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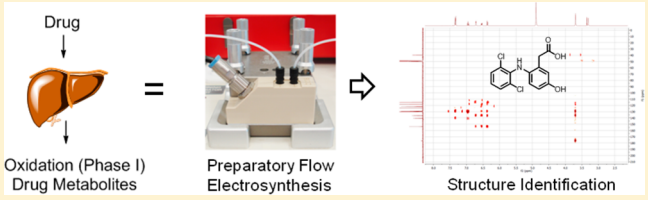
Preparative Microfluidic Electrosynthesis of Drug Metabolites

Romain Stalder and Gregory P. Roth*

Sanford-Burnham Medical Research Institute at Lake Nona, 6400 Sanger Road, Orlando, Florida 32827, United States

Supporting Information

ABSTRACT: In vivo, a drug molecule undergoes its first chemical transformation within the liver via CYP450-catalyzed oxidation. The chemical outcome of the first pass hepatic oxidation is key information to any drug development process. Electrochemistry can be used to simulate CYP450 oxidation, yet it is often confined to the analytical scale, hampering product isolation and full characterization. In an effort to replicate hepatic oxidations, while retaining high throughput at the preparative scale, microfluidic technology and electrochemistry are combined in this study by using a microfluidic electrochemical cell. Several commercial drugs were subjected to continuous-flow electrolysis. They were chosen for their various chemical reactivity: their metabolites in vivo are generated via aromatic hydroxylation, alkyl oxidation, glutathione conjugation, or sulfoxidation. It is demonstrated that such metabolites can be synthesized by flow electrolysis at the 10 to 100 mg scale, and the purified products are fully characterized.



KEYWORDS: *Drug metabolites, electrochemistry, microfluidic synthesis, continuous-flow oxidation*

The first step toward elimination of xenobiotic compounds in vivo occurs predominantly through first pass hepatic oxidation. In the liver, the oxidation takes place at the iron center of the porphyrin that constitutes the catalytic site of the CYP450 family of enzymes.[1,2] The oxidized metabolites (Phase I) may undergo subsequent transformations (Phase II) including, among others, conjugation with glutathione (GSH). This is of particular importance in the design of new drug candidates since their toxicity levels are regulated by such metabolic processes.[3,4] Significant research effort has been devoted to develop synthetic methodologies to simulate the metabolism of drugs. These methodologies fall into four distinct categories: microsomal incubation, porphyrin-catalyzed chemical oxidation, Fenton-type reactions, and electrochemical oxidations.[5,6]

Electrosynthetic transformations offer the advantage of atom economy since the oxidation takes place at the surface of an electrode, which provides the electron from a current source.[7] These transformations are thus heterogeneous reactions by definition. To achieve high conversion, the substrate solution must be conductive, and the ratio of electrode surface-to-solution volume ought to be as high as possible. While electrosynthesis has been extensively performed in batch mode,[8,9] continuous-flow technology provides increased electrode surface ratios by design.[9] Additionally, at the microfluidic scale the distance between the working electrode and the counter electrode is shortened. This can help overcome conversion limitations linked to solution resistivity, and even enable electrolyte-free reactions.[10] In the past few years, the study of drug metabolites has benefited from the emergence of in-line electrochemical/mass spectrometry (EC-MS) systems used to generate phase I and phase II metabolites.[13,14] Unfortunately, full structure elucidation is limited by the analytical nature of the latter technology, where product scale is too low to purify and isolate metabolites for NMR characterization. Moreover, preparative amounts of fully characterized pure drug metabolites can play a significant role in assays and improve the drug development process.

Herein, we report a preparative scale continuous-flow electrosynthesis of the phase I metabolites of several commercial drugs. Phase II glutathione (GSH) adducts are also shown to be synthetically accessible and isolable. A commercially available continuous-flow electrochemical cell was used,[15] within which a diversity of chemical reactivity, functional groups, and solubility were investigated (Figure 1).

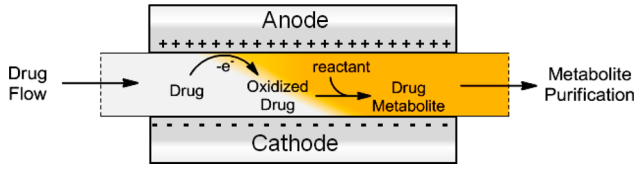


Figure 1. Schematic diagram of the continuous-flow process for electrochemical synthesis of drug metabolites.

Five drugs were used to test the oxidative electrochemistry in flow: diclofenac, tolbutamide, primidone, albendazole, and chlorpromazine. Each drug was selected based on first pass hepatic oxidation at unique metabolic sites. A broad range of oxidative chemistry is targeted: aliphatic oxidation, aromatic hydroxylation, *S*-oxidation, *N*-oxidation, or dehydrogenation.

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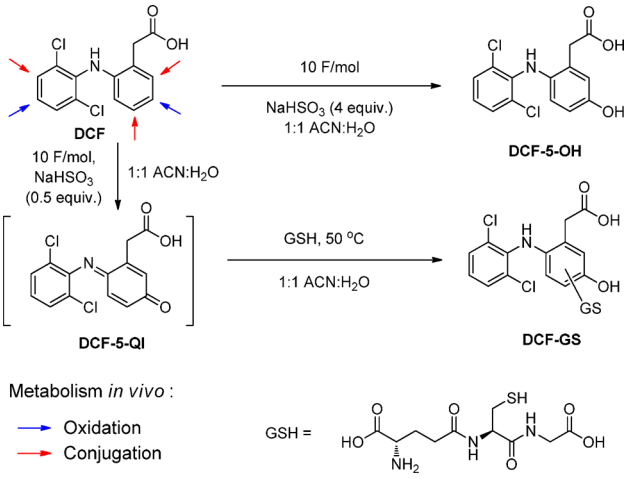
The reactive phase I metabolites can further react in subsequent dealkylation, ring-opening, or glutathione trapping reactions. This study aimed at investigating the versatility of continuous-flow electrosynthesis for the generation, isolation, and full characterization of drug metabolites at the preparatory scale.

Diclofenac (**DCF**) is an anti-inflammatory drug metabolized in the liver via aromatic hydroxylation, which can occur on either of its two phenyl rings para to the nitrogen atom, as shown in Scheme 1.[16] Further conjugation with glutathione in vivo contributes to excretion of the metabolites.

Scheme 1. Electrosynthesis of Phase I Metabolite of Diclofenac (DCF-5-OH) and Its Glutathione Phase II Adduct (DCF-GS) via the Quinone Imine (DCF-5-QI); in Vivo Metabolism of DCF Depicted with Colored Arrows



A 0.1 M solution of **DCF** was prepared in 1:1 acetonitrile/water and flowed through the cell at various flow rates under 113 mA constant current. At constant current, the slower the solution flows through the cell, the more current each unit volume of solution is exposed to. Accordingly, the flow rates were decreased from 0.235 mL/min to 0.088 mL/min to achieve increasing electron equivalents from 3 F/mol to 8 F/mol. Figure 2 shows how the aromatic proton region of the crude ^{1}H NMR spectra evolved with increasing electron equivalents applied to the **DCF** solution. Corresponding to the protons meta to the nitrogen atom on the chlorinated ring, the most deshielded doublet at 7.38 ppm (highlighted in blue) offers a good handle to monitor reaction progress. As the current was increased from 3 to 8 F/mol, the intensity of this **DCF** doublet decreased while a new doublet increased at 7.33 ppm (highlighted in red). A lower intensity doublet also appeared at 7.46 ppm, but remained below 10% of the overall product distribution. At currents greater than 7 F/mol, less than 5% of starting material remained. After purification on silica gel, the major product was identified via 2D-NMR as the hydroxylated metabolite of **DCF** at the 5-position of the phenylacetic acid ring (**DCF-5-OH**), as described in the Supporting Information. In an electrolyte-free environment, a substantial amount of overoxidation occurred as evidenced by formation of precipitates, and the isolated yield was limited to 25%. Reaction condition optimization led to the addition of sodium bisulfite as supporting electrolyte. As a mild reducing agent, sodium bisulfite may also play an important role in preventing overoxidation in the cell, which increased the isolated yield of **DCF-5-OH** up to 46%. Upon isolation, this corresponded to a reaction output of 85 mg of pure metabolite per hour of flow electrosynthesis.

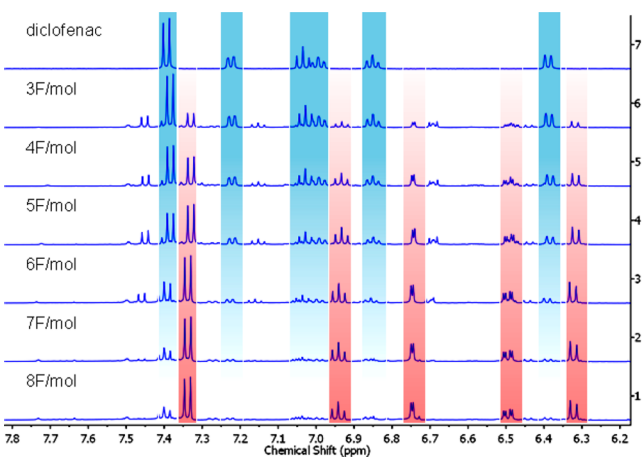


Figure 2. Evolution of the aromatic peaks of diclofenac as a function of increased electron equivalents (from 3 to 8 F/mol) applied to the cell during electrosynthesis of **DCF-5-OH**.

Previously described chemical syntheses of **DCF-5-OH** from **DCF** involve several steps including aryl iodination at the 5-position, conversion to the boron pinacol ester using Miyaura borylation, and subsequent oxidation of the boronate to the alcohol. The overall yield was below 25%.[17,18] In another approach, **DCF-5-OH** was synthesized from 3-methoxyphenylacetic acid in five steps via amidation, iodination, Ullmann coupling, BBr_3-mediated demethylation, and hydrolysis in 42% overall yield.[17,19] In contrast, our conditions enable higher yields in one electrosynthetic step, on an easily scalable platform.

The byproduct observed in Figure 2 from 3 to 6 F/mol with the most deshielded doublet at 7.46 ppm was also isolated as a bright orange solid in 20% yield with an output of 45 mg/h. The compound was characterized by ^{13}C NMR, during which a singlet at 189 ppm was recorded. This is characteristic of a quinone carbonyl downfield shift, and the spectra correspond to the product obtained by previously reported chemical oxidation of **DCF-5-OH**.[18] The byproduct was therefore identified as the oxidized form of **DCF-5-OH**, i.e., the dehydrogenated quinone imine derivative **DCF-5-QI**. Quinone imines are known as first pass hepatic metabolites and are considered toxic due to their electrophilicity.[3] In vivo, glutathione regulates levels of potentially toxic electrophiles by nucleophilic 1,2- or 1,4-addition, affording conjugation metabolites that can be subsequently excreted. Since **DCF-5-QI** was stable enough to be isolated, its reaction with glutathione was attempted, targeting known conjugation metabolites of **DCF**.[17]

The continuous-flow technology is well suited for this purpose because the output of the electrochemical flow cell where **DCF-5-QI** is formed can be directly flowed into a multi-input microfluidic reactor where it is mixed with a flow of glutathione solution. This setup minimizes the idle time between oxidation and conjugation, thus avoiding degradation of the reactive **DCF-5-QI**. Moreover, the stoichiometry of the nucleophilic addition can be precisely controlled throughout the reaction, by adjusting the concentration of the flowing glutathione solution. The electrosynthesis conditions were modified to maintain high levels of the quinone imine at the

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output of the cell by decreasing the stoichiometry of sodium bisulfite. A 0.1 M solution of glutathione was prepared in 1:1 acetonitrile/water to achieve 2 equiv of GSH per mole of **DCF** in the original stock solution. This solution was mixed in flow to the output of the electrochemical cell at the same flow rate of 0.2 mL/min, using a T-shaped connector. The reaction mixture was then heated in flow to 50 °C using a commercially available microfluidic reactor. As shown in Figure 3b, the dark orange

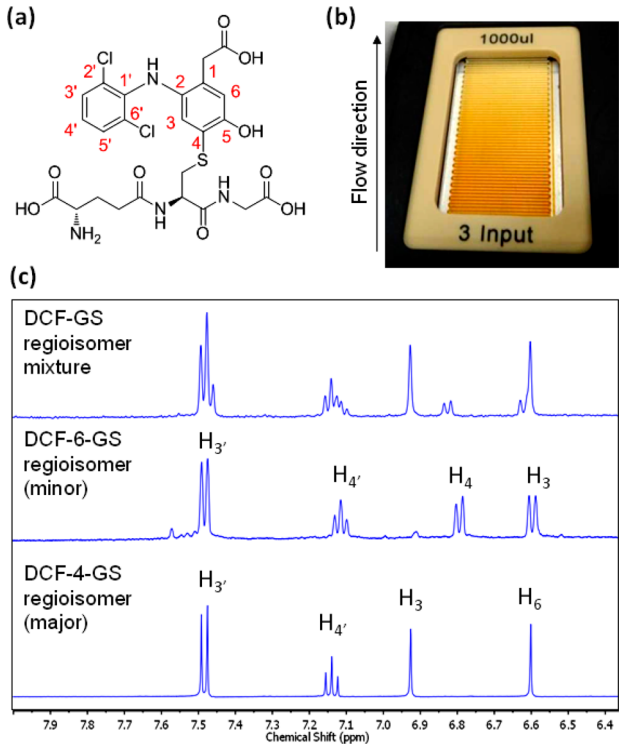
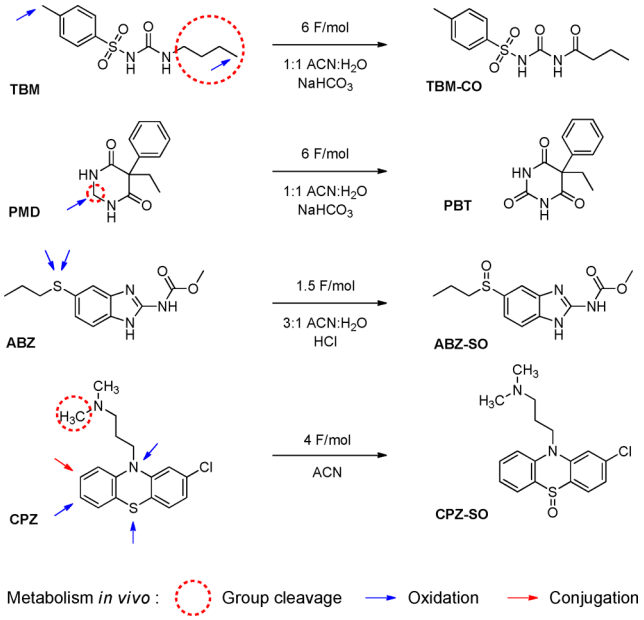


Figure 3. (a) Structure of the major GSH conjugation metabolite **DCF-4-GS**, (b) picture of the 1000 μL reactor where GSH trapping of **DCF-QI** reactive metabolite occurs, and (c) aromatic region of the ^{1}H NMR spectra of the crude mixture and isolated conjugation metabolite regioisomers.

solution at the input (bottom) side of the reactor became light yellow when it reached the output (top) side. Qualitatively, this is indicative of the quinone imine chromophore of **DCF-5-QI** being quenched by reacting with GSH to afford conjugation products. ^{1}H NMR analysis showed that two regioisomers of **DCF-GS** conjugates were formed. The isomer mixture was separated by preparative HPLC. The minor isomer, which accounted for 30% of the mixture, Figure 3c, was identified as the 6-conjugate from the presence of two doublets at 6.59 and 6.78 ppm corresponding to protons H_3 and H_4 on carbons 3 and 4.[17] The major product displayed two singlets at 6.60 and 6.93 ppm, which corresponds to the H_6 and H_3 protons of the aromatic ring, supporting the formation of the 4-conjugate of the **DCF-GS** metabolite displayed in Figure 3a. The isomer mixture was isolated in 28% yield, corresponding to an output of 100 mg/hour.

In a previous study, the electrochemical module used herein was shown to efficiently perform four and six electron oxidations at the benzylic position of selected tolyl substrates.[15] The reaction outcome was shown to depend on the oxidation potential of each substrate: oxidation products were isolated in good yields when a substrate's oxidation occurred below 2.3 V versus the standard calomel electrode (SCE) reference. As depicted in Scheme 2, tolbutamide (**TBM**) is metabolized in

Scheme 2. Oxidation Products of the Flow Electrolysis of Tolbutamide (TBM), Primidone (PMD), albendazole (ABZ), and Chlorpromazine (CPZ); in Vivo Metabolic Sites Are Designated by Colored Arrows and Circles



vivo via benzylic oxidation also, with the hydroxymethyl product reported as a major metabolite.[20,21] The first oxidation of **TBM** occurred at 1.3 V, as measured by cyclic voltammetry (CV). This falls within the potential range at which benzylic oxidation products of tolyl substrates have been isolated. **TBM** was thus subjected to oxidation in flow, targeting the hydroxymethyl metabolite. After liquid chromatography, mass spectrometry revealed one major product with a molecular mass of 285 (MH+), an increase corresponding to the formation of a carbonyl group. ^{1}H NMR analysis of the crude mixture showed no benzaldehyde product. After purification, the major product was identified as **TBM-CO**, resulting from oxidation at the carbon alpha to the urea nitrogen. To the best of our knowledge, this compound has been reported once in the patent literature,[22] but it is important to note that it is not reported as a known metabolite of **TBM**. Nevertheless, this reactivity is consistent with previous reports since oxidation at carbons alpha to amide or urea nitrogens have been commonly utilized in electrosynthesis.[23]

This observation motivated the choice of primidone (**PMD**, Scheme 2) as a test drug for alkyl oxidation in the electrochemical flow cell. One of the two metabolites of **PMD** is phenobarbital (**PBT**), which is formed by oxidation of the methylene linking the two amide nitrogens.[24] A 0.025 M solution of **PMD** in a 1:1 acetonitrile/aqueous buffer was flowed through the electrochemical cell at 0.1 mL/min, with a constant current of 24 mA to achieve 6 F/mol. After purification of the crude mixture by liquid chromatography, **PBT** was isolated in 24% yield. This corresponded to a limited reaction output of 7 mg/h due to the poor solubility of the drug.

The antihelminthic drug albendazole (**ABZ**), depicted in Scheme 2, has two phase I metabolites reported in vivo, which

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result from *S*-oxidation of the thioether: the sulfoxide **ABZ-SO** and the sulfone **ABZ-SO$_2$**.[25,26] A 0.05 M solution of **ABZ** in 3:1 acetonitrile/0.1 M aqueous HCl was flowed though the electrochemical cell at 0.2 mL/min under 24 mA of constant current. This corresponds to 1.5 equiv of electrons, which afforded one oxidized product with a molecular mass of 282 (MH+). At such low current, some starting material remained as evidenced by LC−MS. The methylene protons alpha to the S atom observed at 2.80 ppm in the ^{1}H NMR spectrum of the product become diastereotopic. This is consistent with the structure of the sulfoxide metabolite, where the newly formed S−O bond induces chirality.[27] **ABZ-SO** was isolated in 38% yield after purification with a reaction output of 65 mg/hour. At higher electron equivalents, a distribution of five byproducts was generated as seen by LC−MS. This included a compound with molecular ion mass of 298 (MH+), which presumably corresponds to the sulfone metabolite. Molecular masses indicative of chlorinated byproducts were also observed. Unfortunately, the high number of byproducts hindered isolation of any single compound at such higher currents.

The antipsychotic drug chlorpromazine (**CPZ**) has also been reported to undergo *S*-oxidation upon hepatic metabolism, along with several other metabolites resulting from *N*-oxidation, aromatic hydroxylation, dealkylation, and glutathione conjugation.[28,29] **CPZ** was dissolved in acetonitrile at a concentration of 0.02 M and flowed through the cell at 0.1 mL/min under 12 mA of constant current (4 F/mol). Under such conditions, the flow electrolysis afforded one oxidation product with a molecular ion mass of 335 (MH+), which corresponds to the addition of one oxygen atom to the parent drug. The oxidation product was separated from the unreacted material by liquid chromatography and analyzed by ^{1}H NMR. The number of aromatic protons remained unchanged, but the overall aromatic signals were deshielded by 0.4 to 0.7 ppm compared to the starting material. The oxidation product was identified as the sulfoxide metabolite (**CPZ-SO**), which was isolated in 83% yield with a reaction output of 33 mg/h. This demonstrates a high yielding selectivity for *S*-oxidation in the cell, performed without the addition of an electrolyte.

As summarized in Table 1, several different reactivities simulating the metabolism of drugs in vivo have been demonstrated using a continuous-flow electrochemical cell. Aromatic hydroxylation, alkyl oxidation, sulfoxidation, quinone imine formation, and glutathione conjugation were achieved on a 10 to 100 mg scale of pure isolated metabolites per hour of flow electrolysis.

Table 1. Isolated Metabolites, Corresponding Reactivity, Isolated Yields, and Electrochemical Flow Reactor Output

metabolite	reactivity	yield[a] (%)	output[b] (mg/h)
DCF-5-OH	aromatic hydroxylation	46	85
DCF-5-QI	quinone imine formation	20	45
DCF-GS	GSH conjugation	28	100
PBT	α-amide oxidation	24	7[c]
ABZ-SO	sulfoxidation	38	65
CPZ-SO	sulfoxidation	83	33

[a]Isolated. [b]Calculated after off-line purification. [c]Limited by starting material solubility.

For any specific compound, the product selectivity of electrochemical oxidation is governed by the most redox-active sites on the molecule. For instance, **DCF** was hydroxylated selectively on its phenylacetic acid ring. No hydroxylation was observed on the dichlorobenzene ring, as previously documented.[30] Likewise, electrochemical oxidation of **CPZ** occurred preferentially at the sulfur atom instead of the nitrogen atoms as shown in Scheme 2. Electrosynthesis will not replace analytical biosynthetic techniques such as microsomal incubations for complete drug metabolism pathway studies; but we have demonstrated that electrosynthesis performed in flow can complement biosynthetic studies via convenient synthesis and isolation of drug oxidation products relevant to their in vivo metabolism. The present study focused on known drug compounds as a proof of concept, yet one can envision this useful technology becoming part of the toolbox for the development process of new and unknown drugs.

Moreover, this was achieved at a reaction output higher than that of typical electroanalytical techniques by several orders of magnitude.[13−14] This technology is thus well-suited for complete structural elucidation by NMR, which cannot be fully achieved by relying solely on mass spectrometry techniques. Full structure elucidation is valuable information for medicinal chemists, whereby targeted structural modifications can be deciphered in further structure−activity relationship studies. Within the drug development process, the ability to efficiently isolate drug metabolites at the tens of milligrams scale is also useful to further perform bioassays and study the toxicity of potential drug candidates.

Thanks to new microfluidic technologies, we anticipate that the convenient scale up of oxidation reactions afforded by continuous-flow electrosynthesis may facilitate and speed up the drug discovery process by providing a rapid chemical means to assess new compound scaffolds and advanced lead compounds for phase 1 oxidative liabilities prior to in vitro or in vivo testing.

■ ASSOCIATED CONTENT

Supporting Information

Instrumentation, materials, electrosynthetic methods, compound characterization, and NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*(G.P.R.) E-mail: groth@sanfordburnham.org.

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Notes

The authors declare no competing financial interest.

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