# OXIDATIVE METABOLISM OF ACETAMINOPHEN USING ROXY™ EC SYSTEM

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 pharmaceutics
  - *Acetaminophen*
  - *Artemisinin*
  - *Dihydro-artemisinin*
  - *Artemether*
  - *Etoposide*
  - *8-OH-DPAT*
  - *mesna BNP7787*
  - *Vincristine*

## 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, 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)[1-5]. 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 (urine, plasma, etc.) or in vitro (microsomes) studies.

- Acetaminophen, NAPQI and GSH
- Simulating Cytochrome P450 Oxidation using EC in combination with MS
- Phase I and II Oxidative Metabolism
- Versatile and User-Friendly Platform

## Summary

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

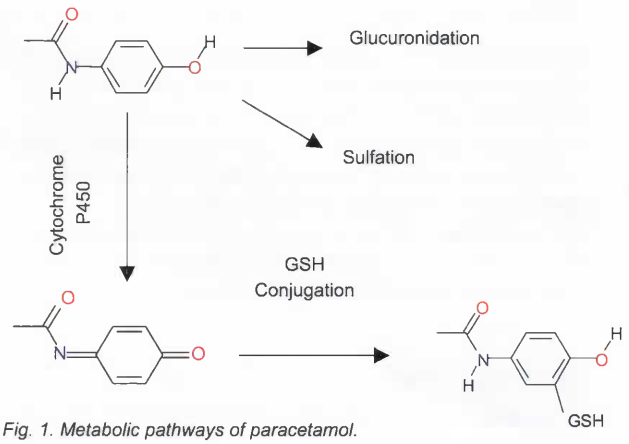


*Fig. 1. Metabolic pathways of paracetamol.*

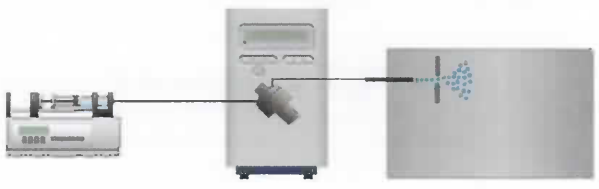

*Fig. 2. ROXY™ EC System including ReactorCell™ and dual syringe infusion pump.*

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) [6]. The other known metabolic pathways of acetaminophen are via glucuronidation and sulfation path-ways (See Figure 1).

## Method

The ROXY EC System (Figure 2) for single compound screening (p/n 210.0070) 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 Glassy Carbon working electrode and HyREF™ reference electrode was used for the generation of acetaminophen metabolites.

Table 1

| Conditions | |
|---|---|
| EC | ROXY™ EC System (p/n 210.0070) |
| Cell | ReactorCell™ with GC WE and HyREF™ |
| Flow rate | 10µL/min |
| Potential | 0 – 1300 mV (100 mV steps) |

The acetaminophen sample was delivered to the system with a syringe pump equipped with 1000 µL 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 10µM paracetamol 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 – 100 m/z |
| Ion polarity | Positive |
| Capillary voltage | -4500 V |
| Nebulizer | 0.4 Bar |
| Dry gas | 4 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 |

## 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 15min. Instrumental set-up of ROXY EC System for oxidative metabolism phase I is shown in Figure 3.

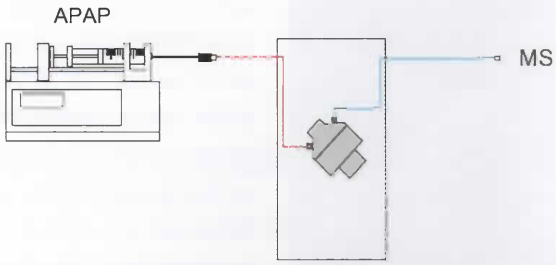


*Fig. 3. Instrumental set-up of ROXY EC System for oxidative metabolism phase I.*

## 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 electro-spray 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 4.

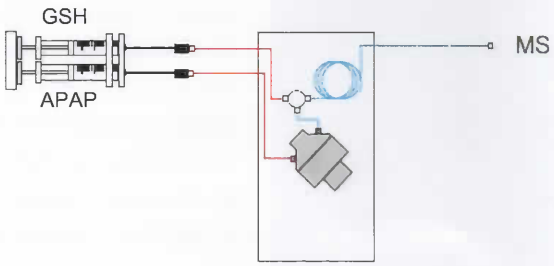


*Fig. 4. Instrumental set-up of ROXY EC System for oxidative metabolism phase II.*

## Results

### Phase I

Table 2 consists of list of compounds related to acetaminophen metabolism and their monoisotopic masses used for mass spectra interpretation. In Figure 5 the mass voltammogram is shown for acetaminophen. The voltammogram was recorded using an event table executed in Dialogue. In appendix 210.001A background information is given about Dialogue and event table programming for automated recording of mass voltammograms.

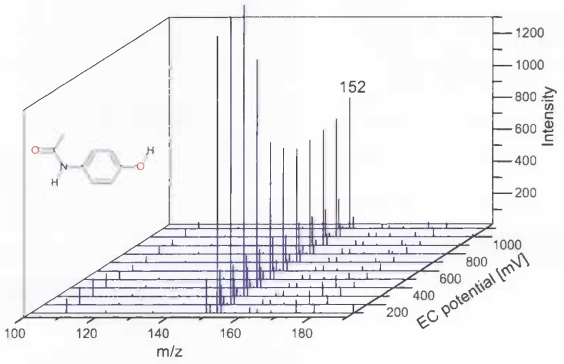


*Fig. 5. Mass voltammogram of acetaminophen. Ion abundance versus m/z as a function of EC potential.*

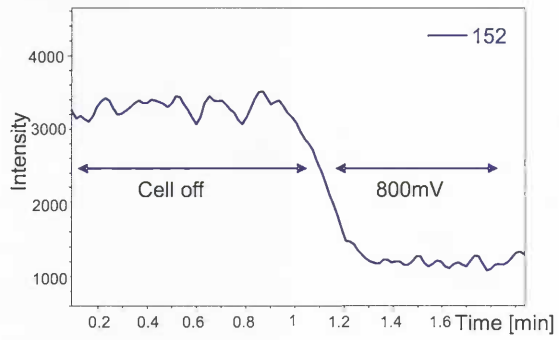


*Fig. 6. APAP abundance vs. EC potential.*

The extracted ion chromatogram for the mass-to-charge ratio (m/z) of 152 (+/- 0.2u), of protonated acetaminophen is shown in Figure 6. 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 ReactorCell.

Table 3

Compounds related to acetaminophen metabolism

| Name | Formula | Monoisotopic mass* [u] |
|---|---|---|
| Acetaminophen | $C_8H_9NO_2$ | 151.063329 |
| NAPQI | $C_8H_7NO_2$ | 149.047678 |
| GSH | $C_{10}H_{17}N_3O_6S$ | 307.083806 |
| NAPQI-GSH | $C_{18}H_{24}N_4O_8S$ | 456.131484 |

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

### 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. 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 range). It is evident that the NAPQI – GSH conjugation product is only present in the spectrum recorded at 800 mV (Fig. 8B).

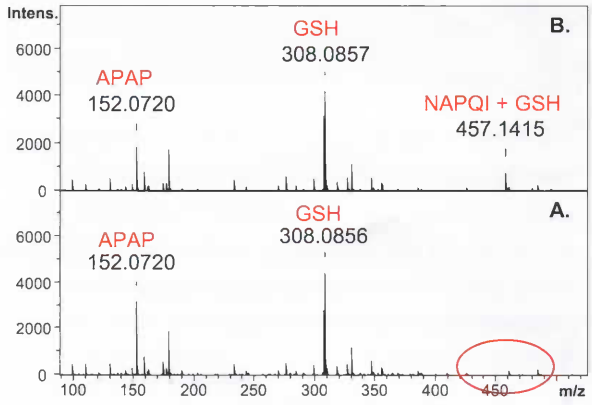


*Fig. 7. Result of conjugation of phase I metabolite of acetami-nophen (APAP) and GSH. (A.) ReactorCell OFF, (B.) Reactor Cell EC=800mV.*

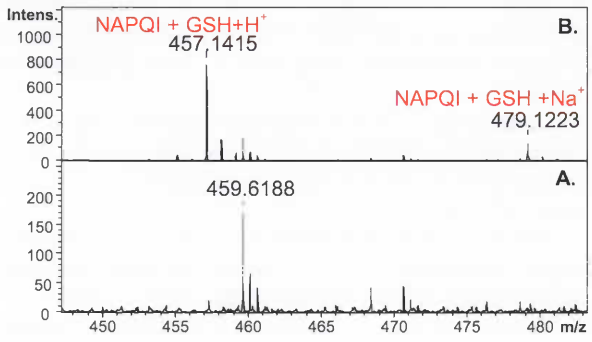


*Fig. 8. Zoom in of mass range from m/z of 447 to 483. (A.) ReactorCell OFF, (B.) Reactor Cell EC=800mV. Peak at m/z of 457.1415 corresponds to protonated ion of conjugation product. The peak of m/z of 479.1223 was identified as its Na+ adduct.*

To confirm that the peak at m/z of 457.1415 is originating from the Acetaminophen-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).

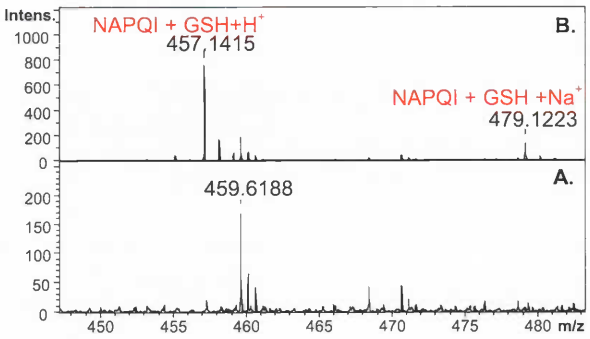


*Fig. 9. Fragmentation spectrum of conjugation product.*


*Fig. 10. ROXY™ EC System.*

## CONCLUSION

**The ROXY™ EC System provides a versatile and user-friendly platform for screening of single target compounds (drugs, pharmaceuticals, herbicides, etc.) in phase I and II metabolomics studies. Mass voltammograms can be recorded automatically to obtain a metabolic fingerprint of the compound of interest in a short time frame.**

| PART NUMBERS | |
|---|---|
| 210.0070 | ROXY™ EC System |

## References

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