Mass Spectrometric Detection of Short-Lived Drug Metabolites Generated in an Electrochemical Microfluidic Chip

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ABSTRACT: The costs of drug development have been rising exponentially over the last six decades, making it essential to select drug candidates in the early drug discovery phases before proceeding to expensive clinical trials. Here, we present novel screening methods using an electrochemical chip coupled online to mass spectrometry (MS) or liquid chromatography (LC) and MS, to generate phase I and phase II drug metabolites and to demonstrate protein modification by reactive metabolites. The short transit time (4.5 s) between electrochemical oxidation and mass spectrometric detection, enabled by an integrated electrospray emitter, allows us to detect a short-lived radical metabolite of chlorpromazine which is too unstable to be detected using established test routines. In addition, a fast way to screen candidate drugs is established by recording real-time mass voltammograms, which allows one to identify the drug metabolites that are expected to be formed upon oxidation by applying a linear potential sweep and simultaneously detect oxidation products. Furthermore, detoxification of electrochemically generated reactive metabolites of paracetamol was mimicked by their adduct formation with the antioxidant glutathione. Finally, the potential toxicity of reactive metabolites can be investigated by the oxidation of carbonic anhydrase I with electrochemically generated reactive metabolites of paracetamol. With this series of experiments, we demonstrate the potential of this electrochemical chip as a complementary tool for a variety of drug metabolism studies in the early stages of drug discovery.

Today’s pharmaceutical industry is facing key challenges of innovation and profitability. Over the past 60 years, the output of new drugs has increased linearly, whereas the costs of developing them have risen exponentially.1 Costs of drug development are for a large part incurred in expensive second and third rounds of clinical trials, where the efficacy and therapeutic effect of candidate drugs are tested. Therefore, failure of a drug candidate at one of these stages results in large financial losses. One possible way to reduce this late-phase risk is to shift resources to the earlier stages of drug development.2 In these stages, selection and prioritization of candidate drug compounds take place using a variety of screening methods, among which is a series of tests related to metabolism.

Drugs are metabolized in reactions that can be classified as phase I and phase II reactions.3 In phase I, the compound is functionalized through oxidation, reduction, or hydrolysis. Specifically, around 75% of the metabolized drugs are oxidized by enzymes from the cytochrome P450 (CYP450) family.4 In phase II reactions, the often lipophilic drug or its metabolites are conjugated to an endogenous compound, forming a more polar product that can be further metabolized or easier excreted via, for example, the kidneys. This overall process, involving one or both of these reactions, results in either the activation or detoxification of a drug compound. Upon activation, however, toxic metabolites may be formed that can cause side effects by modifying proteins, including enzymes.

To minimize total drug development times and costs, compounds from the initial pool of drug candidates that are ultimately destined to fail must be abandoned as soon as possible, thereby limiting the number of compounds that are tested using expensive procedures, such as those involving animals. For this purpose, analytical techniques are being developed that could improve the speed of the in vitro metabolism tests during the early screening process. A short overview of established systems to mimic oxidative phase I metabolism reactions is given below, and more detailed information can be found in a review paper.5

An important class of systems contains CYP450 enzymes to oxidize drug compounds. For example, the use of liver cell extracts, such as human or rat liver microsomes (HLM/RLM), is an affordable way to screen candidate drugs. However, the

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collection of enzymes available in these extracts poorly represents the situation in the human liver, and they are not always capable of producing the largest number of different oxidation products. In addition, primary hepatocytes are widely used, which provide a collection of active phase I and II enzymes that shows more resemblance with the situation in the human liver. Furthermore, a close representation of the in vivo situation is provided by the use of liver slices, especially when used in the controlled environment of a microdevice and supplied with a continuous flow of medium. However, liver slices have a limited lifetime, and ethical aspects related to the use of animal models have to be considered.

Other systems mimic the CYP450 enzyme’s electrochemical oxidation reactions. Here, it should be stressed that each of the following approaches on its own is not capable to completely mimic the enzyme’s activity. However, the three systems most commonly used will provide complementary information, together performing most of the relevant metabolism reactions.

First, synthetic metalloporphyrins can serve as models for CYP450 enzymes due to their ability to catalyze oxidation reactions. The active center of these porphyrins can be iron, but other metals, such as manganese, have also been studied in this context. Second, oxidation reactions can be performed using Fenton’s reagent, in which electrophilic hydroxyl radicals are generated when Fe^{2+} is oxidized to Fe^{3+} by hydrogen peroxide. In an electrochemically assisted Fenton reaction, Fe^{3+} is reduced back to Fe^{2+} at an electrode. Finally, direct electrochemical oxidation can be used to generate drug metabolites in an electrochemical cell, where oxidation takes place at a working electrode surface.

Direct electrochemistry (EC) in an electrochemical flow cell can be combined online with analytical instrumentation such as mass spectrometry (MS) or liquid chromatography (LC)/MS. Studies on electrochemical oxidation pathways of organic compounds were conducted by Hambitzer and Heitbaum and Volk et al., the former being the first to couple electrochemical cells online to thermospray ionization MS equipment for the detection of nonvolatile products. The use of electrospray ionization (ESI) in this context was introduced by Zhou and Van Berkel and applied more recently using various compounds. Speed and ease of use make this an attractive complementary approach for the generation and investigation of drug metabolites in pharmaceutical research. Moreover, this entirely instrumental online setup can be configured to increase the throughput and reduce the time between metabolite generation and detection. This reduced transit time can be exploited when searching for reactive metabolites, which are often short-lived and tend to bind to cellular membranes. Further applications of EC/(LC)/MS systems are described in extensive reviews.

The transit time in an EC/MS system with ESI interface can be reduced by integrating an electrochemical cell into an ESI interface, which is controlled using a potentiostat floating at the ESI voltage. With this approach, a transit time of <2 s between electrochemical oxidation and MS detection was established, but flow rates as high as 30 μL/min were used to achieve this. Furthermore, products generated at the counter electrode were mixed with the working electrode (WE) products and could interfere with the MS signal. This limitation was overcome by reducing the size of the counter electrode (CE), but this resulted in an increased current density which could cause electrolysis of the solvent or the supporting electrolyte.

Alternatively, the electrochemical cell in an EC/MS system can be miniaturized, thereby exploiting the advantages of microfluidics technology to obtain a high electrochemical conversion efficiency while using small amounts of reagent. This can be achieved by optimizing the geometrical parameters of the microchip and the flow rate at which it is operated. A conversion efficiency approaching 100% has been demonstrated using a system containing ~175 nL of reagent, which was able to operate at a large range of flow rates. High flow rates allow rapid detection of metabolites generated at the electrode, while low flow rates enable the use of nanospray, thus increasing ionization efficiency. Design freedom associated with microfluidic cell design allows integration of multiple functionalities in one platform and a high degree of control over the analyte flow, including separation of WE and CE products.

Furthermore, electrospray interfaces have been fabricated on microfluidic chips as well, in various forms. Capillary needles were attached to a microfluidic chip, and multiple chip-based ESI emitters were developed, as described in a published review.

In various studies, the reactivity of phase I metabolites toward different biomolecules was evaluated. Reactive intermediates were generated upon oxidation in an electrochemical cell, and their reactivity was investigated by adding endogenous nucleophiles. The first studies were performed by Getek et al., who oxidized paracetamol (APAP) and identified adducts of the reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI) with glutathione (GSH) and cysteine. Furthermore, in several studies, the reactivity of metabolites toward proteins was investigated. Lohmann et al. demonstrated the potential of electrochemical methods in protein modification studies by showing the formation of different protein adducts of several pharmaceuticals after electrochemical oxidation. Hence, the use of EC in peptide and protein binding studies is of growing interest.

In the first part of this work, we integrated a microfluidic electrochemical cell with an attached ESI needle in our new EC/ESI-MS setup to combine the benefits of well-controlled electrochemistry on-chip, low volumes of reagents, and a short transit time between electrochemical oxidation and MS detection. This was demonstrated by oxidizing the pharmaceuticals chlorpromazine (CPZ), clozapine (CLZ), and amodiaquine (AQ), which are known to form reactive metabolites upon electrochemical oxidation. Following this, real-time mass voltammograms were recorded, providing an easy qualitative assessment of the metabolites that can be formed upon oxidation of a drug compound. Rapid identification of drug metabolites made possible by the described instrumental improvements allowed us to detect a short-lived metabolite of chlorpromazine.

In the second part of this work, we successfully generated phase II metabolites of APAP by identifying the corresponding GSH adduct of the reactive metabolite NAPQI, which was generated electrochemically on-chip. Also, protein modification of carbonic anhydrase I (CAI) with electrochemically generated NAPQI was observed.

With this series of experiments, we show that the microfluidic electrochemical cell can be employed in phase I metabolism studies where rapid detection of metabolites is required and also in studies that need the generation of reactive
species for studying both the detoxification of reactive metabolites by small endogenous nucleophiles and the potential of inducing toxic side effects due to protein modification. This demonstrates the potential of this system to serve as a useful complementary screening tool in the early phases of drug discovery.

■ EXPERIMENTAL SECTION

**Chemicals.** Solutions were prepared of chlorpromazine, clozapine, and amodiaquine, each at a 5 μM final concentration in a buffer containing 50/50 (v/v) 100 mM aqueous ammonium formate (pH 7.4)/acetonitrile, as well as a 100 μM paracetamol solution in the same buffer. Glutathione (10 μM) was dissolved in an 1 mM aqueous ammonium formate (pH 7.4) buffer, and carbonic anhydrase I was used at a concentration of 20 μM in an aqueous buffer containing 6 M guanidinium hydrochloride. All ammonium formate buffers are adjusted to pH 7.4 using a 1% aqueous ammonia solution. Amodiaquine, chlorpromazine, clozapine, paracetamol, glutathione, carbonic anhydrase I, guanidinium hydrochloride, formic acid, and ammonium formate were purchased from Sigma-Aldrich GmbH (Steinheim, Germany). Acetonitrile was purchased from Merck (Darmstadt, Germany). All chemicals were used as provided. Water was purified before utilization with an Aquatron A4000D system (Barloworld Scientific, Nemours, France).

**Setup.** The EC/ESI-MS experiments were carried out using a previously developed microfluidic electrochemical cell, which is shown in Figure 1A and described in detail elsewhere. In short, the device consists of glass substrates containing microfluidic channels (5 μm height) and three integrated platinum electrodes: a working electrode (WE), a counter electrode (CE), and a pseudoreference electrode (RE). Platinum electrodes are known to be easily polarizable, and therefore, they were not expected to perform well as a stable RE. Our hypothesis was that the polarizability of the RE could be lowered by electrodepositing a layer of the conducting polymer poly(3,4-ethylenedioxythiophene) (PEDOT). The potential of the platinum/PEDOT electrode was measured to be −0.17 V vs saturated Ag/AgCl in the NH4formate/ACN buffer used for the experiments. See SI-1, Supporting Information, for the characterization of this electrode.

The chip was designed to provide a high electrochemical conversion efficiency and has an internal volume of ~175 nL. The WE and CE have separate channels, thereby separating the oxidation and reduction products. The frit channels provide a conductive path between the WE and CE over the entire length of the electrodes to reduce the ohmic drop, and fluidic resistors ensure an equal flow rate in both channels.

For oxidation of chlorpromazine, clozapine, and amodiaquine, the microfluidic chip was mounted in a dedicated chip holder (Micronit Microfluidics, Enschede, The Netherlands), to which capillary tubing (100 μm ID) and electrical wires were connected. A 30 mm long stainless steel ESI needle (Thermo Scientific, Breda, The Netherlands) with a 30 μm ID was attached to the outlet of the WE channel using a NanoPort assembly (Upchurch Scientific). The chip holder was mounted in a x−y−z stage to align the ESI tip with the entrance of the time-of-flight MS (micrOTOF, Bruker Daltonics, Bremen, Germany). The setup was placed on a custom-made table that fits the front panel of the MS, and a PMMA cover prevented spray instabilities due to air flow. The setup is shown schematically in Figure 1B; see SI-2, Supporting Information, for more details.

For flow control, a syringe pump (Nemesys, Cetoni, Korbussen, Germany) and valves (Valco, Vici, Houston, USA) were used, which were fitted in a Lab-in-a-Suitcase. The electrochemical chip was controlled using a potentiostat (SP200, Bio-Logic, Claix, France) in floating mode. The ESI needle was connected to the electrical ground of the MS.

Paracetamol was oxidized with the same electrochemical setup. Instead of an ESI needle, an additional capillary was connected to the effluent of the chip. Via a T-piece, a solution of GSH or CAI was added and the mixture was allowed to flow through a reaction coil for 3 min. For the GSH adduct formation, this reaction coil was coupled to the commercial ESI source of the MS. For the protein modification studies, the effluent of the reaction coil was collected in an injection coil of a six-port-valve, which was connected online to an LC system (Alexys, Antec, Zoeterwoude, The Netherlands) prior to MS detection. The setup is shown schematically in Figure 1C.

**Phase I Metabolism.** In both experiments described below, the total flow rate was set to 2 μL/min, which resulted in a 1 μL/min flow toward the MS. The ESI source voltage was set to −3 kV, and the MS was configured to operate in the positive ionization mode under the conditions described in SI-3, Supporting Information. Two types of experiments were carried out using the pharmaceutical compounds chlorpromazine, clozapine, and amodiaquine.

First, the transit time of the system was tested with a chronocoulometric (CA) measurement, which lasted 2 min and was initiated after 2 min of MS recording. In three

![Figure 1. Miniaturized electrochemical cell (A). Adapted from ref 28. Copyright 2012 American Chemical Society. Schematic diagram of the EC/ESI-MS setup used in phase I metabolism studies (B) and schematic diagram of the EC/LC/ESI-MS setup used in phase II metabolism and protein modification studies (C).](image-url)
experiments, the WE potential was successively set to 0.5, 0.8, and 1.2 V. Following that, a real-time mass voltammogram was recorded by applying a linear potential sweep (0–1.2 V with 2 mV/s) while mass spectra were being recorded. For comparison, chlorpromazine was oxidized in a thin-layer electrochemical cell (ReactorCell, Antec, The Netherlands), controlled by a Roxy potentiostat (Antec, The Netherlands), which was connected to the original ESI interface of the micrOTOF MS. The three-electrode electrochemical cell contained a platinum WE, a graphite-doped Teflon CE, and a palladium/hydrogen RE. The transit time between the flow cell and the ESI source was set to 1 min using capillary tubing. Chlorpromazine (2 μM in 50/50 (v/v) 100 mM NH₄formate (pH 7.4)/ACN) was introduced with 10 μL/min using a syringe pump (model 74900, Cole Parmer, Vernon Hills, USA), and a mass voltammogram was recorded by applying a linear potential sweep from 0 to 2.0 V with a scan rate of 10 mV/s.

**Data Processing.** For every experiment, extracted ion chromatograms of the original compounds and detected metabolites are shown on a normalized intensity scale. For each detected metabolite, the transit time was determined from the three extracted ion chromatograms which were recorded with the different WE potentials. First, the average signal intensity during the first 2 min of recording was calculated (when the electrochemical cell was switched off), and this baseline was subtracted from each of the three recorded traces. Then, the point in time was determined at which the resulting signal intensity increased to 0.05 on the normalized scale. The transit time is subsequently reported as the average of these three values with their standard deviation. Mass voltammograms are plotted by synchronizing the mass spectra recorded over time with the linear potential sweep using the transit time determined in the first series of experiments.

**Phase II Metabolism and Protein Modification.** Paracetamol (100 μM in 50/50 (v/v) 100 mM NH₄formate (pH 7.4)/ACN) was pumped into the chip with a flow rate of 2 μL/min, and the potential was set to 1.2 V. Glutathione (10 μM in 1 mM NH₄formate (pH 7.4)) was added to the effluent of the WE channel with a flow-rate of 9 μL/min. The reaction mixture with a total flow rate of 10 μL/min was continuously pumped through a reaction coil (3 min reaction time) and subsequently

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Figure 2. Extracted ion chromatograms (normalized) of three drugs and their detected oxidation products. The potential (0.5 V (blue), 0.8 V (green), or 1.2 V (red)) was switched on for 2 min after 2 min of recording. The transit time of the system is indicated. (A): CPZ (m/z = 319) and oxidation products (m/z = 318 and m/z = 335); (B): CLZ (m/z = 327) and oxidation product (m/z = 325); (C): AQ (m/z = 356) and AQ2I (m/z = 354). Also shown are the oxidation pathways and structures of the drugs and detected oxidation products.

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infused into the MS, which was operated in the positive ionization mode (for detailed MS parameters, see SI-3, Supporting Information).

For the formation of protein adducts, carbonic anhydrase I (10 μM in 6 M guanidinium hydrochloride) was added to the effluent of the WE channel with a flow rate of 1 μL/min. After a reaction time of 3 min, the solution was analyzed by means of online LC/MS. The LC system included two LC 100 pumps, an OR 110 organizer rack with a degasser and a pulse dampener, an AS 100 autosampler, and a Roxy column oven. The mobile phase was composed of acetonitrile and 0.1% formic acid (pH 2.5), and the flow rate was set to 300 μL/min. A binary gradient starting with 25% acetonitrile for 2 min was applied. Afterward, the amount of acetonitrile was increased to 60% in 4 min and was held at this level for 3 min. Subsequently, the amount was decreased to 25% acetonitrile in 3 min and the system was equilibrated for another 3 min. To prevent guanidine hydrochloride from entering the MS, the LC effluent was discarded during the first 2.5 min of separation. MS detection was performed in the positive ionization mode (for detailed MS parameters, see SI-3, Supporting Information).

**Safety Considerations.** Care must be taken with the high-voltage ESI source when the conventional closed ESI interface is replaced with an aluminum table (see SI-2, Supporting Information). Therefore, the MS table and the x-y-z stage were connected to electrical ground, and the table was covered with a PMMA cover.

### RESULTS AND DISCUSSION

**Phase I Metabolism.** To test the transit time of the system and to verify the amount of oxidation taking place in the ESI needle, each experiment started with 2 min of recording with the potential applied to the electrochemical cell switched off (open circuit). The cell was subsequently switched on for 2 min and finally switched off again. In Figure 2, the extracted ion chromatograms are shown for the drugs chlorpromazine (CPZ), clozapine (CLZ), and amodiaquine (AQ) and their detected metabolites, together with the oxidation pathways and structural formulas of the substances involved. The different colors relate to the three different WE potentials (0.5, 0.8, and 1.2 V). All of the traces in these figures follow the expected pattern: when the potential was switched on, the signal intensities for the drug compounds decreased, while the signal intensities for the metabolites simultaneously increased. However, the intensities did not change significantly when the WE potential changes, which is an observation that will be explained further when mass voltammograms are discussed (Figure 3).

Oxidation of CPZ resulted in the detection of two oxidation products (Figure 2A). One was the sulfoxide of CPZ and the other a short-lived radical cation, which was formed by the transfer of one electron. Both oxidation of CLZ and oxidation of AQ produced one metabolite (Figure 2B,C). When CLZ was oxidized, the nitrenium ion of clozapine was detected, and when AQ was oxidized, the dehydrogenated metabolite amodiaquine quinoneimine (AQQI) was detected. Both the nitrenium ion and AQQI are known to be highly reactive toward nucleophilic groups, such as thiol groups of the amino acid cysteine. Therefore, formation and detection of these oxidation products is important for assessing the toxicity of CLZ and AQ metabolites.36

The detected metabolites were identified by their exact mass as determined by MS. On the basis of these exact masses, molecular formulas were determined and subsequently structural formulas were deduced. The detected and calculated m/z together with the relative deviation, the molecular formulas, and the corresponding modifications of the starting compounds are listed in Table 1. The relative deviations are low for every oxidation product, and therefore, the proposed molecular and structural formulas are in accordance to those described in the literature.19,20,40

The transit time (t) in the system was determined for every detected metabolite from the extracted ion chromatograms shown in Figure 2. The resulting values are listed in Table 2, which are based on the time it takes for the recorded MS signal to reach a threshold value upon the start of electrochemical oxidation, as described in the Experimental Section. A possible explanation for the variation in the measured transit times of the different compounds could be a difference in adsorption
characteristics of the substances to the glass channel walls and platinum electrodes. In the traces of Figure 2, a significant rise time can be observed as well, especially for the CLZ nitrenium ion and AQ. This behavior suggests the presence of dead volume which acts as a capacity and could be described by an exponential rise. For the metabolites of CLZ and AQ, these curves are fitted successfully with average time constants of 11 and 18 s, respectively, based on an estimation of the asymptotic values. The traces for the metabolites of CPZ show more variation on their high values, making it difficult to make a good estimate for the time constants. See SI-4, Supporting Information, for more details.

The rapid response of this system provides two advantages. First, it allowed us to further investigate the influence of the WE potential on the oxidation efficiency of the three pharmaceutical compounds. Therefore, a linear potential sweep from 0 to 1.2 V with 2 mV/s was applied to the WE, and mass spectra were recorded at the same time. From these data, real-time mass voltmassograms (MVs) were generated which provide a rapid qualitative analysis of the introduced substance, identifying oxidation products that can be expected confirmed by applying a potential sweep up to 1.5 V (data not shown). The highest oxidation efficiency is observed for CPZ. However, in the MV of CPZ, the signal for the CPZ sulfoxide has a different trend compared to the signal for the radical cation. At lower potentials, the signal for the sulfoxide increases with a more gentle slope, and after the potential exceeds 0.5 V, the signal increases steadily. This suggests that the formation of the sulfoxide requires a higher potential compared to the formation of the radical cation, which is an observation that could not be made in Figure 2A.

Second, the short transit time between metabolite generation and detection allowed us to identify the short-lived metabolite of chlorpromazine, represented by the peaks at \( m/z = 318 \) (Figure 3A). This is confirmed by two observations. First, the absence of a \(^{13}\)C isotope peak at \( m/z = 318.5 \) excludes the possibility that the detected signal is caused by a doubly charged dimer of chlorpromazine. Second, the low deviation of 5.3 ppm between the calculated and detected mass suggests that the proposed structure is correct.

Finally, to confirm that the CPZ radical cation is a short-lived metabolite, CPZ was oxidized in the thin-layer electrochemical flow cell (ReactorCell), which was connected to an original microOTOF ESI interface using a capillary that introduced a time delay of 1 min. A mass voltmassogram was generated by applying a potential sweep to the WE (0−2.0 V, 10 mV/s); see Figure 4. In this MV, the signal intensity for the radical cation (green) compared to the signal intensity for the sulfoxide (blue) is much lower than that observed in Figure 3A, which was recorded using the newly developed system that has a transit time of only 4.5 s. These results show that the relatively lower signal intensity for this metabolite observed in Figure 4 can be attributed to the increased transit time in the system, which is longer than the lifetime of the radical cation. A possible explanation for the detected baseline signals corresponding to oxidation products starting at a WE potential of 0 V is some additional oxidation of CPZ in the ESI emitter. However, these signals have much lower intensities compared to the chip-based system due to the different ESI interface and the higher flow-rate used. Also note the difference in oxidation potentials compared to the MV in Figure 3A, which can partly be explained by the use of a different RE in the flow cell compared to the chip. Our interpretation of this data is supported by observations from Lu et al., who found the CPZ radical cation in an EC/DESI-MS setup, where desorption electrospray ionization was employed. When the flow rate was set to 5 µL/min, the transit time between oxidation and MS detection was approximately 15 s, which they estimated to be

**Table 1.** Masses Detected by MS, Proposed Molecular Formulae of the Oxidation Products of CPZ, CLZ, and AQ, and Modifications of the Starting Compounds by Electrochemical Oxidation

<table>
<thead>
<tr>
<th>detected m/z</th>
<th>calculated m/z</th>
<th>deviation (ppm)</th>
<th>molecular formula</th>
<th>detected ion</th>
<th>modification by oxidation</th>
</tr>
</thead>
<tbody>
<tr>
<td>319.1030</td>
<td>318.9069</td>
<td>0.5</td>
<td>( \text{C}<em>{17}\text{H}</em>{20}\text{ClN}_{2}\text{S} [\text{M} + \text{H}]^+ )</td>
<td>–e(^–)</td>
<td></td>
</tr>
<tr>
<td>335.0985</td>
<td>335.0979</td>
<td>0.3</td>
<td>( \text{C}<em>{18}\text{H}</em>{20}\text{ClN}_{4} [\text{M}^+]^+ )</td>
<td>+H(_2)O, –2H(^+), –2e(^–)</td>
<td></td>
</tr>
<tr>
<td>327.1371</td>
<td>325.1215</td>
<td>5.2</td>
<td>( \text{C}<em>{18}\text{H}</em>{21}\text{ClN}_{3}\text{O} [\text{M}^+]^+ )</td>
<td>–H(^+), –2e(^–)</td>
<td></td>
</tr>
<tr>
<td>356.1524</td>
<td>354.1381</td>
<td>3.7</td>
<td>( \text{C}<em>{18}\text{H}</em>{23}\text{ClN}_{3}\text{O} [\text{M} + \text{H}]^+ )</td>
<td>–2H(^+), –2e(^–)</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2.** Transit Time between Electrochemical Oxidation of Drug Compounds and MS Detection of Drug Metabolites

<table>
<thead>
<tr>
<th>detected metabolite</th>
<th>transit time (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPZ sulfoxide</td>
<td>5 ± 2</td>
</tr>
<tr>
<td>CPZ radical cation</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>CLZ nitrenium ion</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>AQQI</td>
<td>6 ± 2</td>
</tr>
</tbody>
</table>
the lifetime of this metabolite. In comparison, our measurements are characterized by a significantly shorter transit time at lower flow rates and a higher yield of CPZ oxidation products, including the radical cation. With this, significant advantages of using microfluidics technology in phase I drug metabolism studies have been demonstrated.

**Generation of Phase II Metabolites and Protein Modification.** Next, reactive metabolites were generated in the electrochemical chip to evaluate their reactivity toward peptides and larger biomolecules such as proteins. The aim was to investigate the detoxification process of reactive species, to form possible phase II metabolites, and to estimate toxic side effects due to protein binding. Paracetamol (APAP) was selected as a model compound because it forms reactive metabolites in vivo that could also be generated electrochemically.

APAP was oxidized at a constant potential of 1.2 V, and a solution of the endogenous nucleophile GSH was added to the effluent of the WE channel. After a reaction time of 3 min, the mixture was analyzed via MS using the commercial ESI interface. In Figure 5, the pathway of the electrochemical oxidation of APAP and the reaction of the generated reactive metabolite NAPQI with GSH are shown. Via dehydrogenation of APAP, the reactive intermediate NAPQI with its electrophilic structure was formed. In a 1,4-Michael addition, NAPQI can directly react with the free thiol group of the cysteine residue in GSH resulting in the corresponding NAPQI-GSH adduct. Furthermore, the reactive benzoquinone can be formed due to hydrolysis of NAPQI in aqueous solutions. However, no conjugate of GSH with benzoquinone was detected in this study, although the reactivity of benzoquinone toward cysteine containing biomolecules is well-known.

This demonstrates that the reaction time to form the NAPQI-GSH adduct is short enough to avoid hydrolysis of NAPQI taking place. Additionally, polymerization of APAP can occur during electrochemical oxidation.

Figure 5 also shows the extracted ion traces of this experiment. After 4.5 min of MS recording, the potential was switched on and held at 1.2 V for 12 min. After 3 min of reaction time, the ion traces of APAP (m/z = 152) and GSH (m/z = 308) started to decrease. This can be traced back to the electrochemical oxidation of APAP and adduct formation of GSH with reactive oxidation products, since at the same time, a signal for the NAPQI-GSH adduct (m/z = 457) started to appear with increasing signal intensity. Additionally, an adduct of a dehydrogenated dimer of APAP with GSH (m/z = 606) could be identified. This reactive intermediate was formed after dimerization of APAP and was a side product of the electrochemical oxidation. After 12 min of oxidation, the signal intensities of the products decreased and the traces of APAP and GSH started to increase again. Since GSH was modified only one time, the adduct formation selectively took place at the free thiol group of the cysteine residue.

Next, reactive metabolites of APAP were generated on-chip to modify the protein carbonic anhydrase I (CAI), which is an important transport protein for CO2 in the human body. It consists of 260 amino acids and contains one free cysteine moiety with a nucleophilic thiol group. After electrochemical oxidation of APAP at 1.2 V, the protein was added and the mixture was allowed to react for 3 min. Afterward, the solution was analyzed online using HPLC/ESI-MS. By collecting the reaction mixture in an injection coil and switching the six-port-valve, the protein fraction was separated from the salts and small molecules on the HPLC column to achieve a good signal-
phase II metabolites and estimating toxic side effects as a result of protein modification.

**CONCLUSIONS**

This work demonstrates the potential of an online EC/ESI-MS system with an electrochemical chip in the screening of drug candidates for short-lived and potentially toxic metabolites while consuming a small volume of analyte. Real-time mass voltammograms were recorded of the pharmaceuticals chlorpromazine, clozapine, and amodiaquine, which allowed us to identify metabolites generated over a range of oxidation potentials. Although the approach of direct electrochemical oxidation is not capable of mimicking all oxidation reactions carried out by the CYP450 enzymes, this system can serve as a valuable complementary tool in the drug discovery process in the sense that, if a toxic metabolite is detected, it is likely that the candidate drug compound needs to be abandoned. This would save time and costs involved in more expensive testing procedures, such as those involving animal models. The short transit time of our chip with the integrated ESI needle (∼4.5 s) allowed us to detect an unstable radical cation upon oxidation of chlorpromazine, which demonstrates the advantage of our system in the screening for short-lived metabolites. In addition, it was demonstrated that the electrochemical chip can be used for both generation of phase II metabolites and protein modification studies. This allows a further characterization of electrochemically generated metabolites with regard to their reactivity toward different biomolecules such as small endogenous nucleophiles and toxicologically relevant proteins. Therefore, this technology shows promise to be used in valuable assays in the early stages of the drug discovery process.

**REFERENCES**


**ASSOCIATED CONTENT**

5 Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions


Notes

The authors declare no competing financial interest.

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