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Simulating Oxidative Metabolism using on-line Electrochemistry/Mass Spectrometry

Joann Purkerson[1]; Martin Eysberg[2]; Agnieszka Kraj[2]; Hendrik-Jan Brouwer[2]

[1]Antec (USA), P.O. Box 111418, Palm Bay, FL32911, USA; [2]Antec BV, Industrieweg 12, 2382NV Zoeterwoude, The Netherlands

Overview

On-line electrochemistry (EC)/mass spectrometry (MS) has become a powerful tool for the simulation and prediction of the oxidative metabolism of drugs and xenobiotics. Using the ROXY™ EC system on-line with MS results in:

- Fast, synthesis of metabolites (seconds vs. days or weeks using in-vitro and/or in-vivo methods) often accompanied by their intermediates
- Recording metabolic fingerprint
- No isolation steps necessary
- Zero matrix effect (no adduct formation with cell material)

Introduction

The knowledge of the metabolic pathways and the biotransformation of new drugs are crucial for the elucidation of degradation routes of the new active compounds, especially in the area of possible toxicity.
In vitro studies are based on incubating drug candidates with e.g., liver cells (in microsomes activity of cytochrome P450 is high) and isolating and detecting the metabolic products. With the introduction of the ROXY™ EC system oxidative metabolism, as usually occurring in the liver cells by the Cytochrome P450 oxidation, can be simulated successfully within seconds and detected by electrospray mass spectrometry (ESI-MS)[1-4]. Combining the ROXY EC system with MS creates a powerful platform for oxidative metabolite investigations and helps to overcome many of the laborious tasks by isolating the metabolites form in vivo (urine, plasma, etc.) or in vitro (microsomes) studies.

Instrumentation

The ROXY EC system (Fig. 1) for single compound screening includes the ROXY Potentiostat equipped with a ReactorCell™, infusion pump and all necessary fluidic connections. The ROXY EC system is controlled by Antec Dialogue software. The ReactorCell was equipped with a Glassy Carbon working electrode and a HyREF™ reference electrode for the generation of acetaminophen metabolites.

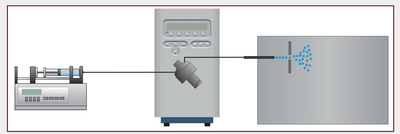

Figure 1: ROXY EC™ system (Antec).

Figure 2: Reactor cell with inlet and outlet (left) and electrode holder with different working electrodes (right).

The acetaminophen sample was delivered through the system with a syringe pump equipped with a 1000 µL gas tight syringe. A MicrOTOF-Q (Bruker Daltonics, Germany) with Apollo II ion funnel electrospray source was used to record mass spectra. MS data were analyzed by Compass software. The method was optimized on a 10 µM acetaminophen solution. Mass spectrometer calibration was performed using sodium formate clusters at the

Oxidative Metabolism – Phase I

A 10µM acetaminophen solution in 10mM ammonium formate (pH 7.4 adjusted with ammonium hydroxide solution) with 25% acetonitrile was pumped at a constant flow rate of 10 µL/min through the ReactorCell using an infusion pump. The outlet of the reactor cell was connected directly (online) to the ESI-MS source. Working electrode potential was ramped from 0 – 1300 mV with incremental steps of 100 mV. After each change of the cell potential mass spectra were recorded. The total run time to record the mass voltammogram was approximately 15 min. The instrumental setup of the ROXY EC system for oxidative metabolism phase I is shown in Figure 3A.

Oxidative Metabolism – Phase II

A 10µM acetaminophen solution in 10mM ammonium formate (pH 7.4 adjusted with ammonium hydroxide solution) with 25% acetonitrile was pumped with a constant flow of 10 µL/min through the ReactorCell using an infusion pump. Adduct formation of acetaminophen and glutathione (GSH) was established using a 100µL reaction coil placed between ReactorCell and the electrospray source. 50µM glutathione in mobile phase was added at the same flow rate via a T-piece into the coil. The reaction time at the specified flow rate is 5 min. The effluent from the reaction coil was injected directly into the ESI-MS. The instrumental set-up of the ROXY EC system for oxidative metabolism phase II is shown in Figure 3B.

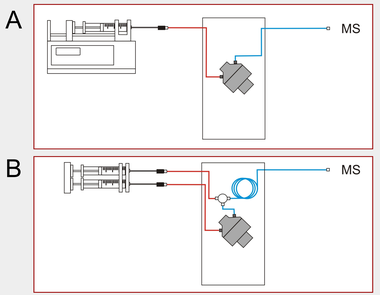

Figure 3: Instrumental set-up of ROXY EC system for oxidative metabolism: A) phase I: oxidation of the compound. B) phase II: adduct formation (conjugation) of the oxidized compound with target (e.g. protein, GSH, etc.).

Results

Acetaminophen (paracetamol; APAP; IUPAC: N-(4-hydroxy phenyl)acetamide) was chosen as model drug to investigate oxidative metabolism using the ROXY EC system.
Acetaminophen is a non-narcotic, analgesic and antipyretic drug, widely used as a pain relief medicine. Acetaminophen is metabolized in the liver by enzyme cytochrome P 450 to a highly reactive metabolite – N-acetyl-p-benzoquinoneimine (NAPQI), which can cause acute hepatic necrosis if not followed by conjugation with glutathione (GSH). The other known metabolic pathways of acetaminophen are via glucuronidation and sulfation pathways. The electrochemical conversion of the acetaminophen into reactive phase I metabolites and the NAPQI – GSH phase II conjugate (adduct) was successfully achieved within a few minutes using the ROXY EC system.

Results (cont.)

Phase I

In Figure 4 the mass voltammogram is shown for acetaminophen. The voltammogram was recorded using an event table executed in Dialogue software (Antec). The extracted ion chromatogram for the mass-to-charge ratio (m/z) of 152 (+/- 0.2u), of protonated acetaminophen is shown in Figure 5A. A significant drop in response is observed in the potential range between 400 and 800 mV which is attributed to the oxidation of acetaminophen in the cell.

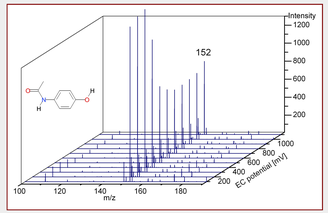

Figure 4: Mass voltammogram of acetaminophen, m/z 152. Ion abundance versus m/z as a function of EC potential. The mass voltammogram is metabolic fingerprint of the molecule.

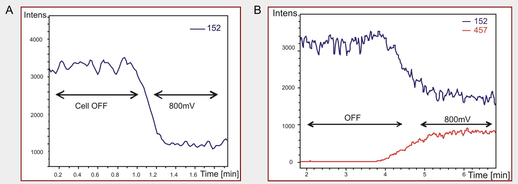

Figure 5: A) Phase I experiment. APAP abundance vs. EC potential (EIC of m/z =152.1 Th). B) Phase II experiment. APAP abundance (blue trace) and NAPQI –GSH conjugate (red trace) vs. EC potential (EICs of m/z=152.1Th and 457.1Th, respectively).

Phase II

The toxic metabolite N-acetyl-*p*-benzoquinoneimine (NAPQI) created in phase I experiment, in the presence of thiol containing molecules like GSH, is quenched by adduct formation. To confirm the presence of the conjugation product of the NAPQI and GSH, mass spectra were acquired with the ReactorCell off and at Ec = 800 mV. Figure 6 shows the spectra with the ReactorCell off (Fig. 6A) and on at 800 mV (Fig. 6B). Figure 7 shows zoom in of the mass spectrum from Figure 6 (the red circle range). It is evident that the NAPQI – GSH conjugation product is only present in the spectrum recorded at 800 mV (Fig. 5B). To confirm that the peak at m/z of 457.1415 is originating from the Acetaminophen-GSH adduct, the fragmentation spectrum was acquired and the chemical formula of the adduct was calculated using Smart Formula (Bruker Daltonics software). The correct formula was found with relative error of 0.8 ppm. The fragmentation pattern confirmed loss of Glycine and Glutamate, which are building block of glutathione (Glu-Cys-Gly) (Data not shown in the poster).

Results (cont.)

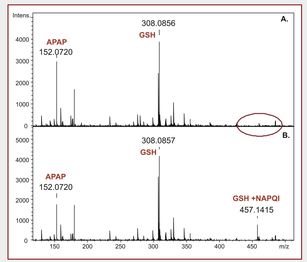

Figure 6: Result of conjugation of phase I metabolite of acetaminophen (APAP) and GSH. (A.) ReactorCell OFF, (B.) Reactor Cell EC=800mV.

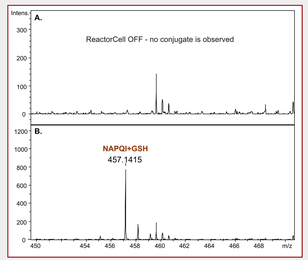

Figure 7: Zoom in of mass range from m/z of 447 to 483. (A.) ReactorCell OFF, (B.) Reactor Cell EC=800mV. Peak at m/z of 457.1415 corresponds to protonated ion of conjugation product.

Conclusions

Acetaminophen was successfully used as model drug to mimic the oxidative metabolic detoxification pathway in the human liver by on-line EC/MS. Phase I and II metabolites, which were already known from the literature as detoxification products in vivo, were generated in the EC reactor cell and on-line identified by MS using either acetaminophen alone or in the presence of glutathione.
These results clearly illustrate the potential of EC/MS as a powerful tool for predicting metabolic processes.
The ROXY™ EC system provides a versatile and user friendly platform for studying phase I and II metabolism of target compounds (drugs, pharmaceuticals, herbicides, etc.). Mass voltammograms can be recorded automatically to obtain a metabolic fingerprint of the compound of interest in a short time frame.

Acknowledgements

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