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Understanding the degradation of electrochemically-generated reactive drug metabolites by quantitative NMR

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ABSTRACT

Phenacetin is known to be metabolized to N-AcetylParaBenzoQuinoneImine (NAPQI), which is a common metabolite of paracetamol (also called acetaminophen or APAP). The electrochemical conversion of APAP to NAPQI was shown in 1989 by Getek and co-workers, thus demonstrating the capacity of electrochemistry to mimic the formation of NAPQI from APAP as well as from phenacetin. This study focuses on a preparative electrochemical electrolysis associated with quantitative ^{1}H NMR. On one hand, this method is able to synthesize reactive metabolites in sufficient concentrations and amounts for NMR analysis. On the other hand, NMR allows the simultaneous detection and quantification of all chemical species, in contrast to mass spectrometry. The combination of electrochemistry with quantitative NMR is thus presented as a relevant method for elucidating the degradation of reactive metabolites and may be considered a valuable complementary tool to EC-MS.

1. Introduction

The progress made in the development of both instrumentation and methodology has allowed the constant implementation of new spectroelectrochemical associations, such as the combination of electrochemistry (EC) with ESR (Electron Spin Resonance), mass spectrometry, infra-red spectroscopy, and Nuclear Magnetic Resonance (NMR), among others [1]. For example, electrochemistry coupled to mass spectrometry has been widely investigated, particularly in the field of drug oxidative metabolism [2–4]. It has been reported that electrochemistry may be used to understand oxidative metabolism reactions initiated by one electron transfer and catalyzed by P450 enzymes better [4–6] while the use of electrochemical cells was found useful for generating the hepatotoxic quinone imine metabolite of paracetamol [7,8]. The quinone imine structure was also observed during the oxidation of various drugs such as diclofenac, amodiaquine, phenacetin and acebutolol [7–13]. Hepatotoxicity, often attributed to the production of reactive metabolites, is one of the most important aspects in oxidative metabolism studies [14]. Thus, elucidating the chemical structure of quinone imine derivatives during oxidative metabolism is vital to predict detrimental effects or to provide a better understanding of the formation of adducts by binding to proteins [15]. However, the direct detection of quinone imine species by ESI-MS seems to suffer from its low ionization rate and often requires the use of trapping agents such as glutathione [16,17]. Moreover, the identification is not always unambiguous and the exact structure elucidation of reactive metabolites is therefore required to understand their modes of interaction with the biological environment better. The chemical elucidation of unstable metabolites is often a tedious process because of their short lifetime, and a huge effort is usually required to synthesize and isolate metabolites which are not commercially available [9,17,18]. It is worth noting that the whole process is very difficult to transpose to reactive compounds such as quinone imine. For this reason, identification and structure elucidation in the absence of reference standards (metabolites) require new approaches departing from classic methodologies. *In situ* NMR may be an appropriate tool for the simultaneous identification and elucidation of chemical structures. The most relevant and recent examples of coupling electrochemistry with NMR measurements concern paracetamol and phenacetin [19,20]. Simon et al. have recently reported the on-line combination of electrochemical and NMR flow cells for monitoring the oxidative degradation of paracetamol [19]. By using an *in situ* spectroelectrochemical method [20], we monitored the real-time oxidation of phenacetin leading to the production of quinone imine. Both on-line and *in situ* spectroelectrochemical NMR demonstrate the usefulness of monitoring the formation of reactive metabolites for their structural elucidation. In fact, the combination of electrochemistry and NMR enables the unambiguous determination of the chemical structures of generated metabolites. Therefore, implementing an

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electrochemical cell inside an NMR instrument may be the most appropriate EC-NMR combination to observe reactive intermediates before their degradation, even if the most unstable species with the shortest life times, *i.e.* a few milliseconds, cannot be detected by EC-NMR yet. Nevertheless, reactive metabolites such as quinone imines with longer life times can be observed through the off-line association of electrochemistry and NMR.

Here, we focus our efforts on an off-line association of electrochemistry and quantitative NMR (qNMR) for the *in situ* monitoring of quinone imine degradation. A device based on carbon microfiber electrodes was used as a preparative electrochemical tool for generating reactive metabolites without the need for a preconcentration and/or extraction process. The degradation pathway of quinone imines was elucidated and is discussed.

2. Materials and methods

2.1. Chemicals

Phenacetin, APAP and lithium acetate were purchased from www.sigmaaldrich.com. Deuterated solvents were purchased from www.eurisotop.com.

2.2. Electrochemical parameters

The electrodes were manufactured as described in an earlier study [20]. The starting solution consisted of phenacetin and lithium acetate, dissolved to reach respective concentrations of 10 and 50 mM in an acetonitrile-D_3/D_2O 1/1 solution. 800 μL of the starting solution was placed in an isolated compartment in the presence of the working electrode facing the Pd/H_2 reference electrode. The anodic and cathodic compartments were isolated by a porous wall made of vycor glass (from www.bio-logic.info). The cathodic compartment contained a counter electrode placed in a 50 mM lithium acetate solution (acetonitrile-D_3/D_2O 1/1). The electrolysis was monitored for 4 h at 1300 mV vs. the Pd/H_2 reference electrode by a VMP multichannel potentiostat piloted by ECLab software 10.18 (from www.bio-logic.info). An optimum electrolysis time of 4 h was determined under our conditions to obtain the highest concentration of NAPQI, based on the monitoring of NAPQI signals during the previously reported *in situ* EC-NMR oxidation [20]. Then, the oxidized solution was transferred manually to the tube for NMR analysis. The total oxidation of phenacetin can be reached with the electrochemical device described; nevertheless, too long an electrolysis would favor the degradation of NAPQI.

2.3. NMR parameters

Quantitative NMR experiments were carried out at 298 K on a Bruker Avance III 500 spectrometer monitored by Topspin 2.1 software (from www.bruker.com), at a frequency of 500.13 MHz with a cryogenically cooled probe including *z*-axis gradients and $PW_{90}=7.86$ μs for ^1H. Transverse relaxation times (T_1) were estimated on the initial solution thanks to an inversion-recovery sequence. The highest relaxation time was estimated at 4.0 s thus the repetition time was fixed at 20 s (repetition delay $> 5T_{1max}$) to reach a theoretical precision of 1%.

^1H NMR spectra were obtained with 16 repetitions of a pulse sequence consisting of a single 90° pulse on the ^1H channel followed by an acquisition with GARP ^{13}C decoupling to remove satellite peaks. Free induction decays (FIDs) were recorded with 15*k* points and an acquisition time (Aq) of 1.50 s. FIDs were weighted with an exponential apodization function (line broadening 0.3 Hz). After manual phase adjustment, the baseline was automatically corrected between 0.5 and 9.5 ppm by a third degree polynomial.

The signal areas were finally determined by integration. The concentrations were estimated by using the CH_3 signal of the support electrolyte (i.e. acetate, whose 50 mM concentration did not vary in the course of time) as an internal reference. The NAPQI concentration was calculated from its three signals (acetyl singlet and two aromatic doublets). The BQ concentration was obtained from the singlet area at 7.27 ppm. The APAP concentration was determined from its two aromatic signals (7.24 and 7.73 ppm).

3. Results and discussion

In our previous work, we reported that *in situ* NMR spectroelectrochemistry (EC-NMR) constitutes a useful technique for the elucidation of the chemical structure of unstable intermediate species [20]. In the case of the electrochemical oxidation of phenacetin, we reported insights into the formation of NAPQI and EtOH (Fig. 1). The mechanism involved in the hydrolysis of aromatic ethers such as phenacetin may be compared to that already elucidated for the electrochemical hydrolysis of acebutolol

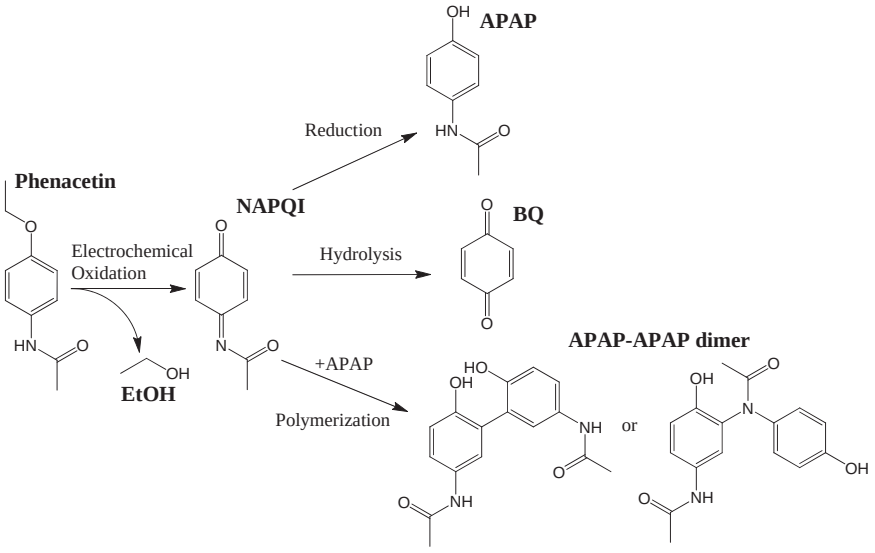

Fig. 1. Summary of the electrochemical generation of NAPQI through the cleavage of phenacetin aromatic ether and the different ways of NAPQI degradation.

[12,21]. To sum up, after abstracting two electrons and one proton, the cationic intermediate undergoes a water addition, then a rearrangement leads to the formation of a quinone imine (NAPQI) and an alcohol through aromatic ether bond cleavage [17].

As has already been reported and expected in this study, the NAPQI structure is not stable so performing *in situ* monitoring of the degradation of NAPQI in solution should bring relevant hints about its degradation pathway. For this purpose, the phenacetin solution was first oxidized *ex situ*, using the electrochemical device described in our previous work [20] except here the anodic compartment was separated from the cathodic one, then the degradation of NAPQI was monitored by quantitative NMR.

To understand the degradation of NAPQI after its electrochemical formation better, it should be noted that the quinone imine structure can be degraded according to three different pathways (Fig. 1). First, quinone imine can be reduced at the counter electrode or in solution in the presence of some reductive species as recently proposed in the electrochemical study of acebutolol [12]. Second, NAPQI can also undergo hydrolysis leading to the formation of a benzoquinone structure (BQ). Third, NAPQI can react with APAP through a nucleophile addition reaction [22] or through the binding of aromatic carbons [22,23] leading to the formation of dimeric and polymeric structures. To argue the proposed mechanism displayed in Fig. 1, we first examined how possible the formation of the reduced form (APAP) is under the electrochemical oxidative mode. Intuitively, the formation of APAP may be explained if the electrochemical reduction of NAPQI is taken into account at the counter electrode when the cathodic and anodic compartments are not separated. However, the formation of APAP is still observed in spite of the use of an electrochemical cell with separated anodic and cathodic compartments. For instance, Fig. 2 displays how the NMR signals change as a function of time once the electrolysis process has been stopped. The spectrum carried out at the initial stage, i.e. before electrolyzing the phenacetin solution, displays two doublets at 7.34 and 7.82 ppm attributed, respectively, to the two ^{1}H in ortho and the two ^{1}H in meta from the aromatic ether group of phenacetin. The two protons H_a at 7.34 ppm are coupled (^3J=9.1 Hz) with the two protons H_b at 7.82 ppm. This result shows that, after electrolyzing the phenacetin solution, a significant decrease in the resonance peak intensities was observed for aromatic ring protons of phenacetin, accompanied by the appearance of new signals in the aromatic region. Among these peaks, higher doublets were obtained at 7.13 and 7.51 ppm while other weaker doublets were obtained at 7.24 and 7.73 ppm. The formation of significant amounts of NAPQI (7.13 and 7.51 ppm) and a small fraction of APAP (7.24 and 7.73 ppm) shows that the latter was not electrochemically formed since both cathodic and anodic compartments were separated during the electrochemical process. It is important to notice that both APAP and BQ were formed as minor components whereas EtOH signals were observed at the expected chemical shifts with stoichiometric concentrations compared to the consumed amount of phenacetin (Supporting information S1 and S2). In addition, the spectrum recorded 5 h after the end of the electrolysis shows a significant increase in the concentration of both BQ and APAP to the detriment of the NAPQI signal (see also Supporting information S3a), while both EtOH and phenacetin concentrations remain unchanged. Except for dimeric species, which may exist in solution in weak amounts because of their low solubility, all the chemical structures, namely NAPQI, APAP, EtOH and BQ compounds, were identified by ^{1}H NMR.

The concentrations of NAPQI and its two main degradation products, namely BQ and APAP, were monitored by ^{1}H quantitative NMR and plotted as a function of time in Fig. 3. As already mentioned, the concentration of phenacetin and EtOH remained constant throughout the experiment so, for greater clarity, these two species were not plotted. The concentration of NAPQI decreased as a function of time and its signal became almost undetectable after 5 h. Fig. 3 also indicates that the concentration of both APAP and BQ increased and reached a plateau showing a good correlation with the change observed for the NAPQI concentration. Without elucidating the degradation pathway of the reduction of NAPQI, Novak et al. have already reported a half-life for NAPQI of about 30 min in neutral phosphate buffer [24]. So the remaining question is the presence of the reduced form such as APAP under oxidative conditions. We have already reported the same behavior in the case of the electrochemical oxidation of acebutolol in solution [12]. In the same way, we point out the involvement of quinone imine species in a Michael addition reaction that leads to a substituted phenol amide with a lower oxidation potential compared to that of APAP. To justify the presence of APAP, we hypothesized that, in the bulk of the reaction mixture, NAPQI may act as both the precursor and the oxidizing agent of dimeric and polymeric species. On the basis of this hypothesis, we propose that the reducing character of the solution

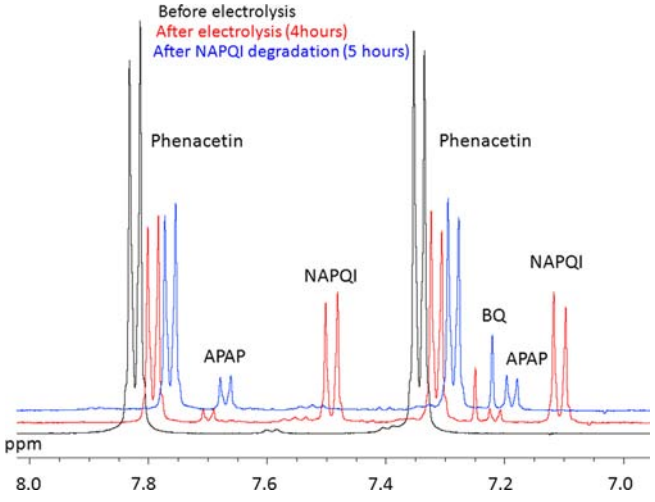

Fig. 2. Stacked view of the ^{1}H NMR aromatic area of a phenacetin solution at different reaction stages. The spectrum represented in black was recorded before electrolysis, whereas the red spectrum was taken at the end of the electrolysis. The blue spectrum was obtained after NAPQI degradation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

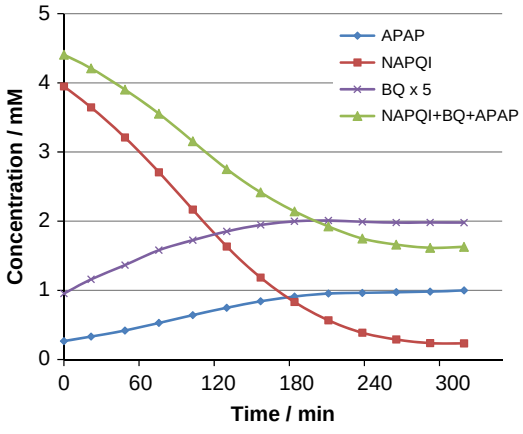

Fig. 3. ^{1}H NMR monitoring of NAPQI degradation (red) and its two degradation products, APAP (blue) and BQ (purple, upscaled five times). The green curve represents the added concentrations of these three species. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

mixture increases with an increase in the amount of dimeric and polymeric species, thus leading to the reduced form of NAPQI, i.e. APAP. This hypothesis is supported by our previous work where we reported the formation of a brown precipitate during the degradation of NAPQI at a higher concentration (10 mM), mentioning the possibility of dimer and polymer formation. In addition, high resolution mass spectrometry experiments supported the formation of APAP–APAP and phenacetin–APAP dimers. This process should lead to the chain polymerization of NAPQI–APAP derivatives until precipitation due to their low solubility, as shown in Fig. 4. A redox reaction between phenacetin and NAPQI may also be considered in order to explain the formation of APAP under the oxidative mode. From the experimental data point of view, this should lead to a decrease in the phenacetin signals in solution to the benefit of those of APAP and EtOH. However, under our experimental conditions, we did not notice a decrease in the phenacetin or EtOH resonance signals. Fig. 3 shows that the sum of the concentrations of NAPQI, BQ and APAP was found not to be constant during the degradation process. This indicates a loss of at least 3 mM of monomeric aromatic compounds thus indicating the polymerization process. Potter et al. identified (by NMR) APAP polymeric structures, C_{arom}–C_{arom} or C_{arom}–N bonded dimers, in the presence of HRP enzymes. The authors mentioned two possibilities for the polymer oxidation into a quinone imine equivalent; an enzymatically catalyzed reaction (HRP enzymes) or redox reactions in solution [22]. Then, Nematollahi et al. proposed a mechanism involving the attack of APAP on electrochemically-generated NAPQI leading to the bonding of aromatic carbons of the two species [23]. The structure of the dimer was also confirmed by NMR analysis of the dimer adsorbed at the working electrode surface. Kauffmann et al. also discussed NAPQI dimerization in the presence of APAP thanks to micro-electrosynthesis using screen-printed electrodes [25].

It is also important to note that the phenacetin and EtOH concentrations remained unchanged during NAPQI disappearance, and are assumed to not be involved in the degradation of NAPQI, either as a reactant or as a product. Moreover, it has been reported that the consumption of NAPQI is faster when the APAP concentration increases [24]. This assumption was supported by adding APAP to 800 μL of oxidized solution to reach the amount of 4 mM; the NMR monitoring of NAPQI degradation under these conditions is plotted against time and displayed in Fig. 5. The degradation of NAPQI was found to be at least 4 times faster in the APAP-spiked solution. Based on the stability of phenacetin and EtOH NMR

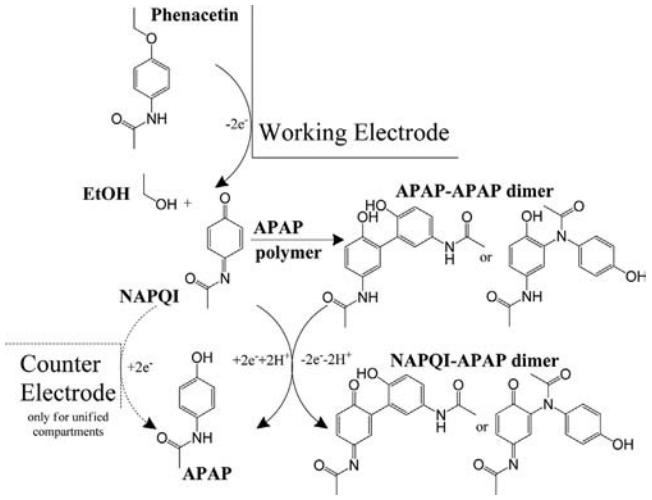

Fig. 4. Overview of the electrochemical reaction occurring at the electrode (s) surface(s) and redox reactions occurring in the bulk solution, dimers structures were tentatively assigned based on literature.

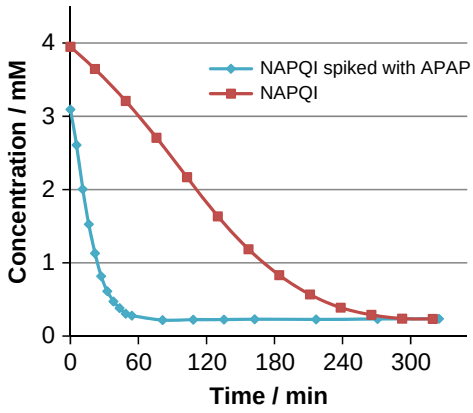

Fig. 5. ^{1}H NMR monitoring of NAPQI concentration estimated by signal integration. The NAPQI concentration after split compartment electrolysis is plotted in the absence of additive (red) and after addition of 0.4 mg of APAP (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

signals (Figs. 2, S1 and S2) and on the influence of APAP on the stability of NAPQI (Fig. 5), it is clear that the polymerization of NAPQI is more favored by APAP than by phenacetin. As discussed above, NAPQI and APAP can polymerize according to two pathways: (i) bonding of aromatic carbons in *ortho* of the phenol/quinone functions [23] or (ii) bonding of aromatic carbons in *ortho* of the quinone (from NAPQI) function and nitrogen (from APAP) [22].

These observations are in accordance with previous works dealing with NMR monitoring of the electrochemical generation of NAPQI on-line as well as *in situ* [19,20]. In the case of *in situ*, a correlation between the decrease in the NAPQI signals and the increase in the APAP ones was observed, suggesting a dependence of NAPQI stability on APAP concentration. In the on-line case, authors reported a transfer time of 8 min between NAPQI generation and its NMR detection. In addition to an unachieved electrochemical conversion of APAP, they also reported the formation of BQ in sufficient quantity to be observed by NMR, thus confirming the fast decay of the NAPQI generated [19]. The presence of APAP tends to place NAPQI in a more favorable situation for polymerization. Consequently, with the aim of implementing a preparative electrochemistry method for the NMR analysis of reactive metabolites, it is advisable to use an electrochemical device with an isolated counter electrode in order to avoid the presence of a polymerization inducer.

4. Conclusion

Carbon microfiber electrodes were used for the electrochemical generation of NAPQI at a preparative scale leading to a sufficient quantity for NMR quantitative studies. These experiments showed evidence of the formation of APAP and BQ from NAPQI without electrochemical intervention. Moreover, the polymerization of NAPQI with APAP was clearly identified as the source of its non-elucidated reduction.

On one hand, we can assume that the electrochemical reduction of NAPQI into APAP enables its long-term stabilization. On the other hand, the production of APAP through the reduction of NAPQI dramatically affects the short-term stability of the latter. Nevertheless, this observation demonstrates the capacity of our device to synthesize reactive metabolites in sufficient quantity for qNMR acquisition. Access to the most reactive metabolites, such as quinone imine, is achieved by the use of a synthetic process without extraction and/or pre-concentration steps.

The coupling of electrochemistry with more sophisticated NMR methods, such as ultra-fast 2D NMR, would certainly help solve the remaining overlap issues [26].

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.07.026.

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