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Guideline for Metabolite Synthesis using the µ-PrepCell

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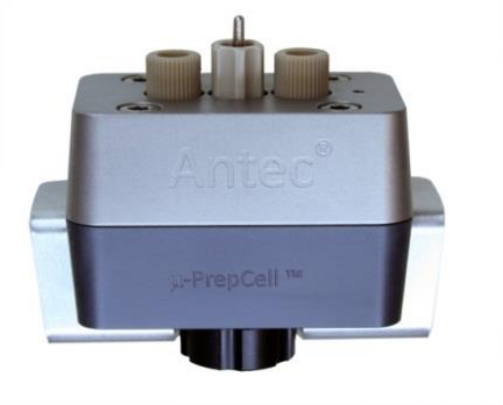

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Introduction

Combining the ROXY™ EC system with MS creates a powerful platform for oxidative metabolite studies and helps to overcome many of the laborious tasks by isolating the metabolites form *in vivo* (urine, plasma, *etc.*) or *in vitro* (microsomes) studies. The oxidation of target compounds occurs in the electrochemical cell within seconds and the similarity between reactions catalyzed by cytochrome P450 in nature and simulated in the electrochemical cell is high [1, 2, 3, 4, 6, 7].
The µ-PrepCell (Fig. 1) is a thin-layer electrochemical cell designed for high yield synthesis of metabolites. The active surface of the working electrode is about 10 times the size of a standard ReactorCell, and guarantees a significantly higher efficiency in formation of reactive metabolites.
A concentrated parent drug solution (200-500µM) is typically used for metabolite synthesis and generated metabolites are collected off-line, e.g., by fraction collector and analyzed by LC/MS. Furthermore, µ-PrepCell can be easily connected to MS for on-line screening or optimization of synthesis parameters.
Dedicated Dialogue software enables to control the potentiostat and syringe pump. The user defined programs can be adjusted according the current requirements, including a pulse and scan mode in automated manner.

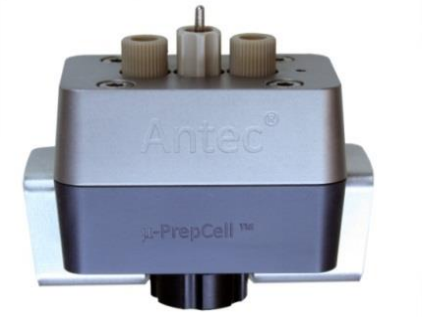

Fig. 1. µ-PrepCell.

Objective

This document is describing a generic optimization protocol for metabolites synthesis using µ-PrepCell. A square wave pulse is applied and optimization is performed based on scanning voltammetry and on-line mass spectrometric measurements.

C H A P T E R 2

Methods

Experiments

Step 1: Scanning voltammetry with electrochemical detection
Step 2: Scanning voltammetry with MS detection
Step 3: Optimization of square wave pulse parameters
Step 4: Metabolite synthesis with off-line samples collection

Instrumental set up

A ROXY EC System for single compound screening (p/n 210.0070) equipped with a ROXY potentiostat and µ-PrepCell™ with Glassy Carbon (GC) working electrode, infusion pump and all necessary LC connections was used in all experiments (Fig. 2). The ROXY EC System was controlled by Antec Dialogue software.

The samples were collected offline, followed by MS analysis of the collected fractions. A 5 mL syringe was used to deliver the sample in off line experiments. The flow rate used in the synthesis experiments was 50 µL/min. The metabolites were synthesized in pulse mode with the settings as described in Table I. The details of the settings used in optimization experiments are described in the next sections of this document.

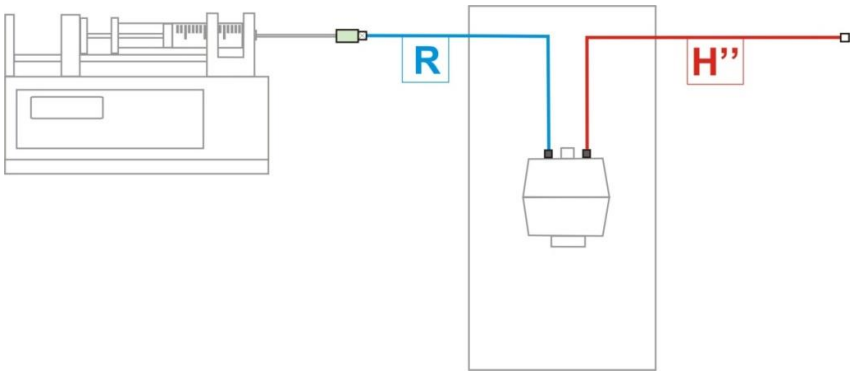

Fig. 2. Instrumental set up. Inlet tubing was 0.254 mm I.D. PEEK (R, blue), as outlet 1 m of 0.127mm I.D. PEEK (H", red) was used.

The µ-PrepCell was used at 35°C (oven temperature of the potentiostat) during all experiments. In the optimization steps (Step 2 and 3) the outlet tubing was and connected to ESI source via grounding union.

A LTQ-FT (Thermo, Germany) mass spectrometer equipped with electrospray (ESI) source was used to monitor the oxidation products during the optimization steps and to confirm the presence of the metabolites in the control samples. The MS spectra were collected in a centroid mode and acquired by LTQ. MS data were analyzed using Xcalibur software.

Samples

- 250µM Verapamil in 20mM ammonium formate (pH 7.4) in 50% acetonitrile for off-line experiments and synthesis (STEP 1 and 4).
- 2.5µM Verapamil in 20mM ammonium formate (pH 7.4) in 50% acetonitrile was used in on-line MS experiments in optimization phase (STEP 2 and 3).
- Control samples collected in STEP 4 were diluted 100x in 30% MeOH containing 0.1% formic acid (FA) and analyzed by MS.

Potentiostat EC settings

Table I. µ-PrepCell settings.

	Description
Step 1 and 2 Scan	E1= 0 mV; E2= 1500mV Scan rate: 20mV/s Cycle: continuous or half
Step 3 Pulse optimization step	E1= 600 - 1200mV; E2= 200 - 800mV t1= 1990ms; t2= 1010ms ts= 40ms E3= t3= 0
Step 4 Pulse synthesis (optimized)	E1= 800mV; E2= 400mV t1= 1990ms; t2= 1010ms ts= 40ms E3= t3= 0

All measurements were done in range of 200µA without filter.

Synthesis

The metabolites were synthesized continuously for 100 minutes. The pulse settings were as described in the Table I (STEP IV). The synthesis was repeated after reactivation of electrode surface by pulsing in mobile phase and activation scan in oxidative range of 0-1.5V (Table II). The cell was refilled with the sample according protocol [5].

Table II. Activation pulse and scan settings.

	Description
Activation Pulse	E1= + 2000 mV; E2= - 2000 mV t= 1000ms; t2= 1000ms ts= 40ms E3= t3= 0 Run time = 5 min
Activation scan	E1= 0mV; E2= 1500mV continuous scan rate 50mV/s run time = 10 min

Flow rate was set to 50µL/min.

C H A P T E R 3

Results

STEP 1 Scanning voltammetry with electrochemical detection

In step 1, a working potential range and maximum potential were established. The oxidation reaction takes place within the working potential range. The maximum potential represents the highest value of potential, which can be applied without evoking mobile phase electrolysis. Electrolysis can result in oxygen or hydrogen evolution and aggregation of the gas bubbles inside the cell. Bubbles in the µ-PrepCell are a major source of poor reproducibility!

The working and maximal potential can be established by means of scanning voltammetry in flow injection conditions. This method allows for fast identification of redox potentials of any electro-active drug. In this experiment potential is ramped and a current peak is indicating oxidation (or reduction) of elecro-active component.

Fig. 3 shows a scanning voltammetry plot of Verapamil. A broad potential range was chosen to evaluate the behavior of the current. The potential was scanned from 0V to 1.5V with 20mV/s rate in continuous cycle. The advantages of using continuous cycle are: a.) measurements are repeated until defined run time will elapse; b.) the first measurements can be discarded or treated as an equilibration of the electrode surface. The measurements can be represented as function of time (Fig. 3 A) or converted to E/I plot (Fig 3 B).

For sake of clarity only one scan cycle of Verapamil is presented in Fig 4. It is clearly seen that with potentials higher than 1.2V, Icell is in “overload” and E_{aux} was displaying an extreme value (-9.9V, not shown; the Eaux value can be read in Dialogue). Non measurable I_{cell} and E_{aux} values could be an indication of electrolysis and lower potential should be applied to prevent electrolysis.
The redox potential of Verapamil is shown in red color in the Figure 4. A current peak occurs between 600 and 1000mV and point out the oxidation of the Verapamil occurring in this potential range.

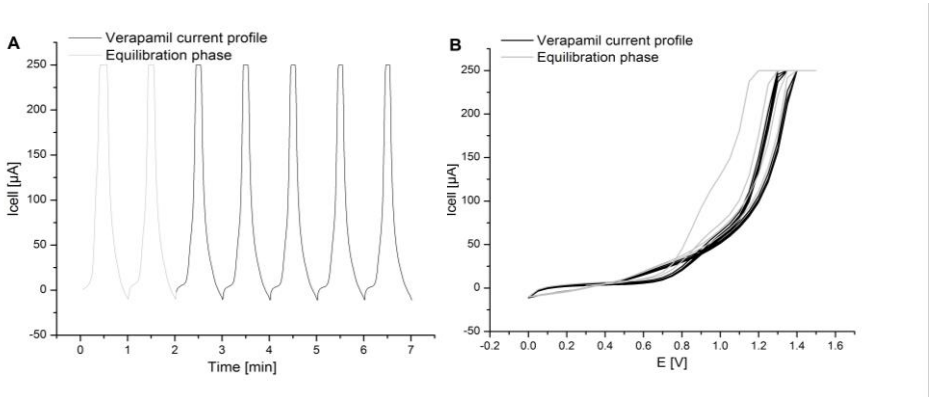

Fig. 3. A scanning voltammetry plot of Verapamil. A. Continuous scan from 0 to 1.5V with 20mV/s scan rate; flow rate 50µL/min. I_{cell} vs. time. B. The same plot exported to I/E plot (Current (I) vs. potential (E)).

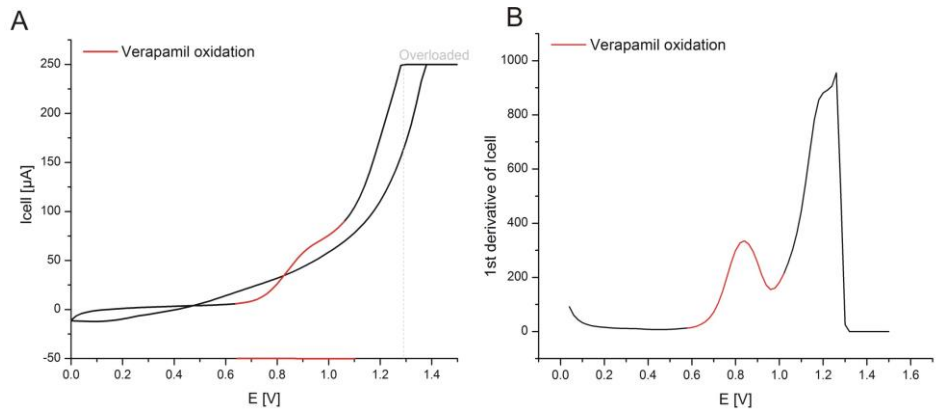

Fig. 4. A) A single (full) scan cycle of Verapamil. Conditions: E1=0V; E2=1.5V; cycle: continuous; rate: 20mV/s; flow rate: 50µL/min. B) The same plot but 1st derivative of the current is drawn to visualize the region where oxidation take place

The scanning voltammetry of Verapamil was used to identify:

- The potential range for oxidation of Verapamil (600-1000mV)
- Estimation of the highest potential that should be apply in further experiments to prevent electrolysis (E < 1200mV)

Furthermore, redox reactions occur on the surface of the electrode and a flux of electrons is detected at the moment of reaction. The I_{cell} measured in the voltammograms corresponds to the real time changes of potential and no delay must be considered. This experiment does not require MS, and can be carried in off-line mode in exact conditions of synthesis, whereas direct infusion of highly concentrated samples is not recommended for MS.

STEP 2: Scanning voltammetry with MS detection

In step 1, only the working potential range was determined for the oxidation of Verapamil. It is not possible to identify the metabolites based on the current profile alone. Therefore, a scanning voltammetry experiment with on line MS detection is mandatory for unambiguous identification of the synthesized metabolites. Mass spectrometry provides valuable information for metabolite synthesis and for characterization of all oxidation products as, for example, fragmentation spectra or elemental composition if a high resolution instrument is used. With on-line MS experiment confirmation can be obtained whether or not the working potential range determined in step 1 is applicable for synthesis of Verapamil metabolites.
Moreover, on-line MS measurements allow fine-tuning of the synthesis conditions and optimization of the yield of individual metabolites that may appear during an oxidation process.
In the first experiment, 2.5 µM Verapamil was oxidized by applying a scan from 0-1.5 V with the same parameters as for acquisition of the voltammograms (20 mV/s; continuous cycle). The results of the test are presented in Fig. 5. The metabolites of Verapamil are synthesized. Although the MS Voltammogram shows EIC traces as a function of time, the time scale can be recalculated to corresponding potential values as indicated by arrow below the plot (Fig. 5 A). It is important to note that metabolites will be detected in MS with a delay determined by:

- The dead volume between µ-PrepCell and the ion source of the mass spectrometer.
- The volume of the µ-PrepCell (12µL).
- The flow rate of the syringe infusion pump, in this case it was 50 µL/min.

In fact, a longer delay time should be taken than based on the theoretical calculation to compensate for the tolerance in tubing inner diameter (affects volume) and dilution in the tubing (Poiseuille flow profile), and in the cell itself.

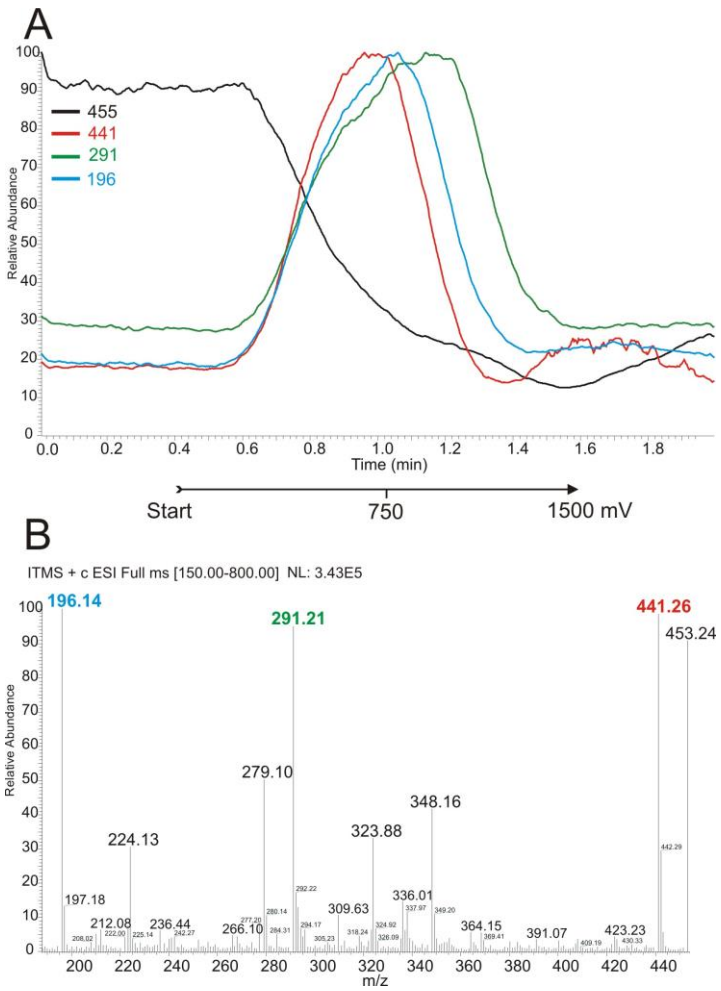

Fig. 5. A) MS Voltammogram of Verapamil. B) Mass spectrum corresponding to 0.7 – 1.2 min of EIC from A. Spectrum was corrected by subtracting background. All three metabolites are synthesized at almost the same potential range. Maximum signal was detected ca. 750mV and it is consistent with ECD experiment performed in step 1.

So MS allows direct monitoring of metabolites synthesized and gives direct feedback on the optimization process of the metabolite synthesis.

STEP 3: Optimization of a square wave pulse parameters

There are differences between electrochemical detection and synthesis of metabolites using an electrochemical cell. Only small volumes of sample (usually very low concentrations) are injected onto a LC column and detected on a working electrode of an electrochemical flow cell, whereas concentrated solution of drugs, typically 200-500 µM, are used in metabolites synthesis. On the contrary to ECD, the cell is continuously fed with large concentrations of drugs during metabolite synthesis, undergoing continuous conversion on the surface of the working electrode. The activity of the WE can be attenuated due to adsorption onto the electrode surface during the course of experiment, resulting in a decrease in yield over time when operating in DC mode (direct current).
Application of a square wave pulse is beneficial for metabolite synthesis. The main advantage of this technique is a stable current over a long period of time and more constant metabolite synthesis (Fig. 6). The electrode surface is continuously reactivated during the run, reducing adsorption/fouling . Pulsing in the range of the redox potential guarantees efficient metabolites synthesis from concentrated samples. Additionally, the pulse form can be easily adapted to the user needs and programmed in the event table.

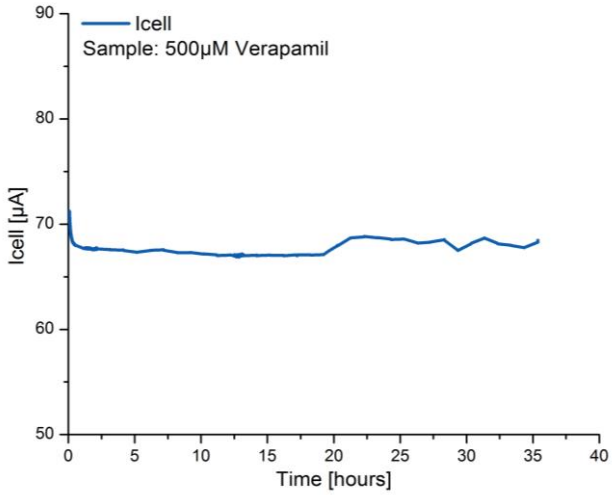

Fig. 6. Cell current stability experiment in off line mode. 500µM Verapamil was oxidized by applying square - wave potential pulses with following parameters: E1=1200mV, t1 = 1990ms; E2 = 800mV, t2 = 1010ms; E3 = 0, t3=0; ts=20ms. Flow rate was 50µL/min.

In STEP 1, the redox potential suitable for Verapamil oxidation was established between 600 - 1000mV. In STEP 2, formation of the Verapamil metabolites was confirmed by mass spectrometry.
In STEP 3, settings for a square wave pulse for metabolite synthesis will be optimized. It is recommended to run this experiment with on-line MS

detection to monitor on-line the synthesis efficiency when different pulse settings are applied.
The midpoint of the potential range determined in step 1, 800 mV, was taken as the optimal potential. The Pulse mode of the ROXY potentiostat is used for the application of the square wave pulse. E1 and E2 values are used (Fig. 7) with a difference of 400 mV. The pulse time t1 was set to 1990 ms while t2 was set to 1010 ms (t3 was set to 0 ms). Four different square wave pulses were tested with:

(I) E1 slightly below optimal potential
(II) E1 equal to the optimal potential
(III) E1 and E2 centered around the optimal potential
(IV) E1 much higher than optimal potential.

The detailed settings of all tested pulses are described in Table III. For further fine-tuning of the synthesis conditions also the time intervals could be optimized.

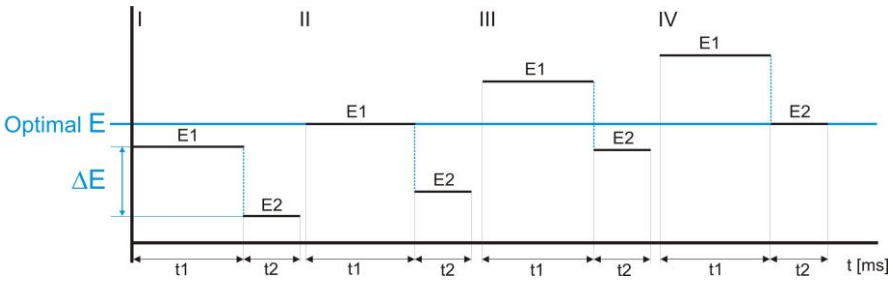

Fig. 7. Pulse mode optimization. The potentials were chosen for a regime where reaction was occurring (STEP 1). Different pulse potentials were tested (e.g., I, II, III, IV). Suggested settings are: ΔE = 400mV, t1 = 2s and t2 = 1s. ΔE, t1 and t2 may be optimized.

Table III. Pulse settings (t1 = 1990ms, t2=1010ms).

	E1 [mV]	**E2 [mV]**
I	600	200
II	800	400
III	1000	600
IV	1200	800

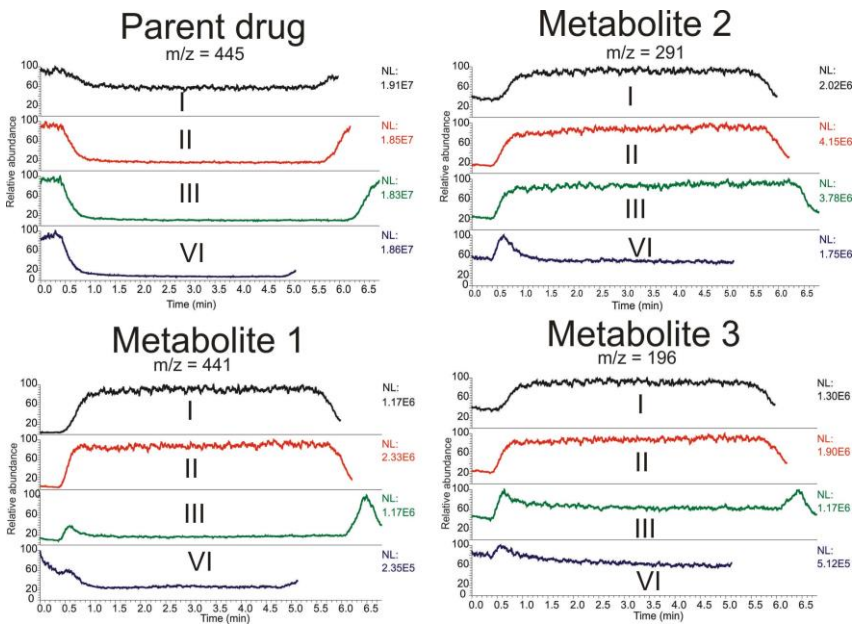

Fig. 8.The extracted ion currents of Verapamil (m/z 455) and its three metabolites (m/z 441, 291, 196) measured with I, II, III and IV settings (Table III

Table III). See details in the text.

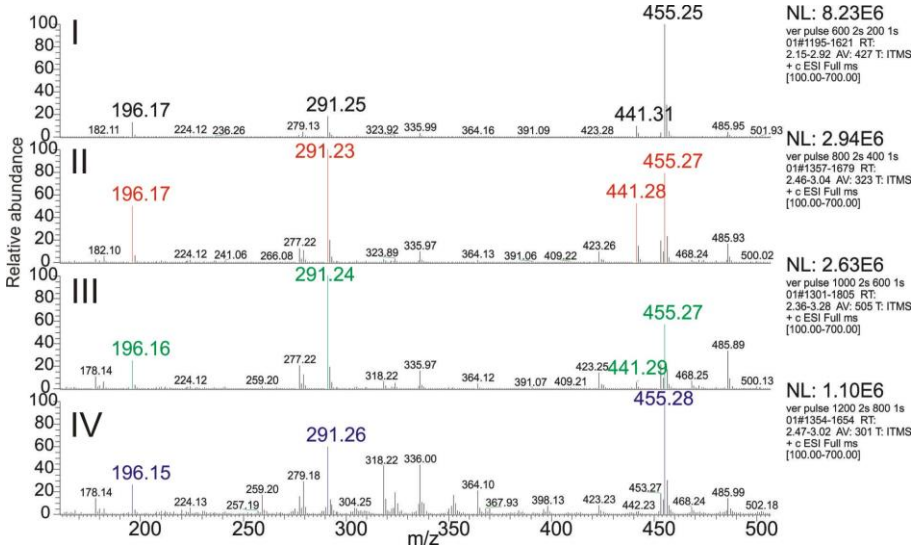

Fig. 9. Mass spectra corresponding to the pulses I, II, III, IV. Spectra are an average of ca. 300 scans beginning with second minute. See details in the text.

Fig. 8 shows the extracted ion currents of Verapamil (m/z 455) and its three metabolites (m/z 441, 291, 196) measured with I, II, III and IV settings. Mass spectra corresponding to the pulses I, II, III, IV are shown in Fig. 9.

Although all three metabolites were detected for each tested pulse settings, eventually, the pulse at E1=800mV and E2=400mV (II) was chosen for the synthesis of Verapamil metabolites. The abundances of all three investigated metabolites were the highest for this pulse settings (Fig. 10). Moreover, synthesis of the investigated metabolites was stable during the measurement (Fig. 8). However, more metabolites were detected, e.g., 178, 182, 259, 323, 277, 318 [1] in the mass spectra whereas pulse IV was tested. The optimal pulse settings must be found in order to their synthesis.

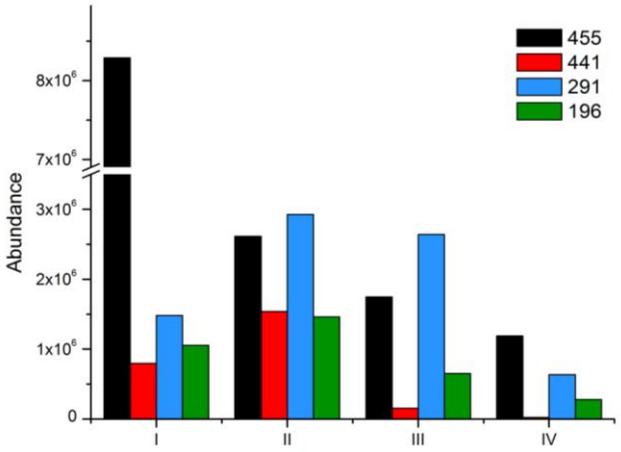

Fig. 10. Ion abundances corresponding to Verapamil (m/z 455) and its three metabolites (m/z 441, 291, 196) measured with I, II, III, IV pulse settings.

STEP 4: Metabolite synthesis with offline samples collection

In STEP 4, metabolites of Verapamil (m/z of 441; 291 and 196) were synthesized by applying the square wave pulse (
Fig. 11). Control samples were collected after 15, 45, 75, and 100 minute of synthesis. The control samples were diluted 100 x before injection into the MS.

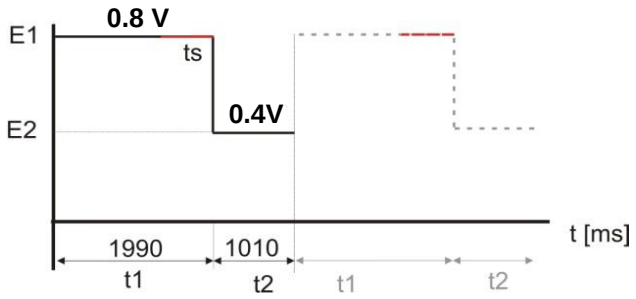

Fig. 11. The square wave pulse settings used for Verapamil metabolites synthesis.

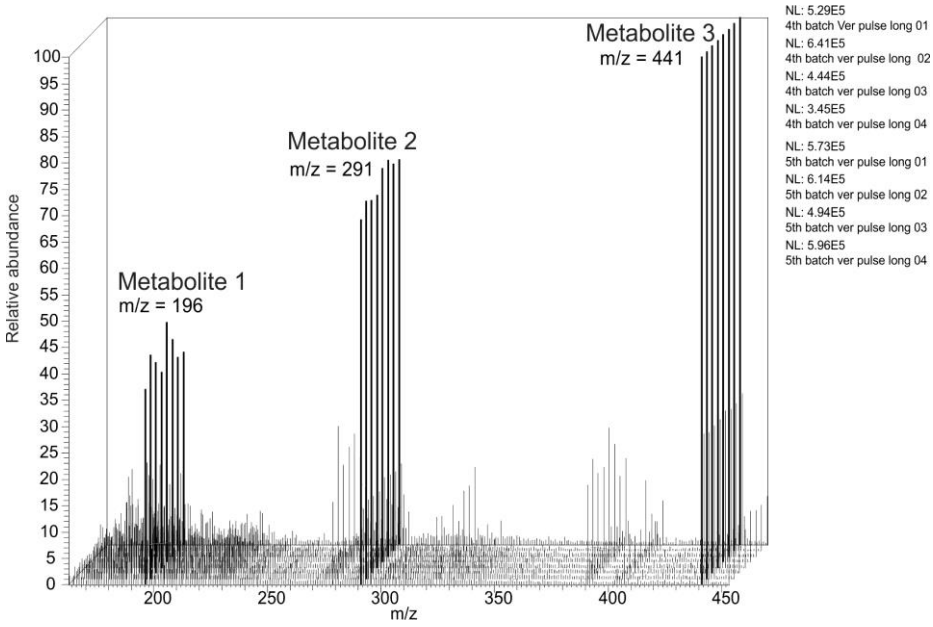

Fig. 12. An overlay of 8 mass spectra corresponding to the control samples measurements.

The experiment was performed in duplicate. Fig. 12 shows an overlay of mass spectra corresponding to all 8 control samples. The abundances of m/z values corresponding to three metabolites of Verapamil are plotted in the Fig. 13.

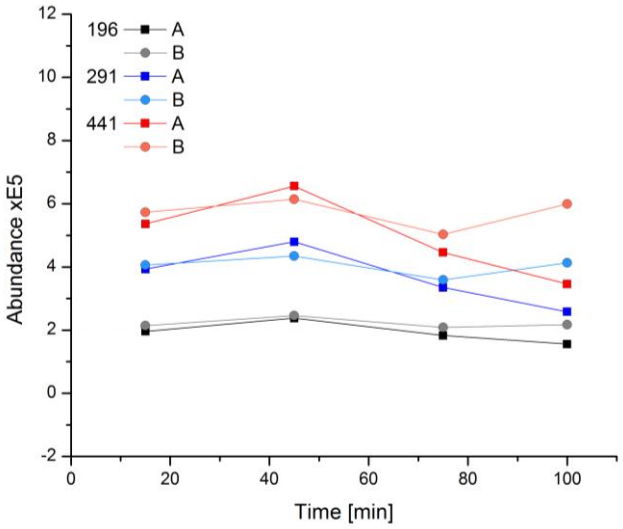

Fig. 13. The abundances of Verapamil metabolites (m/z of 441; 291; 196) synthesized using µ-PrepCell. A and B corresponds to duplicate experiments. Before both experiments the electrode was activated by pulse and activation scan as described in the methods section.

C H A P T E R 5

Conclusions

A generic approach towards optimal metabolite synthesis is described using Verapamil as a model compound. A square wave pulse is applied to achieve stabile continuous metabolite generation over long periods of time. Optimization of the metabolite synthesis is performed based on scanning voltammetry and on-line MS measurements. In a four step experimental approach:

Step 1: Scanning voltammetry with electrochemical detection to determine the potential range for oxidation of Verapamil.
Step 2: Scanning voltammetry with MS detection to confirm metabolite formation in the potential range determined in step 1.
Step 3: Optimization of the square wave pulse parameters for continuous metabolite synthesis.
Step 4: Metabolite synthesis with off line samples collection using the optimized square wave pulse form.

For 100 minutes Verapamil metabolites were synthesized and their presence confirmed by MS analysis to demonstrate the effectiveness of the approach.

This generic approach can be used as a guideline to establish the optimal conditions for metabolites synthesis for your compounds of interest.

C H A P T E R 5

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