Fast Mimicking of Phase 1 and 2 Metabolism of Acetaminophen using the ROXY™ EC System

- Acetaminophen, Paracetamol, Tylenol, Panadol, APAP
- Simulating CYP450 oxidative metabolism in <10 min.
- Controlled oxidation (phase 1) and detoxification (phase 2) reactions
- Fast and easy in use, complements HLM, RLM

Introduction

The knowledge of the metabolic pathways and the biotransformation of new drugs are crucial for 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, which usually occurs in the liver cells by Cytochrome P450 oxidation, can be simulated successfully within seconds and detected by electrospray mass spectrometry (ESI-MS) [1-5].

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 studies, e.g., urine, plasma, etc., or in vitro studies, e.g., rat liver microsomes (RLM) or human liver microsomes (HLM).
**Summary**

Acetaminophen (paracetamol; APAP; IUPAC: N-(4-hydroxy phenyl)acetamide) was chosen as model drug to investigate oxidative metabolism using the ROXY EC System. Electrochemical conversion of the acetaminophen into reactive phase I metabolite – N-acetyl-p-benzoquinoneimine (NAPQI) and the NAPQI – GSH phase II conjugate was successfully achieved.

All three pathways yield final products that are inactive, non-toxic, and excreted by the kidneys. In the third pathway, however, the intermediate product NAPQI is toxic. NAPQI is primarily responsible for the toxic effects of acetaminophen, causing acute hepatic necrosis. Production of NAPQI is primarily due to two isoenzymes of cytochrome P450: CYP2E1 and CYP3A4. At usual doses, NAPQI is quickly detoxified by conjugation with glutathione (phase II reactions).

**Method**

The ROXY™ EC System (Figure 2) for single compound screening includes the ROXY potentiostat equipped with a ReactorCell™, infusion pump and all necessary LC connections. The ROXY EC System is controlled by Antec Dialogue software. The ReactorCell equipped with a Glassy Carbon working electrode and a HyREF™ reference electrode was used for the generation of acetaminophen metabolite.

**Acetaminophen Metabolism**

Acetaminophen is a non-narcotic, analgesic and antipyretic drug, widely used as a pain relief medicine. Acetaminophen is metabolized primarily in the liver, into toxic and non-toxic products. Three metabolic pathways are known (see Figure 1). The non-toxic Glucuronidation which accounts for 45-55% and the Sulfation (sulfate conjugation) which accounts for 20–30%. N-hydroxylation and dehydration, then GSH conjugation, accounts for less than 15%. The hepatic cytochrome P450 enzyme system metabolizes acetaminophen, forming a minor yet significant alkylating metabolite known as NAPQI (N-acetyl-p-benzoquinoneimine). NAPQI is then irreversibly conjugated with the sulphydryl groups of glutathione (GSH) [6].
Acetaminophen (paracetamol; APAP; IUPAC: N-(4-hydroxyacetamido)anisole) is a widely used pain relief medicine. Acetaminophen is primarily responsible for the toxic effects of acetaminophen, however, the intermediate product NAPQI is toxic. NAPQI is established using a 100μL reaction coil placed between the ReactorCell and the electrospray source and 50μM glutathione (GSH) 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 and the effluent from the reaction coil was injected directly into the ESI-MS. The instrumental set-up of the ROXY EC System for adduct formation (phase II) is shown in Figure 4.

### Oxidative metabolism – Phase I

A 10μM acetaminophen solution in 10mM ammonium formate (pH 7.4 adjusted with ammonium hydroxide solution) in 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 (on-line) 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 MS voltammogram was approximately 10min. Instrumental set-up of ROXY EC System for oxidative metabolism phase I is shown in Figure 3.

### Detoxification (GSH adduct formation) – 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 the ReactorCell and the electrospray source and 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 and the effluent from the reaction coil was injected directly into the ESI-MS. The instrumental set-up of the ROXY EC System for adduct formation (phase II) is shown in Figure 4.

### Table 2

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
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<tbody>
<tr>
<td>Mass range</td>
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<tr>
<td>Ion polarity</td>
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<tr>
<td>Capillary voltage</td>
<td>-4500 V</td>
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<tr>
<td>Nebulizer</td>
<td>0.4 Bar</td>
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<tr>
<td>Dry gas</td>
<td>4 L/min</td>
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<tr>
<td>Temperature</td>
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<tr>
<td>Funnel 1 RF</td>
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<tr>
<td>Funnel 2 RF</td>
<td>200 Vpp</td>
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<tr>
<td>ISCID energy</td>
<td>0 eV</td>
</tr>
<tr>
<td>Hexapole</td>
<td>100 Vpp</td>
</tr>
<tr>
<td>Ion energy</td>
<td>5 eV</td>
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</table>

### Figure 3: Instrumental set-up of ROXY EC System for oxidative metabolism phase I.

### Figure 4: Instrumental set-up of ROXY EC System for adduct formation (Phase II reactions) by adding GSH via a T-piece after the ReactorCell. Mimicking the detoxification reaction of NAPQI by forming the NAPQI-GSH adduct.
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Results

Phase I

Table 3 consists of a list of compounds related to acetaminophen metabolism and their monoisotopic masses used for mass spectra interpretation. The mass voltammogram for acetaminophen (Figure 5) was recorded using an event table executed in Dialogue. In the Appendix 210.001A the background information is given about Dialogue and event table programming for automated recording of MS voltammograms.

Table 3

<table>
<thead>
<tr>
<th>Compounds related to acetaminophen metabolism</th>
<th>Name</th>
<th>Formula</th>
<th>Monoisotopic mass* [u]</th>
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<tbody>
<tr>
<td>Acetaminophen</td>
<td>C8H9NO2</td>
<td>151.063329</td>
<td></td>
</tr>
<tr>
<td>NAPQI</td>
<td>C8H7NO2</td>
<td>149.047678</td>
<td></td>
</tr>
<tr>
<td>GSH</td>
<td>C10H17N3O6S</td>
<td>307.083806</td>
<td></td>
</tr>
<tr>
<td>NAPQI-GSH</td>
<td>C18H24N4O8S</td>
<td>456.131484</td>
<td></td>
</tr>
</tbody>
</table>

* In ESI ions are created by the loss or gain of a proton (Monoisotopic mass of proton: 1.00727646677 u).

A significant drop in response is observed after the potential above 400 mV is applied. The drop of abundance is attributed to the oxidation of acetaminophen in the ReactorCell and the formation of reactive metabolite. The extracted ion chromatogram representing the mass-to-charge ratio (m/z) of 152 (+/- 0.2u), of protonated acetaminophen is shown in Figure 6.

![Figure 6: APAP abundance vs. EC potential. EC=800mV was applied to oxidize acetaminophen.](image)

Phase II

To confirm the presence of the conjugation product of acetaminophen reactive metabolite (NAPQI) and GSH, mass spectra were acquired with the ReactorCell off and at Ec = 800 mV, when phase II instrumental set up was used. Figure 7 shows the spectra with the ReactorCell off (Fig. 7A) and on at 800 mV (Fig. 7B). Figure 8 shows zoom in of the mass spectrum from Figure 7 (the red circle). It is evident that the NAPQI – GSH conjugation product is only present in the spectrum recorded at 800 mV (Fig. 8B).

![Figure 7: Result of conjugation of phase I metabolite of acetaminophen (APAP) and GSH. (A.) ReactorCell OFF, (B.) ReactorCell EC=800mV.](image)
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Figure 8: Zoom in of mass range from m/z of 445 to 490 (Red circle in the Figure 7). (A) ReactorCell OFF, (B) ReactorCell EC=800mV. Peak at m/z of 457.1432 corresponds to protonated ion of conjugation product. The peak of m/z of 479.1245 was identified as its Na+ adduct.

To confirm that the peak at m/z of 457.1432 is originating from the NAPQI-GSH adduct, the fragmentation spectrum (Fig. 9) was acquired and the chemical formula of the adduct was calculated using Smart Formula (Bruker Daltonic 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).

Figure 9: Fragmentation spectrum of conjugation product.

Conclusion

The on-line coupling of the ROXY™ EC System with MS (EC/MS) provides a versatile and user-friendly platform for fast screening of target compounds (drugs, pharmaceuticals, pollutants, etc.) for oxidative metabolism (phase 1 reactions), thereby mimicking the metabolic pathway of CYP450 reactions.

MS voltammograms can be recorded automatically to obtain a metabolic fingerprint of the compound of interest in less than 10 min.

In addition, rapid and easy studies of adduct formations can be performed simply by adding GSH after the ReactorCell (phase II reactions).
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References
Figure 8: Mass spectra obtained with different potentials and a ReactorCell pump and ReactorCell.

Figure 10: Schematic view of the system consisting of ROXY Potentiostat, dual syringe pump, ReactorCell, and Mass Spectrometer.

Ordering number 210.0070A ROXY™ EC system, incl. dual syringe pump, ReactorCell, and Mass Spectrometer.

All parts included for described Electrochemical experiments.

References

Conclusion
Simulating CYP450 oxidative metabolism in <10 min.

Acetaminophen, Paracetamol, Tylenol, Panadol, APAP

Purposeful degradation
Purposeful degradation

Degradation & persistence
Degradation & persistence

Surface & drinking water
Surface & drinking water

Transformation products
Transformation products

Degradation & persistence
Degradation & persistence

Phase II reactions
Phase II reactions

The knowledge of the metabolic pathways and the biotransformation of new drugs are crucial for drug development. In vitro studies are based on incubating drug candidates with, e.g., liver cells (in microsomes) or isolated enzymes (CYP450). In vivo studies, e.g., rat liver microsomes (RLM) or human liver microsomes (HLM), provide information about the metabolic stability and toxicity of new drugs.

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 the complex biological matrix.

References