Application of high resolution multistage mass spectral trees for identification of electrochemically synthesized metabolites

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Introduction

Combining electrochemistry with MS creates a powerful platform for oxidative metabolite studies and helps to overcome many of the laborious tasks by isolating the metabolites formed in vivo (urine, plasma, etc.) or in vitro (microsomes) [1, 2].
Furthermore, the electrochemical cell can be used for preparative synthesis of reactive metabolites in a short period of time. The cell can be hyphenated to MS or LC/MS to perform separation and identification of the created oxidative compounds i.e., intermediates, (reactive) metabolites, etc. Alternatively, the cell can be used off-line and the generated metabolites can then be collected for supplementary research such as NMR.
Here, highly efficient metabolite synthesis by applying square-wave potential pulses is presented. Metabolites are generated continuously for more than 2 hour using a highly concentrated sample (250 µM) and collected off-line. Mass spectra are measured to confirm the presence of Verapamil metabolites (Figure 1) in the collected samples.
Furthermore, electrochemically-synthesized metabolites of Verapamil are identified using the high resolution multistage spectral trees method [3]. Therefore, comparison of fragmentation patterns of parent drug and its metabolites was performed. A data-dependent acquisition protocol is applied to register spectral trees of Verapamil (m/z 455) and its metabolites (m/z 441 and 291). Fragmentation trees up to MS5 are acquired. High mass accuracy measurements allow assigning the elemental composition to the fragments and help with their structural elucidation.

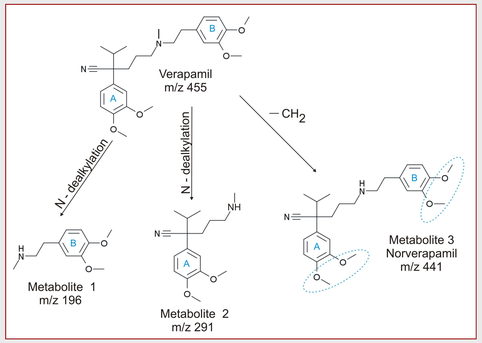


Figure 1: An excerpt of the Verapamil metabolic pathway. Blue dotted ellipses are indicating other places of possible loss of CH_2 [4].

Methods/Instrumental

A preparative electrochemical cell (Antec) equipped with a Glassy Carbon (GC) working electrode was used for synthesis of metabolites. 250 µM solution of the Verapamil in 20mM ammonium formate, pH 7.4 in acetonitrile (50/50, v/v) was used as a model drug. The electrochemically synthesized metabolites of Verapamil were collected off-line, followed by MS analysis of the collected fractions (Figure 2C). The flow rate used in the synthesis experiments was 50 µL/min. A square wave pulse was applied to achieve stabile continuous metabolite generation over long periods of time. Optimization of the metabolite synthesis was performed based on scanning voltammetry. An LTQ-FT (Thermo, USA) 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 TriVersa NanoMate (Advion, USA) system operating in Chip-Based Infusion mode connected to LTQ-Orbitrap (Thermo, USA) was used to record the multistage mass spectral trees of Verapamil and its selected metabolites. The five most intense ions were selected for MS^2 and MS^3, and the three most intense ions for the rest of the MS levels (Figure 2D). RAW data were converted to mzXML format using ReadW software (Institute for Systems Biology, Seattle, US). All MS^n data of the reference compounds were processed with the so-called MEF tool [3].

Instrumental set up

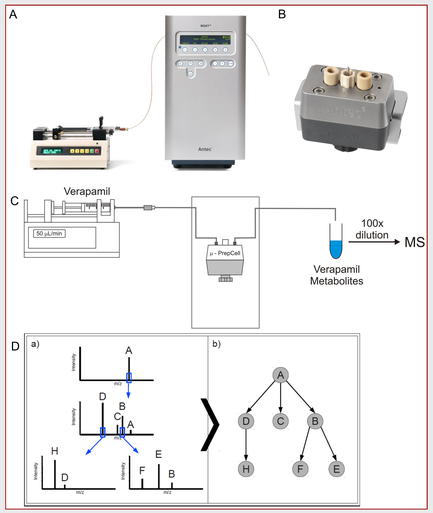


*Figure 2: **A:** ROXY EC system (Antec). **B:** µ-PrepCell (Antec). **C:** Instrumental set-up of used for metabolite synthesis. **D:** Correlation between the spectral tree graph representation (a) and the fragmentation tree graph representation (b) [3].*

Results

We propose 4 steps protocol for an optimized, electrochemical synthesis of drug/xenobiotic metabolites:

Step 1: Scan Voltammetry with electrochemical detection

Step 2: Scan Voltammetry with mass spectrometric detection

Step 3: Optimization of square wave pulses parameters

Step 4: Metabolite synthesis with off line samples collection

In **Step 1**, a working potential range and maximum potential are established (Figure 3A). It is not possible to identify the metabolites based on current profile acquired in Step 1. Therefore, an scan voltammetry experiment with on line MS detection is mandatory for unambiguous identification of the synthesized metabolites (**Step 2**, Figure 3B). MS allows direct monitoring of metabolites synthesized under the current experimental conditions and speed up process of optimization. In **Step 3**, settings for a square wave pulse for metabolite synthesis are optimized. It is recommended to run this experiment with on-line MS detection to monitor on-line the synthesis efficiency while different pulse settings are applied.
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 a constant metabolite synthesis. The electrode surface is continuously reactivated during the run, reducing adsorption/fouling, while high sample concentration is recommended for efficient synthesis. Additionally, the pulse form can be easily adapted to the user needs and programmed in the event table of Dialogue software. In **Step 4**, metabolites of Verapamil (m/z of 441; 291 and 196) were synthesized by applying the square wave pulse. The control samples were collected in 15, 45, 75, and 100 minute of synthesis. The control samples were diluted 100 x before injection to MS.

Results

Step 1: Scanning Voltammetry with electrochemical detection

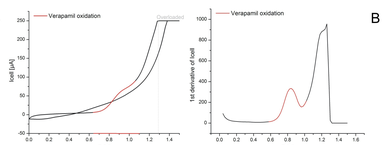


*Figure 3: **A:** A single (full) scan cycle of Verapamil. Conditions: E1=0V; E2=1.5V; cycle: continuous; rate: 20mV/s; flow rate: 50µL/min. **B:** The same plot but 1st derivative of the current is drawn to visualize the region where oxidation take place.*

Step 2: Scanning Voltammetry with mass spectrometric detection

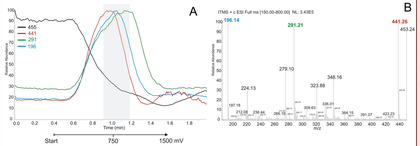
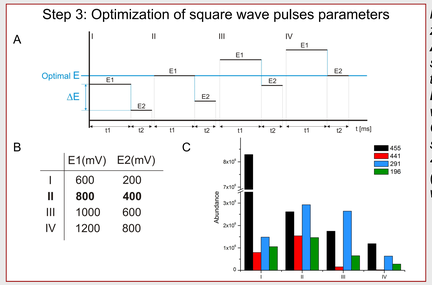


*Figure 4: **A:** MS Voltammogram of Verapamil. **B:** Mass spectrum corresponding to gray area of EIC from A (background was subtracted). Maximum signal was detected at 750mV and was consistent with experiment performed in Step 1.*

Step 3: Optimization of square wave pulses parameters



	E1(mV)	E2(mV)
I	600	200
II	**800**	**400**
III	1000	600
IV	1200	800

*Figure 5: Pulse mode optimization. **A:** The potentials were chosen for a regime where reaction was occurring (STEP 1). **B:** Different pulse potentials were tested (I, II, III, IV). **C:** 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 off-line samples collection

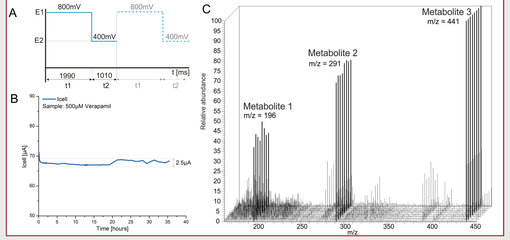


*Figure 6: **A:** Optimized square wave pulses settings. **B:** Long term current stability experiment. **C:** An overlay of 8 mass spectra of the control samples. The control samples were collected in 15, 45, 75, and 100 minute of synthesis. The control samples were diluted 100 x before injection to MS.*

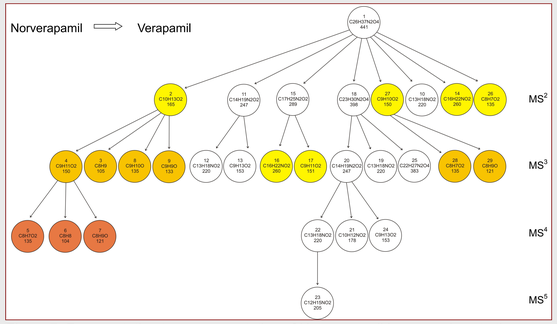
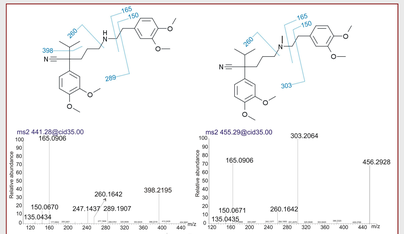


Figure 7: Fragmentation tree representation of Norverapamil in comparison to the parent drug (Verapamil). Coloured nodes indicate common fragmentation pattern for both compounds. The white nodes are the unique fragmentation patterns of Norverapamil. The similarity is based on comparing the elemental compositions. Elemental composition was calculated using MEF tool [3]. Fragmentation of the peak at m/z = 289 (Norverapamil) and m/z = 303 (Verapamil, not shown here) results in the same fragmentation pattern (peaks nr 16 and 17).



Type of modification	MS^2 fragment ions
N - demethylation	398, 289, 260, 247, 165, 150, 135
O-demethylation (ring A)	289, 246, 165, 150, 135
O-demethylation (ring B)	303, 291/289, 260, 248, 233, 165, 151

Figure 8: MS^2 fragmentation spectra of Norverapamil (A) and Verapamil (B). Presence of peaks at m/z = 398 and 247 indicates formation of Norverapamil. MS^2 fragment ions corresponding to demethylation of Verapamil are presented in the table [4].

Conclusions

A long-lasting, stable and efficient electrochemical synthesis of metabolites is possible by applying a square wave pulse. Spectral trees comparison can automate and accelerate identification of unknown metabolites or confirm the origin of the known ones. Electrochemistry, MS, and a reliable data analysis protocol can create a complete platform for investigation of drug/xenobiotic metabolism.

Acknowledgements

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References

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