min
Detect : ECD with Diamond electrode, Applied voltage 1600mV
(On-Line Reproduction 4000mV for 1min.)
injection : 10uL
Pre-Treatment : deprotonation using $HClO_4$ + diluted with solvent

Evaluation of Linearity:Spiked Plasma Sample

Criteria of FDA Guidance :Accuracy :85 - 115%

Cys		GSH		Hcy		Cys-Cys	
Conc. (umol/L)	Accuracy (%)	Conc. (umol/L)	Accuracy (%)	Conc. (umol/L)	Accuracy (%)	Conc. (umol/L)	Accuracy (%)
6	98.3	3	100.4	6	103.9	15	99.4
12	104.3	6	99.7	12	96.3	30	102.4
30	104	15	100.2	30	99	75	97
60	102.4	30	100.2	60	102	150	86.8
120	96.9	60	98.8	120	99.4	300	
300	91	150	100.5	300	100	750	108.4
Waight	$1/X^2$	Waight	$1/X^2$	Waight	$1/X^2$	Waight	$1/X^2$

< How to estimate accuracy of endogenous compound>

Low limit quantification should be set at 1/2-1/3 of endogenous concentration.

Spike
Endogenous
} Measured Value of Spiked sample
−
Endogenous
} Measured Value of Blank sample

Spike
} Theoretical Value

<Evaluation of Intra-day Precision : Spiked plasma samples>

Criteria of FDA Guidance : Accuracy 85 - 115% , Precision <15%

Cys			GSH			Hcy			Cys-Cys		
Conc. (umol/L)	Accuracy (%)	Precision (%)	Conc. (umol/L)	Accuracy (%)	Precision (%)	Conc. (umol/L)	Accuracy (%)	Precision (%)	Conc. (umol/L)	Accuracy (%)	Precision (%)
12	99.7	6.9	6	86	7.5	12	96.2	3.7	30	100.5	6.7
60	103.2	2.2	30	99.4	3.5	60	103.5	2.5	120	112	9.7
300	93	5.5	150	101.8	3.5	300	100.9	3.4	750	110.3	3.6

Normal concentrations in rat Plasma
Cys:10~15umol/L、GSH: <10umol/L、Hcy:<1umol/L(Under LOD)、Cys-Cys:20~30umol/L

The quantitative ability for Cys, GSH and Cys-Cys were demonstrated at normal concentration level of rat plasma.
>>> Slight variation of concentration were observed because of pathological condition or dosing would be detected

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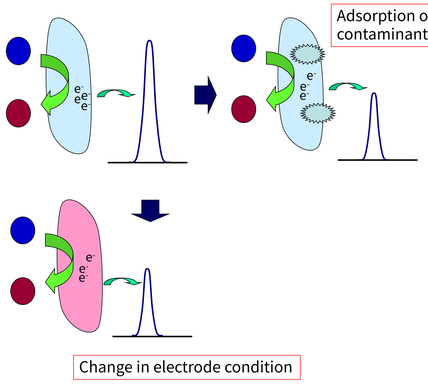
Overcome the existing problems of ECD by the state-of-the-art technology !!

Major causes led to irreproducibility of electrochemical detector

During redox reactions, the electrode surface can be deteriorated/contaminated by reduced or oxidized products, resulting in low sensitivity and unstable response.

Model of irreproducible results caused by deterioration/contamination of the electrode surface



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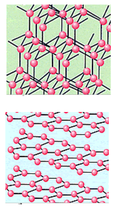
New technology 1： On-Line Cleaning using High Voltage

Advantage of Diamond electrode : Solidity for high voltage!!

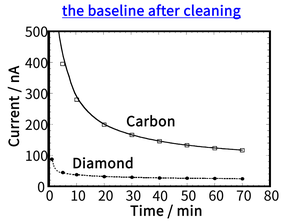
Diamond
High potential
⇨ SP^3 carbon structures

Carbon
Limited potential
⇨ SP^2 carbon structures



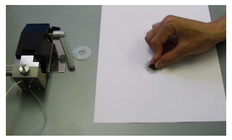
Ref : http://www.courtside.co.jp/racket/dunlop/rim40.htm

Extremely fast to stabilize the baseline after cleaning



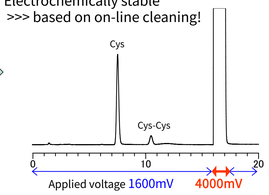
Regeneration of electrode activity

Conventional carbon electrode
⇩
mechanical or manual polishing



Time-consuming mechanical or manual polish.
Polishing is an off-line process,
and a long time is necessary to get a stable baseline.

Diamond electrode
⇩
Electrochemically stable
>>> based on on-line cleaning!



On-Line cleaning
At a high potential, the on-line cleaning process is automatically performed using a time sequence between analytical runs.

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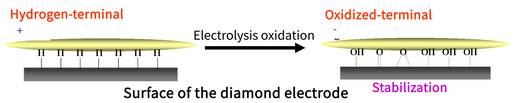
New technology 2： Stabilization of the electrode surface

On-line electrochemical polarization

The diamond electrode can exhibit two status including a hydrogen-terminated and an oxidized surfaces. Generally, while original hydrogen-terminated surface is changed to other oxidized surface, the response is unstable and irreproducible, leading to decrease in peak area.

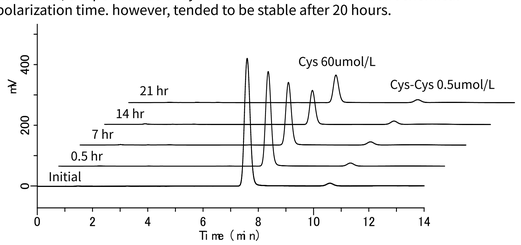
The results demonstrate that on-line electrochemical polarization can accelerate conversion of the hydrogen-terminated surface to oxidized surface, and achieve long-term stability and excellent analytical results.

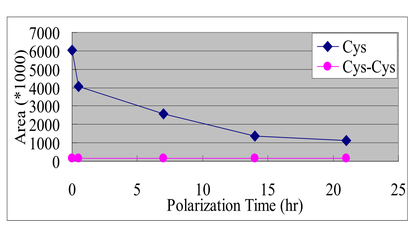


Diamond electrode oxidizing condition : Applied voltage 5000mV

Electrochemical polarization time and peak area

As a result, the peak areas of Cys decreased as increased electrochemical polarization time. however, tended to be stable after 20 hours.





Column : Inertsil ODS-3 3 mm i.d.X150mm 3um(GL Sciences)
* without pre-column and valve swathing. Other conditions : See Slide 14

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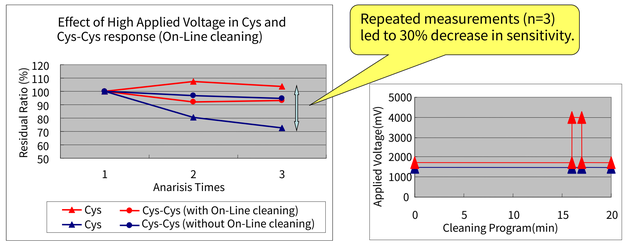
Advantage of New technologies ⇒ Taking usability as UV detector !!

Efficiency of on-line cleaning



Repeated measurements (n=3) led to 30% decrease in sensitivity.

Without on-line cleaning, the sensitivity for Cys was obviously decreased

>>> The electrode may have been deteriorated/contaminated by oxidized products.

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Conclusion

1. Established an ECD-HPLC system equipped with a special stabilization-treated conductive diamond electrode by a column switching method enabling a simultaneous analysis of cysteine and cystine.
2. To assure the robustness of this electrode used in this system, surface treatment (stabilization method) and On-line cleaning methods were established.This led to a phenomenal robust electrode.
3. The robustness of this electrode was proved again as the sensitivity did not vary even conducting a continuous analysis of biological samples for 2 weeks.
4. This system enables high-precision and selectivity in less time for the specification test of cysteine and cystine in infusion solutions.
5. Also enables simultaneous high throughput/precision analysis of thiol and disulfide, and the trace amount measurement of varying sensitivity of SAA in biological samples.

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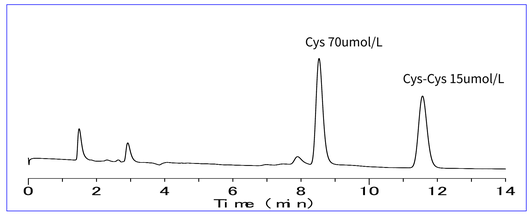
Typical System

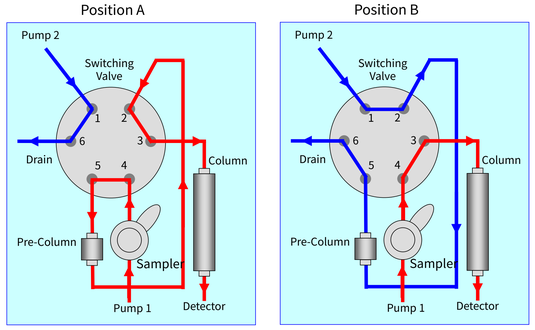
Cys and Cys-Cys analysis in rat plasma



Conditions

Column	: Inertsil ODS-3 3 mm i.d.X 150mm 3um(GL Sciences)
Pre-Column	: Inertsil ODS-3 3 mm i.d.X 33mm 3um(GL Sciences)
Column temp.	: 40 C
Solvent	: 50mM NaH_2PO_4-5mM OSA* Buffer pH2.2 /CH_3CN = 97.5/2.5 (w/w)
Flow rate	: 0.4mL/min
Detect	: ECD with Diamond electrode, Applied voltage 1600mV
	* On-Line cleaning 4000mV for 1min. (15-16min)
Valve Switching: Initial	position A
Program	2min position B
Pretreatment	: deprotonation using $HClO_4$
	*OSA: Octanesulufonic Acid

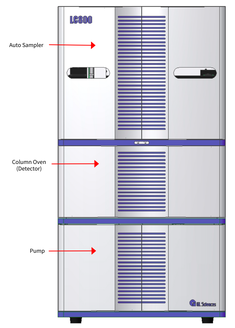
Flow diagram

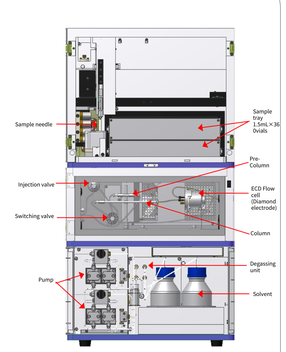


Electro chemical detector ED703 pulse (GL Sciences)

■Measuring method : Pulsed amperometric, Amperometric, Scan
■Working electrode : Diamond, Gold,
■Reference electrode : Ag/AgCl
■Oven : 20 to 45 degree C

HPLC System LC800 (GL Sciences)





The new HPLC system featured that all units including injector, switching valve, column and flow cell of the electrochemical detector were installed into an oven to achieve high reliability of the analytical results.

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