

μ-PrepCell – A NOVEL TOOL FOR EFFICIENT METABOLITE SYNTHESIS

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 pharmaceuticals

Amodiaquine
Amiodarone
Irinotecan
Mesna BNP7787
Vincristine

INTRODUCTION

Structural elucidation of reactive metabolites plays an important role in drug development. Xenobiotics such as pharmaceuticals are often metabolized by cytochrome P450 enzymes (CYP) to reactive metabolites. A number of publications [1-6] have shown that the *vivo* formation of these metabolites can be mimicked successfully *in vitro* by electrochemical oxidation in a flow cell. Generating these metabolites *in vitro* has a number of advantages over *in vivo* formation, most important: it is much quicker and cleaner. Metabolites are formed instantaneously in a well-defined matrix. In this note a novel flow cell, the μ-PrepCell™, is presented aimed for high efficiency synthesis of metabolites. The active surface of the working electrode is about 10 times the size of a standard ReactorCell™ resulting in significantly higher conversion efficiencies in formation of reactive metabolites. A ROXY™ EC system equipped with μ-PrepCell™ can be hyphenated to MS or a LC/MS system to perform separation and identification or the created metabolites can be collected off-line and used in supplementary research.

- Quick and clean alternative for in-vivo studies
- Synthesize micrograms of metabolites
- Collect fractions for MS or structural elucidation by NMR
- High efficiency conversion

Summary

The novel μ-PrepCell™ is designed for high efficiency metabolite generation. The cell can be used in either oxidative or reductive mode in combination with the ROXY™ Potentiostat or integrated in the ROXY EC and ROXY EC/LC system for the synthesis of metabolites. Applications are:

- Metabolite synthesis in clean and well-defined matrix for use as reference material by MS or for structural elucidation by NMR.
- High yield oxidation/activation reactions, i.e. drug-protein binding studies, oxidative stress of biopolymers (DNA, Proteins, etc.).

In this application note the performance of the μ-PrepCell is presented in terms of conversion efficiency, yield and reproducibility. The performance is compared to that of the ReactorCell for the oxidative metabolism of the pharmaceutical drugs Amiodarone, Amodiaquine and Irinotecan.



Fig. 1. μ-PrepCell™.

Furthermore, important factors governing the conversion efficiency such as flow rate, pH and choice of electrode material were studied and the results are discussed in this note. These parameters are important tools to optimize the conversion efficiency and yield of formed metabolites.

The rate of metabolite formation was quantified in terms of mass per unit time ($\mu\text{g}/\text{min}$) using the electrochemical reduction of Glutathione disulfide (GSSH) into GSH as a model reaction. The results presented in this note demonstrate that rates of $\mu\text{g}/\text{min}$ are in principle attainable.

μ -PrepCell™

A photograph of the μ -PrepCell™ is shown in Figure 1. The μ -PrepCell™ consists of a PEEK working electrode block (lower half of the cell) facilitating easy exchange of working electrodes. The cell can be equipped with for example a Glassy Carbon or conductive diamond electrode (Magic Diamond™).

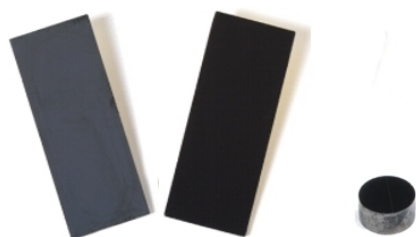


Fig. 2. Working electrodes of μ -PrepCell™. Left: Magic Diamond (MD) electrode, Middle: Glassy Carbon (GC) electrode. Right: GC electrode of the ReactorCell™.

The inert Magic Diamond electrode can be used at high working potentials (> 2 Volt) for the most demanding reaction conditions. The working electrode size is 30×12 mm and the active area of the μ -PrepCell electrode is more than 12 times larger compared to that of the ReactorCell. For reference the GC electrode of the ReactorCell is also shown in figure 2. The Titanium inlet block is equipped with the maintenance-free HyREF™ (Pd/H₂) reference electrode. The Titanium block itself serves as the auxiliary electrode. The cell volume can be varied with a set of 50 and 100 μm stainless steel spacers supplied with the cell. In all experiments presented in the note the effective spacer thickness was approximately 50 μm corresponding with a cell volume of 11 μL . An effective sealing construction of the cell based on a Viton O-ring allows backpressures up to 75 bar in combination with the MD electrode (max pressure with GC electrode 25 bar).

Method

The following set-up (Fig. 3) is used for the experiments: ROXY EC System (p/n 210.0070) consisting of the ROXY potentiostat equipped with a ReactorCell™ or μ -PrepCell™ (Fig. 1), infusion pump and all necessary LC connections. The ROXY EC System is controlled by Antec Dialogue software. The ReactorCell and μ -PrepCell equipped with Magic Diamond™ or Glassy Carbon

working electrode and HyREF™ reference electrode were used for the generation of the metabolites from the compounds of interest (amiodarone, amodiaquine, irinotecan, GSH, and GSSG).



Fig. 3. ROXY™ EC System including ROXY potentiostat, μ -PrepCell™ and dual syringe pump.

Table 1	
Conditions	
EC	ROXY™ EC System (p/n 210.0070)
Cell	ReactorCell™ with MD/GC WE and HyREF™ μ -PrepCell™ with MD/GC WE and HyREF™
Flow rate	5-200 $\mu\text{L}/\text{min}$
Potential	0 – 3200mV (for oxidation) -500 – -2700V (for reduction)

The sample was delivered to the system with a syringe pump equipped with 1000 or 500 μL Hamilton gas tight syringe. A MicrOTOF-Q (Bruker Daltonik, Germany) with Apollo II ion funnel electrospray source was used to record mass spectra. MS data were analyzed by Compass software. The relevant mass spectrometer parameters are listed in the Table 2. The method was optimized on a 10 μM sample solution. Mass spectrometer calibration was performed using sodium formate clusters at the beginning of the measurements.

Table 2	
MS settings	
Parameter	Value
Mass range	50 – 1000 m/z
Ion polarity	Positive
Capillary voltage	-4500 V
Nebulizer	1.6 Bar
Dry gas	8 L/min
Temperature	200 °C
Funnel 1 RF	200 Vpp
Funnel 2 RF	200 Vpp
ISCID energy	0 eV
Hexapole	100 Vpp
Ion energy	5 eV

Phase I metabolite generation

All experiments were conducted to generate phase I metabolites of amiodarone, amodiaquine or irinotecan in the μ -PrepCell or ReactorCell and compare the performance. The sample concentrations and matrices are listed in table 3.

Table 3

Samples description		
Name	Concentration	Buffer/Organic modifier
Amiodarone	10 μ M	10-20mM ammonium formate; pH 7.4 (NH ₄ OH)/ 50 % ACN
Amodiaquine	5-10 μ M	10-20mM ammonium formate; pH 7.4 (NH ₄ OH)/ 50 % ACN
Irinotecan	5 μ M	10mM ammonium formate; pH 3.3 (0.1% Formic Acid)/ 50% ACN

The samples were pumped at 5-200 μ L/min flow rate through the ReactorCell or μ -PrepCell using an infusion pump. The outlet of the cell was connected directly (on-line) to the ESI-MS source. The working electrode potential was applied in range from 0 – 3200 mV to find the optimal conditions for metabolite generation. After each change of the cell potential mass spectra were recorded. The instrumental set-up of ROXY EC System for oxidative metabolism phase I is shown in Figure 4.

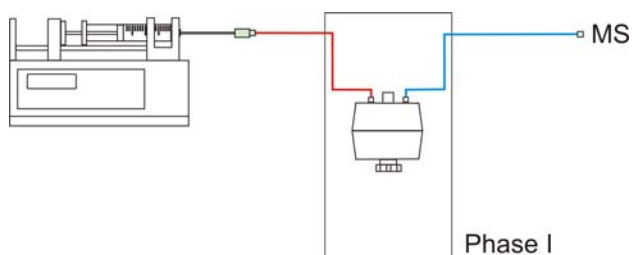


Fig. 4. Instrumental set-up of ROXY EC System with μ -PrepCell for oxidative metabolism phase I.

Quantification

For the quantification experiment GSH or GSSG (Table 4) was introduced into an LC system as shown in Figure 6. A syringe pump was used to transfer the sample through the μ -PrepCell into an injection loop connected to the LC system. The μ -PrepCell was switched off during the analysis of the samples for the calibration curve. For reduction experiment the voltage range was -500mV to -2700mV to find the optimum potential. MD electrode was used.

Table 4

Samples description		
Name	Concentration	Buffer
GSH	5-50 μ M	20mM ammonium acetate
GSSG	5-50 μ M	20mM ammonium acetate

The mobile phase contained 20mM ammonium acetate (pH 7.0) and 5% MeOH, and to separate GSH and GSSG isocratic flow of 300 μ L/min was used. A BetaSil Phenyl column 250mm x 3mm (Thermo) was used for the separation.

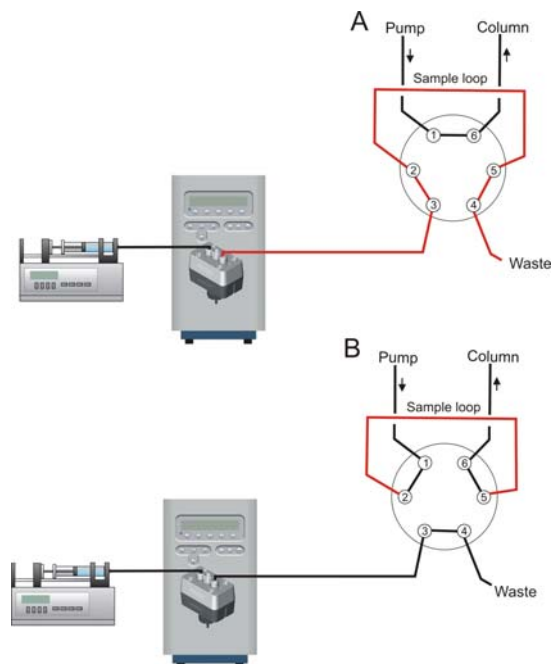


Fig. 5. Instrumental set-up for the quantification experiment. Injections were established using a manual valve connected to LC gradient pumps and column. Loop was filled with syringe pump (A). Position B shows the valve configuration during Injection.

AMIODARONE

Amiodarone (IUPAC: (2-butyl-1-benzofuran-3-yl)-[4-(2-diethylaminoethoxy)-3, 5-diiodophenyl] methanone) was chosen as model drug to evaluate the μ -PrepCell™ and compare its performance with that of the ReactorCell. Amiodarone is an antiarrhythmic drug, which is metabolised in the liver by N-deethylation, hydroxylation, O-dealkylation and deiodination. Part of the metabolic pathway of amiodarone is shown in Figure 7. Electrochemical oxidation of amiodarone into phase I metabolites was successfully achieved in both the ReactorCell and μ -PrepCell.

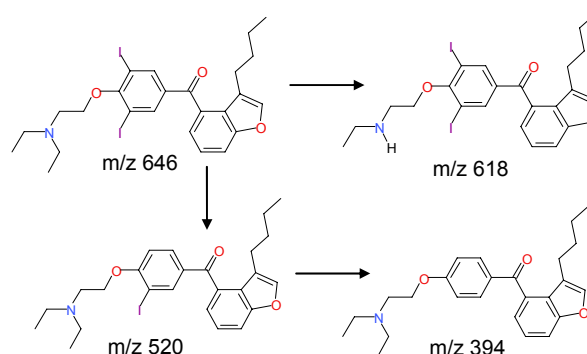


Fig. 6. Metabolic pathways of Amiodarone. Selected metabolites.

Table 5 consists of a list of compounds related to amiodarone metabolism and their monoisotopic masses used for mass spectra interpretation. The MS voltammogram obtained with the μ -PrepCell with MD electrode is plotted in Fig. 8.

Table 5

Amiodarone and its metabolites		
Name	Formula	Monoisotopic mass [u]
Amiodarone (AM)	C ₂₅ H ₂₉ I ₂ NO ₃	645.023680
N-deethylated AM	C ₂₃ H ₂₅ I ₂ NO ₃	616.992380
Mono-deiodinated AM	C ₂₅ H ₂₉ I ₁ NO ₃	519.127037
Bis-deiodinated AM	C ₂₅ H ₂₉ NO ₃	393.230394

The presence of amiodarone and its de-ethylated, mono-deiodinated and bis-deiodinated metabolites was investigated. Based on the obtained MS voltammograms a working electrode potential of 1 and 3V was selected for further evaluation.

At a potential of 1V the de-ethylated metabolite of amiodarone was detected ($m/z = 618$) and a potential of 3V potential was chosen as the optimal potential for the generation of the mono- and bis-deiodinated metabolites ($m/z=520$; 394, respectively). During a typical experiment mass spectra were acquired first with the cell off (no potential applied) and then with the selected voltage applied. The data were saved in one MS analysis file to simplify comparison between applied conditions ("OFF-ON" measurement).

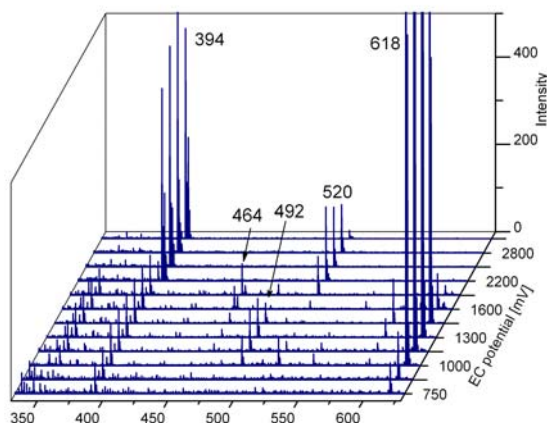


Fig. 7. MS voltammogram of amiodarone obtained for μ -PrepCell with Magic Diamond electrode. (Flow rate: 50 μ L/min). Ion abundance versus m/z as a function of EC potential.

PERFORMANCE μ -PREPCELL vs. REACTORCELL

To compare the performance of the μ -PrepCell and ReactorCell the signal attenuation of the amiodarone ion ($m/z=646$) was studied (Fig. 8 -10) at 1 and 3 Volt as a function of flow rate. The study confirmed that the attenuation of the amiodarone signal was significantly stronger for the μ -PrepCell. For example at a flow rate of 20 μ L/min and applied potential of 3V a decrease of 27% only of the amiodarone signal was observed with the ReactorCell (Fig 9). Under the same conditions (Fig 8) a decrease of 98% of the amiodarone signal was reached with the μ -PrepCell. The magnitude of signal attenuation is depended on the applied cell potential (Fig 10.). For both the μ -PrepCell and ReactorCell the signal attenuation (conversion of Amiodarone) reaches higher values at 3 Volt.

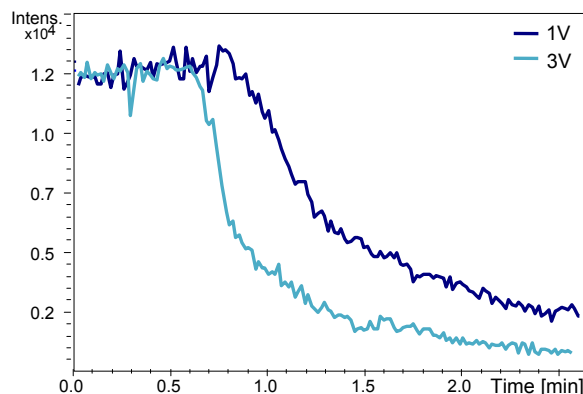


Fig. 8. μ -PrepCell - Extracted ion current of $m/z = 646$ acquired at Ecell of 1V (dark blue) and 3V (light blue). Flow rate 20 μ L/min. At $t = 0 - 0.6$ min the cell is off (un-attenuated signal).

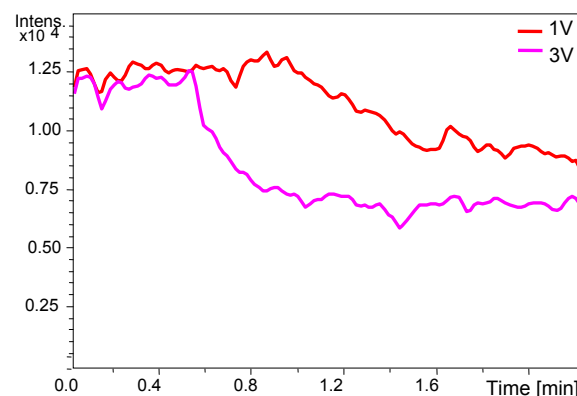


Fig. 9. ReactorCell - Extracted ion current of $m/z = 646$ acquired at Ecell of 1V (red) and 3V (pink). Flow rate 20 μ L/min. At $t = 0 - 0.6$ min the cell is off (un-attenuated signal).

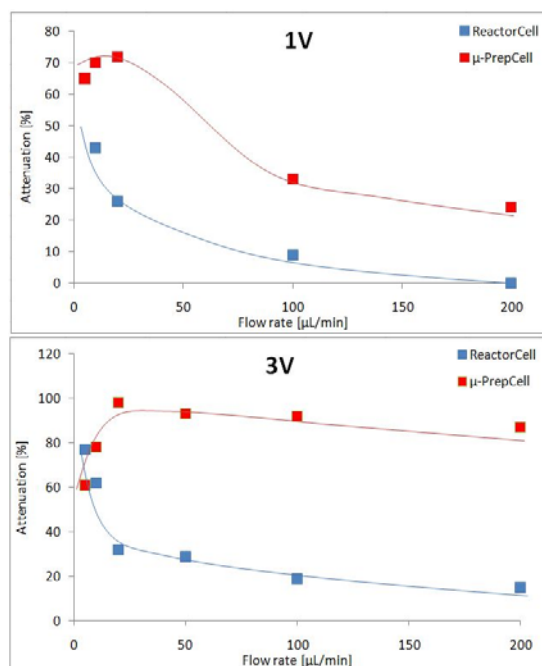


Fig. 10. Comparison of amiodarone signal attenuation for different flow rates and potential applied. Top: $E=1V$, Bottom: $E=3V$.

It is evident from figure 10 that the μ -PrepCell outperforms the ReactorCell with respect to conversion efficiency over the complete flow rate range. Even at a relatively high flow rate of 200 μ L/min at 3 Volts almost 90% of signal attenuation is reached for Amiodarone.

RELATIVE METABOLITE YIELD

The relative yield of formation of the de-ethylated, mono-deiodinated and bis-deiodinated metabolites of Amiodarone were also evaluated as a function of flow rate for both cells. The MS spectra were acquired with the cell switched off (no potential) and at an applied potential of 1V or 3V to generate the de-ethylated metabolite of amiodarone ($m/z=618$), or mono- and bis-deiodinated metabolites ($m/z=520, 394$), respectively ("OFF-ON" measurement).

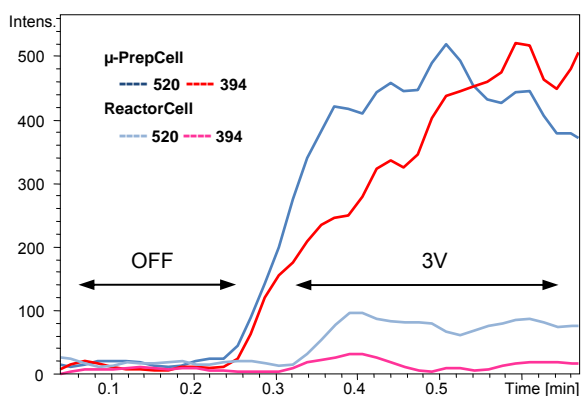


Fig. 11. Metabolite generation in μ -PrepCell and ReactorCell at a flow rate of 200 μ L/min. EIC for m/z of 520 and 394.

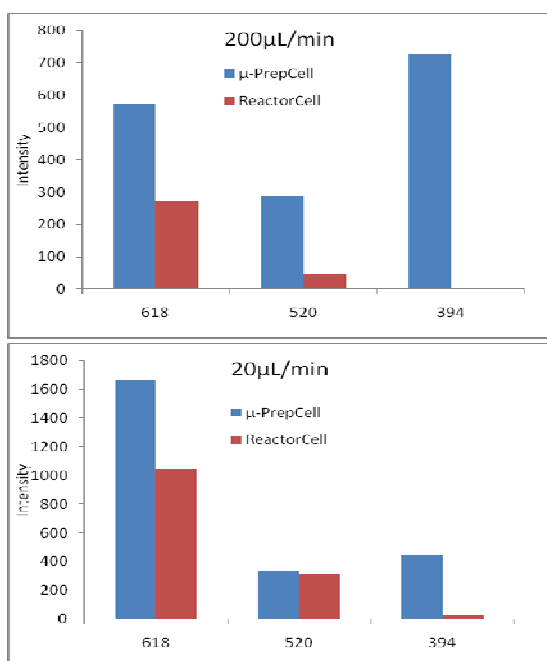


Fig. 12. Metabolite yield in μ -PrepCell and ReactorCell. Upper panel 200 μ L/min; Bottom panel 20 μ L/min.

At lower flow rates (see figure 12) the μ -PrepCell shows higher (m/z 618 and 618) or the same level (m/z 520) of metabolite formation as the ReactorCell. For the bis-deiodinated metabolite with m/z 394 the yield is approximately 180x higher at 20 μ L/min. At the highest flow rate (200 μ L/min) the yield of metabolite formation is 2x ($m/z=618$) and 8x ($m/z=520$) higher for the μ -PrepCell compared to the ReactorCell. At such high flow rate the bis-deiodinated metabolite (m/z 394) cannot be generated using the ReactorCell while in the μ -PrepCell it can be easily synthesized.

One factor which can influence the metabolite yield is adsorption of 'sticky' metabolites on the electrode surface, as was seen with amiodarone. In such cases its advised to use higher concentration of organic modifier (~50% acetonitrile) in the mobile phase. Furthermore, higher flow rates can also be applied to effectively minimize adsorption on the surface of the electrode (Fig. 13) and increase the yield.

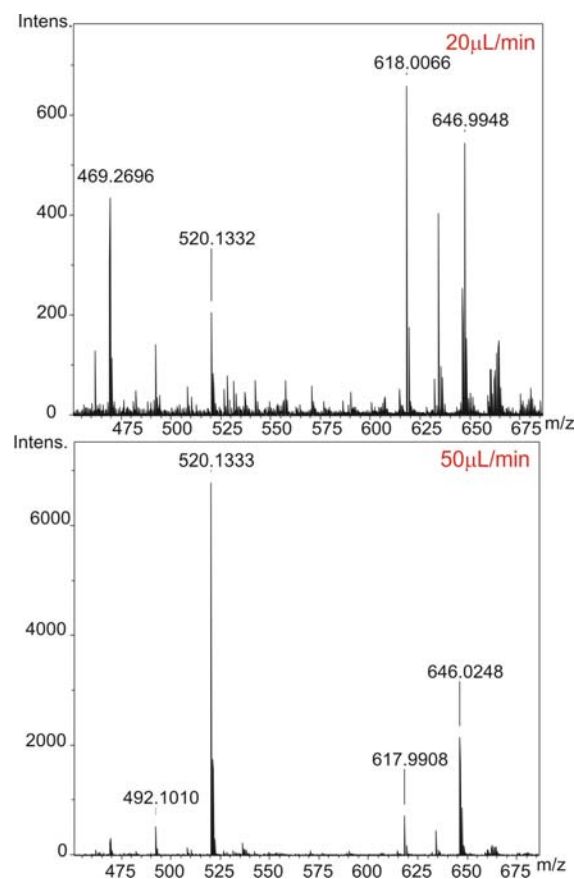


Fig. 13. Mass spectra acquired with μ -PrepCell at 50 μ L/min (bottom panel) and 20 μ L/min (upper panel). With the higher flow rate, more abundant signals from metabolites were registered because of minimized adsorption on the electrode surface.

The oxidation of amiodarone was repeated 6 times to check the repeatability (Fig. 14). The de-ethylated metabolite of amiodarone ($m/z=618$) signal gave similar response for each measurement. The long-term stability was studied by monitoring the signal of the de-ethylated metabolite formed at a cell potential of 1.2 Volt. It is evident from figure 15 that the metabolite signal was stable for

over 40 min. This experiment indicates that reproducible sample collection is possible over long time periods if required.

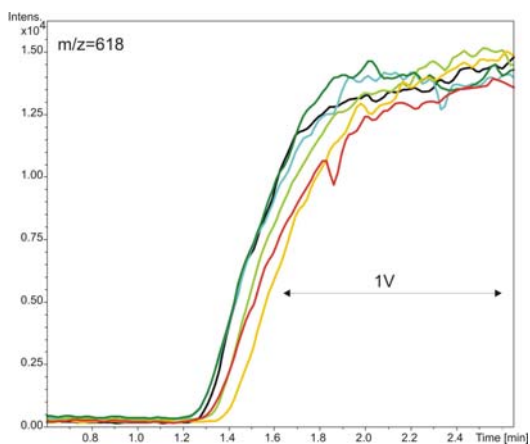


Fig. 14. Repeatability of the amiodarone oxidation: De-ethylated metabolite of amiodarone ($m/z=618$) generated at 1 Volt.

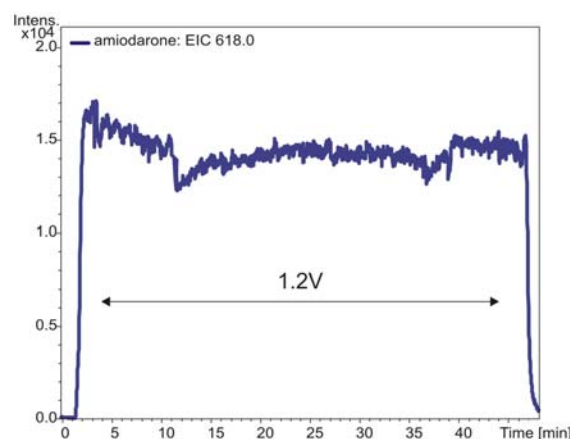


Fig. 15. Long-term signal stability for the de-ethylated metabolite of amiodarone ($m/z=618$) formed at 1.2 Volt.

AMODIAQUINE

Amodiaquine (AQ) is an anti-malarial agent, used against *Plasmodium falciparum*, which can cause cerebral malaria. The drug however was withdrawn from the market because of its hepatotoxicity. Amodiaquine is metabolized to reactive electrophilic metabolites, which are difficult to detect in-vivo since they are short-lived, and can undergo further reactions resulting in stable product. Amodiaquine can metabolize to amodiaquine quinoneimine, mono- and bis- de-ethylated amodiaquine quinoneimine (Table 6 and Fig. 18).

The Amodiaquine metabolism was studied using a Glassy Carbon working electrode. A MS voltammograms (Fig. 17) was recorded to determine the optimum potentials for metabolite formation. A cell potential of 0.9V was selected for the formation of the metabolites with m/z of 326; 299; 297. To synthesize the quinone amodiaquine metabolite (m/z of 354) a much lower potential of 0.4 Volt was chosen.

Table 6

Amodiaquine and its metabolites

Name	Formula	Monoisotopic mass* [u]
Amiodaquine (AQ)	$C_{20}H_{22}ClN_3O$	355.14514
1 (quinoneimine)	$C_{20}H_{20}ClN_3O$	353.12949
2 (desethyl; quinoneimine)	$C_{18}H_{16}ClN_3O$	325.09819
3 (bis desethyl; quinoneimine)	$C_{16}H_9ClN_2O_2$	296.03526
4 (bis desethyl; aldehyde)	$C_{16}H_{11}ClN_2O_2$	298.05091

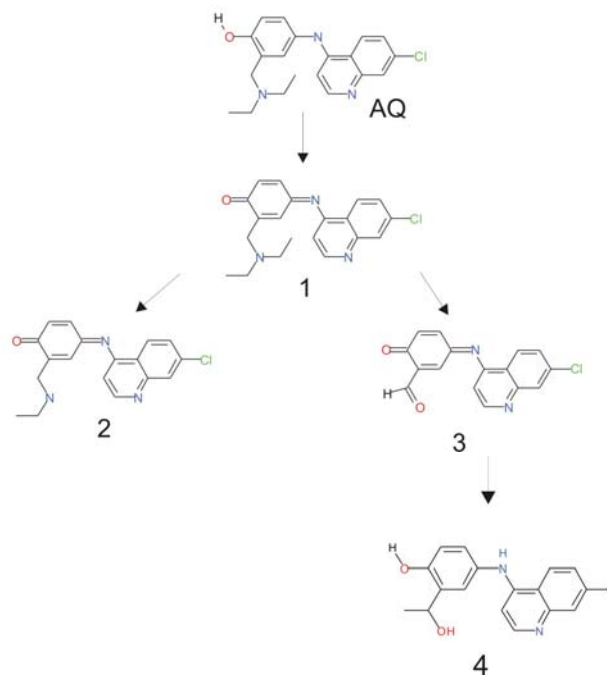


Fig. 16. Fragment of metabolic pathway of Amodiaquine.

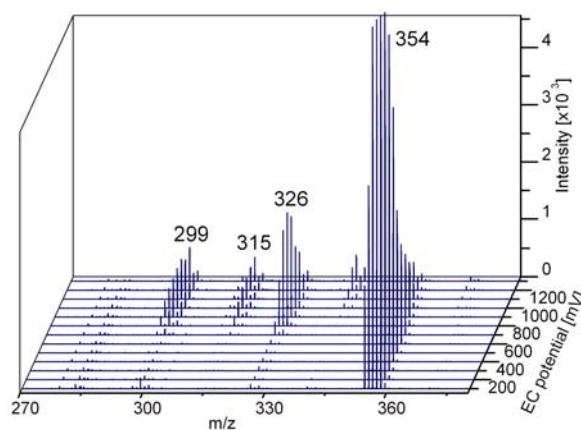


Fig. 17. MS voltammogram of Amodiaquine obtained with a μ -PrepCell with GC electrode. Ion abundance versus m/z as a function of applied EC potential.

A solution of 10 μ M amodiaquine was infused in the ROXY EC system using both the μ -PrepCell and ReactorCell. It is clear from

figure 18 that the yield of metabolite 2 (m/z 326; Table 6) using the μ -PrepCell is significantly higher than that of the ReactorCell even though the ReactorCell is operating in its optimal flow rate range ($10\mu\text{L}/\text{min}$).

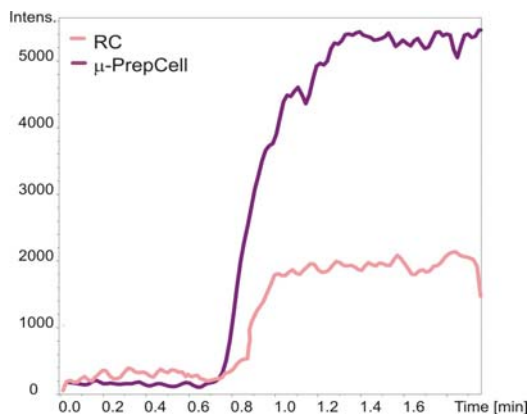


Fig. 18. μ -PrepCell vs. ReactorCell. Flow rate was $10\mu\text{L}/\text{min}$. $E=0.9\text{V}$. EIC of $m/z=326$ is plotted for both cells.

Mass spectra obtained for both type of cells operating at 0.9 Volt and a flow rate of $20\mu\text{L}/\text{min}$ are shown in figure 19. For both cells the same oxidation pattern is observed.

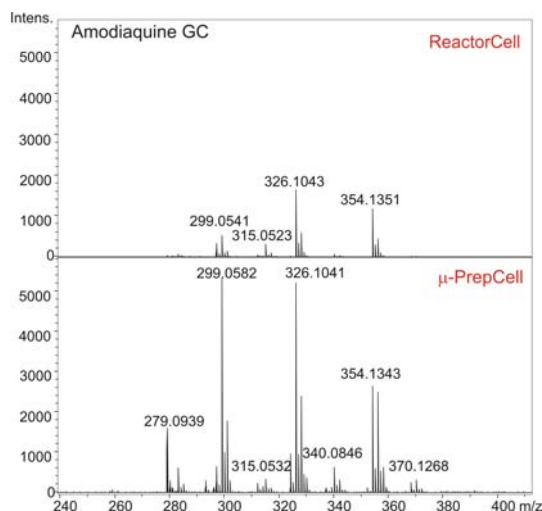


Fig. 19. Mass spectra for amodiaquine oxidized in the μ -PrepCell and ReactorCell. The intensity scale is the same for both cells ($20\mu\text{L}/\text{min}$; Glassy Carbon electrode at 0.9 Volt).

Furthermore, the increase in the abundance of another amodiaquine metabolite (nr. 4 in the Table 6) is evident (Fig. 21).

The long-term stability of the amodiaquine metabolite generation was evaluated by monitoring the MS signal of the different metabolites of amodiaquine formed at 0.9 Volt over a longer time period (Fig. 20). The signal was stable for more than 40min (only 20 min is shown). This observation is in agreement with the results obtained with Amiodarone indicating that efficient and reproducible metabolite synthesis is also possible over long periods of

time using the μ -PrepCell in combination with a Glassy Carbon electrode.

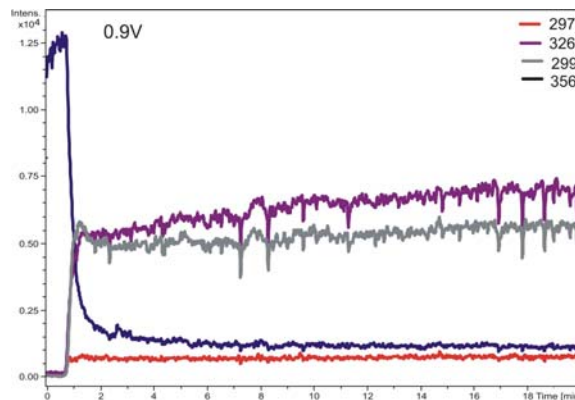


Fig. 20. Long-term signal stability for the amodiaquine metabolites formed at 0.9 Volt and a flow rate of $10\mu\text{L}/\text{min}$. For the EIC description see legend in the figure.

pH DEPENDENCY

The effect of pH was studied for the amodiaquine metabolite formation using both type of working electrodes: Glassy Carbon and Magic Diamond. Two mobile phases were tested with a different pH, one at pH 7.4 (10mM ammonium formate/50% acetonitrile, pH adjusted with NH_4OH) and one at pH 3.3 (10mM ammonium formate/50% acetonitrile, pH adjusted by 0.1% of formic acid).

Figures 21 and 22 shows the amodiaquine metabolites formed at different pH using a GC electrode in the μ -PrepCell. A significant higher intensity is observed for metabolite 4 ($m/z=299$) when a mobile phase at pH 3.3 is used, especially at lower flow rates.

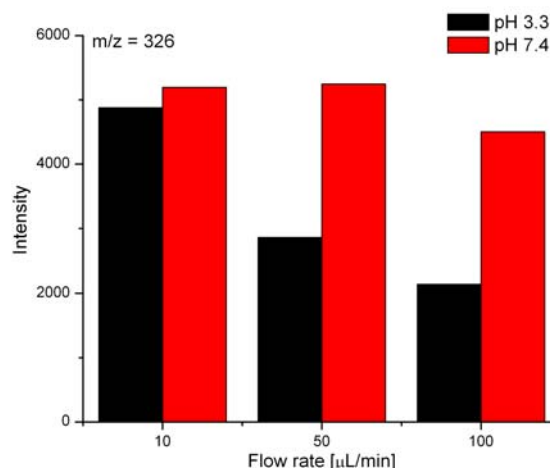


Fig. 21. Response as a function of flow rate at pH 3.3 and 7.4 for the Amodiaquine metabolite 2 (m/z 326, table 6) generated in a μ -PrepCell with Glassy Carbon working electrode.

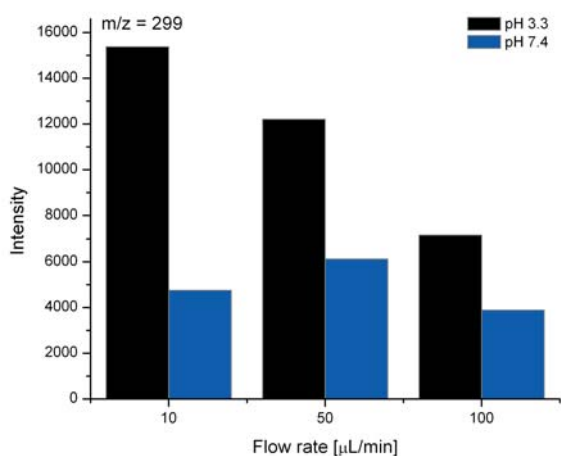


Fig. 22. Response as a function of flow rate at pH 3.3 and 7.4 for Amodiaquine metabolite 4 (m/z 299, table 6) generated in a μ -PrepCell with Glassy Carbon working electrode.

The formation of metabolite 2 (m/z 326) is favoured at pH 7.4. Furthermore, a difference in flow rate dependence is observed. At pH 3.3 a significant decrease in intensity for both metabolites is observed as function of increasing flow rate (to roughly half of its initial intensity at 100 μ L/min). At pH 7.4 the decrease in intensity as a function of flow rate is less significant in the range up to 100 μ L/min.

Figures 23 and 24 shows the amodiaquine metabolites formed at different pH on the MD electrode. With Magic Diamond the difference in intensity for metabolite 4 (m/z 299) between pH 3.3 and 7.4 is even larger. For metabolite 2 (m/z 326) at pH 7.4 more abundant peaks are seen.

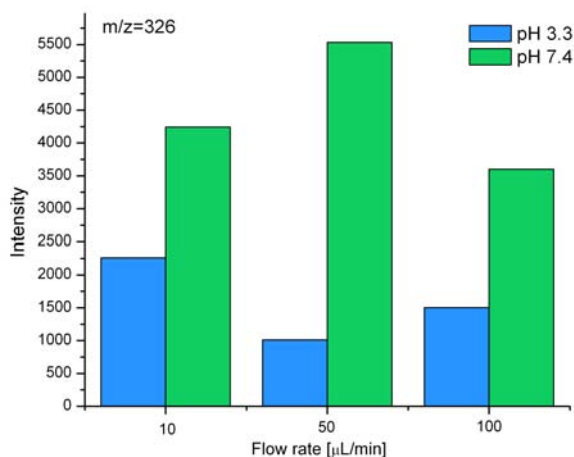


Fig. 23. Response as a function of flow rate at pH 3.3 and 7.4 for the Amodiaquine metabolite 2 (m/z 326, table 6) generated in a μ -PrepCell with Magic Diamond working electrode.

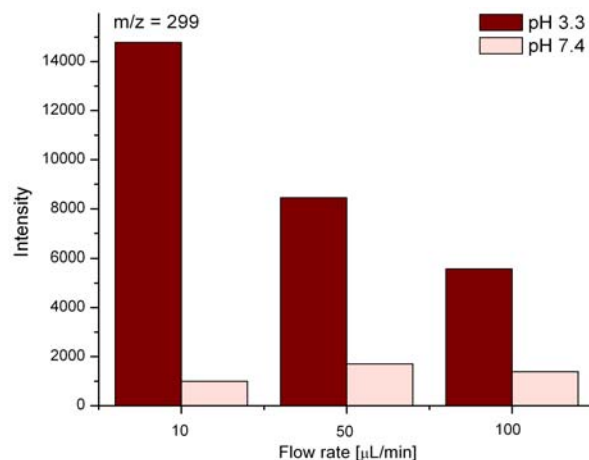


Fig. 24. Response as a function of flow rate at pH 3.3 and 7.4 for Amodiaquine metabolite 4 (m/z 299, table 6) generated in a μ -PrepCell with Magic Diamond working electrode.

These experiments demonstrate that the formation of metabolites is pH dependent. pH is therefore an important factor for the optimization of the conversion efficiency of individual metabolites.

IRINOTECAN

Irinotecan is an antineoplastic agent of the topoisomerase I inhibitor class and it is used as a first line therapy in the metastatic carcinoma of the colon or rectum (Fig. 25).

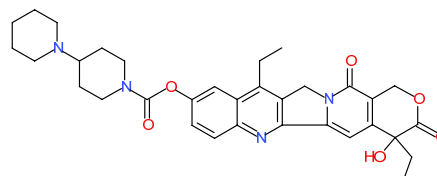


Fig. 25. Chemical structure of irinotecan.

The electrochemical conversion of Irinotecan was evaluated using the μ -PrepCell. Both Glassy Carbon and Magic Diamond was used as working electrode and the performance compared.

A MS voltammograms (Fig. 26) was recorded to determine the optimum potentials for metabolite formation. Based on the recorded MS voltammograms an optimal potential of 1V was selected for the GC electrode and 1.4V for the MD electrode.

ELECTRODE-DEPENDENT OXIDATION PROFILES

For Glassy Carbon and Magic Diamond a significantly different oxidation pattern was observed for the oxidation of Irinotecan (Fig. 27). The metabolites 3 and 4 (Table 7) were formed with MD as working electrode. With the GC electrode the metabolites 1 and 2 were formed. This phenomenon was observed with Irinotecan only, for amiodarone or amodiaquine the oxidation profile was the same for both electrodes. This example clearly demonstrates that the use of different electrode materials can lead to different oxidation profiles and metabolites.

Table 7

Irinotecan and its metabolites		
Name	Formula	Monoisotopic mass* [u]
Irinotecan (I)	C ₃₃ H ₃₈ N ₄ O ₆	586.2791
1 (Dehydrogenation)	C ₃₃ H ₃₆ N ₄ O ₆	585.2707
2 (Oxidation to ketone)	C ₃₃ H ₃₆ N ₄ O ₇	601.2657
3 (Hydroxylation)	C ₃₃ H ₃₈ N ₄ O ₇	603.2813
4 (Di-hydroxylation)	C ₃₃ H ₃₈ N ₄ O ₈	619.2762

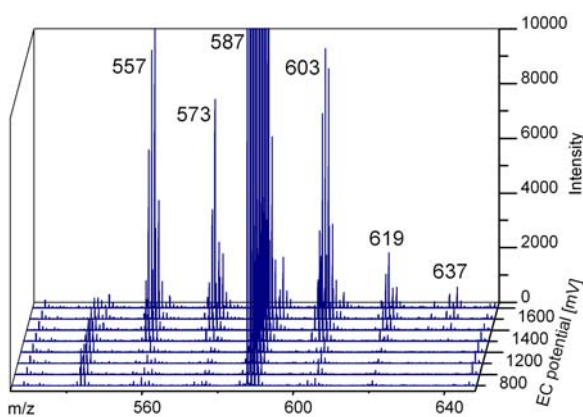


Fig. 26. MS voltammogram of Irinotecan obtained with a μ-PrepCell with MD electrode. Ion abundance versus m/z as a function of EC potential.

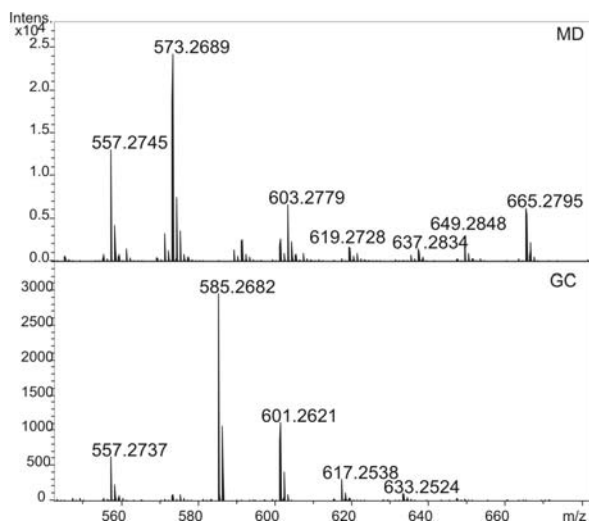


Fig. 27. Comparison of Irinotecan oxidation using MD and GC as working electrode. Flow rate 20 μL/min.

Also in the case of the oxidation of Irinotecan stable metabolite formation can be achieved over long time periods (see figure 28).

Table 7

GSH and GSSG		
Name	Formula	Monoisotopic mass* [u]
GSH (1)	C ₁₀ H ₁₇ N ₃ O ₆ S	307.083806
GSSG (2)	C ₂₀ H ₃₂ N ₆ O ₁₂ S ₂	612.151962

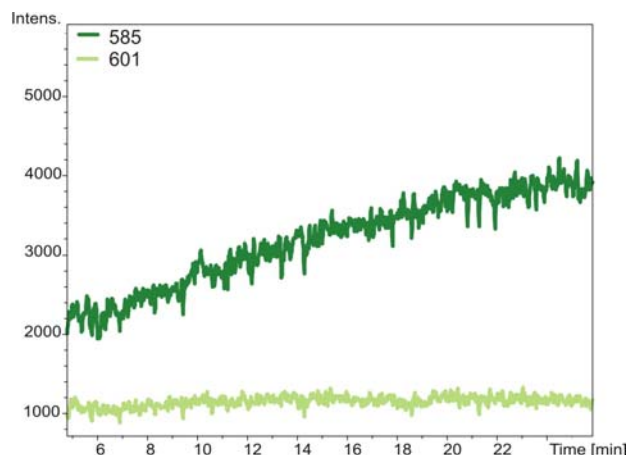
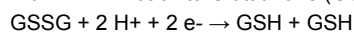


Fig. 28. Long-term signal stability of Irinotecan metabolites formation at 1.0 Volt using μ-PrepCell with GC electrode. Note: the conversion efficiency for metabolite 2 is increasing over time.

REDUCTION OF DIGLUTATHIONE

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.

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). Glutathione disulfide (GSSG) is reduced by glutathione reductase with NADPH back to Glutathione (GSH).



RATE OF METABOLITE FORMATION

This enzymatic reaction can also be simulated in a ROXY EC system with μ-PrepCell operating in reductive mode. In this study the electrochemical reduction of GSSG into GSH was used as a model reaction to quantify how much reaction product (metabolites) can be produced with the μ-PrepCell per unit time. Note, that the results presented below are indicative and could be different in the case of other reactions/metabolic pathways.

For this study Magic Diamond was used as working electrode. A cell potential of -1.2 Volts was applied to reduce GSSG to GSH using Magic Diamond. A 50 μM GSSG solution in 20mM ammonium acetate at pH 7.0 was used for the reduction experiments.

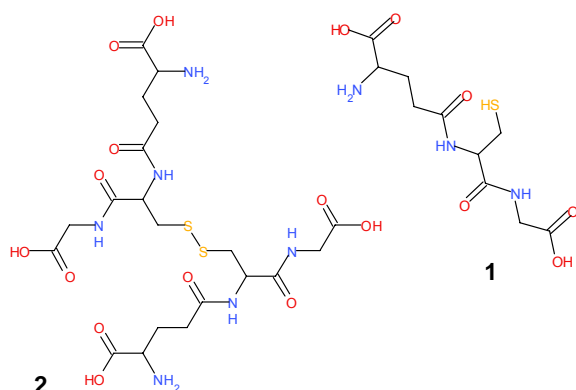


Fig. 29. Molecular structure of Glutathione (GSH) and Di-glutathione (GSSG).

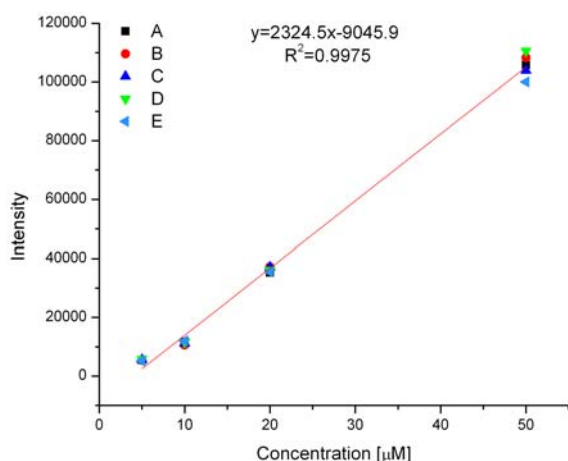


Fig. 30. The calibration curve for glutathione in the range 5-50 μ M. linear regression results are determined using the average intensity of 5 injections per concentration.

GSH concentration	
Formed GSH peak area	Calculated concentration [μ M]
60751	30.0266
58084	28.8792
58681	29.1361
49930	25.3714
48512	24.7614
Average: 55191	Average: 27.6347

A calibration curve of GSH was recorded in the concentration range between 5-50 μ M, see figure 30. This calibration curve was used to calculate the amount of GSH formed during the reduction of the GSSG solution with the μ -PrepCell.

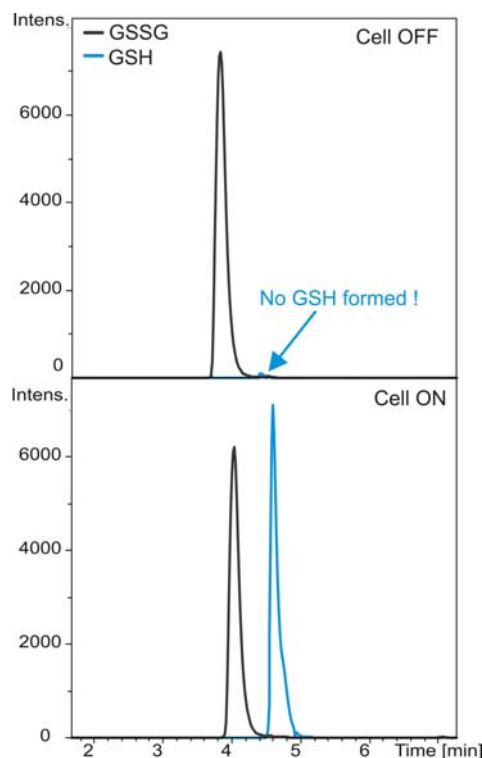


Fig. 31. EIC of GSH and GSSG for Cell OFF (top) and Cell ON (bottom).

In figure 31 the Chromatograms are shown for the experiment with the cell off (top) and with the cell on at -1.2 Volt (bottom). The EIC traces of GSSG and GSH were plotted with 0.002Da window. The exact masses of GSH ($m/z=308.0917$) and GSSG ($m/z=613.1590$) corresponds the theoretical masses with error of 3ppm for GSH and 1.2ppm for GSSG, and that confirms the origin of the peaks with high confidence as GSH and GSSG, respectively. The mass spectra corresponding to the chromatographic peaks from figure 31 are shown in figure 32. GSSG is observed as single and double protonated ion, and the number of the charge is indicated in the figure.

It is evident that only in the case when the μ -PrepCell is switched on at -1.2 Volt GSH is formed. In table 8 the results are shown of 5 consecutive measurements with the cell on. The average peak area of the formed GSH corresponds to a concentration of 27.6 μ M and an absolute amount of 84.8ng (10 μ L injection volume). The loop is loaded with a flow rate of 20 μ L/min, so under steady state conditions (continuous feed of 50 μ M GSSG into the μ -PrepCell) it corresponds to a rate of formation of 0.2 μ g/min GSH. This example illustrates that efficient metabolite generation in the range of μ g's per minutes is achievable with the μ -PrepCell.

Taken the reaction stoichiometry into consideration (for every GSSG molecule 2 GSH molecules are formed) approximately 28% of the GSSG is converted into GSH under these conditions.

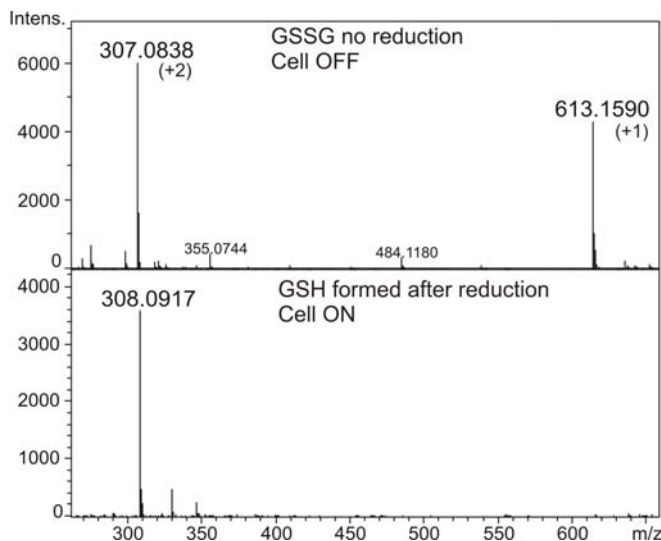


Fig. 32. Mass spectra reactor cell OFF (upper) and reactor cell ON (bottom). Spectra are average of the chromatographic peaks corresponding the GSSG (cell OFF) and formed GSH that appears only when cell is ON (-1.2V)

It is anticipated that the rate of formation can be increased when slightly higher concentration of GSSG are used or the flow rate is increased. However, It should be stated that the results are indicative and could be different in the case of other reactions/metabolic pathways. For every drug compound the relevant conversion parameters (cell potential, max. concentration of drug solution, flow rate, choice of mobile phase, pH and electrode material etc.) need to be optimized to achieve the best yield. With an appropriate purification technique the collected reaction product(s) can be worked-up for NMR analysis.

CONCLUSION

The μ -PrepCell is designed for efficient synthesis of metabolites in combination with the ROXY EC system. Micrograms of metabolites/reaction products can be generated in a short time period and collected offline for structural elucidation or other research purposes.

References

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Fig. 33. ROXY™ EC System.

PART NUMBERS

PART NUMBERS	
210.0070	ROXY™ EC System
204.4300	μ -PrepCell™ incl. kit (with GC and MD electrode)
204.5007	μ -PrepCell™ WE GC
204.5050	μ -PrepCell™ WE MD

