Chapter 5

The oxidative metabolism of the dopamine agonist N–0923 mimicked by EC/MS and electrochemically assisted Fenton reaction.

The two on–line oxidation systems described earlier, the EC/MS system and the EC–Fenton system, have been applied to the dopamine agonist S(—)-(N-propyl-N-2-thienylethylamine)-5-hydroxytetralin (N–0923). The oxidative metabolism previously reported from rat liver perfusion experiments was partly mimicked by both methods. The EC/MS system gave a mimic of the N–dealkylation while the oxidation of the phenol function was not fully mimicked since the catechols (or p–hydroquinones) formed were immediately oxidized to the corresponding quinones. By collecting the oxidized sample solution and injecting it again at a reductive potential, the corresponding catechols (or p–hydroquinones) could be generated.

Oxidation in the EC–Fenton system gave rise to five different products, all showing a gain of oxygen. Interpretation of the product ion spectra suggests that three of these products are hydroxylated at the phenolic ring or at the benzylic carbons 1 or 4. Two other compounds were assigned as the N–oxide and a compound with the oxygen located at the thienylethyl group. The results give a clear indication that the EC/MS system and the EC–Fenton system give different products and that they can be complementary to each other.

5.1 Introduction

The EC/MS system described if chapter 2 and the EC–Fenton system described in chapter 3 are both useful tools for the generation of oxidative metabolites. Both systems have their limitations. Since the mechanism of oxidation is different in these two systems, it is interesting to investigate their individual limitations and the possibility that they may be complementary to each other. We have therefore chosen to investigate to what extent a combination of the two systems can be used to generate metabolites of a drug with a known metabolism pattern.

The drug 2-(N-propyl-N-2-thienylethylamine)-5-hydroxytetralin (N–0437) was first synthesized by Horn et al.1 In vitro as well as in vivo studies have proven it to be a potent and selective D–2 receptor agonist.2,3 N–0437 is a racemic mixture and it was
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shown that the R(+)–enantiomer, N–0924, is inactive in reducing dopamine release following i.p. injection while the S(−)–enantiomer, N–0923 (Figure 5.1), acts as an agonist at both pre- and postsynaptic receptors and might be of therapeutic use in Parkinson's disease.\textsuperscript{4,5} The metabolism of N–0437 has been studied extensively.\textsuperscript{6-8} The S(−)–enantiomer, N–0923, was available in our laboratory and was considered a suitable candidate for a test in the EC/MS system as well as the EC-Fenton system.

![Figure 5.1 2S(-)-(N-propyl-N-2-thienylethylamino)-5-hydroxy-tetralin, N-0923](image)

5.2 Experimental

**Chemicals**

N–0923 is the S(−)–enantiomer of the dopamine agonist N–0437 [2-(N-propyl-N-2-thienylethylamino)-5-hydroxytetralin] and has been synthesized earlier.\textsuperscript{1} The following chemicals were obtained commercially: Iron(III)chloride hexahydrate, 98 %, Aldrich; ethylenediaminetetraacetic acid disodium salt (EDTA), 99 %, Merck; hydrogen peroxide, 35 % solution in water, Aldrich; ammonium acetate, p.a., MERCK; glacial acetic acid, p.a., MERCK; lithium trifluoromethane sulfonate, 99.995%, Aldrich; trifluoromethane sulfonic acid, 98%, Aldrich.

The water used in the experiments was purified in a Maxima Ultrapure water system (ELGA, High Wycombe, Bucks, U.K.) and was sonicated for about 15 minutes before use.

**Instrumentation**

The EC/MS system was set up as described in chapter 2 and the EC–Fenton system was used in the same way as described in chapter 3. All reported cell potentials are versus a palladium reference electrode. In all experiments where an HPLC–column was used to separate the oxidation products a splitter was introduced between the HPLC–column and the mass spectrometer to reduce the flow to approximately 40 µl/min. The voltammograms
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and mass chromatograms presented in Figures 5.4, 5.6, 5.7, 5.9 and 5.11 have been smoothed by "Kalman smooth" in Multiview 1.4 (MDS–Sciex, Concord, Ontario, Canada) prior to reconstruction in DeltaGraph 4.5 (SPSS Inc., Chicago, Illinois, USA)

**Electrochemical reaction conditions in the EC/MS system (method 1)**
N–0923 was tested in pure water and in the following supporting electrolytes: acetic acid, ammonium acetate, lithium trifluoromethane sulfonate, and trifluoromethane sulfonic acid. The supporting electrolytes were used in 0.1, 1 and 10 mM concentrations. The electrolytes did not affect the oxidation reaction pathway for N–0923. A high electrolyte concentration gave a better conversion of the sample but also gave increased background ion signals and decreased the sample ion signal.

**Electrochemical reaction conditions in the EC–Fenton system (method 2)**
The sample solution injected through the electrochemical cell contained 1 mM N–0923, 1 mM FeCl$_3$, 1.02 mM EDTA (to prevent precipitation of ferric hydroxide) and 10 mM hydrogen peroxide. The final concentration of acetonitrile in the sample solution was 21%. The hydrogen peroxide was kept in the refrigerator and was added just before the injection. The “inactive” Fenton mixture was injected at a flow of 50 µl/min. After 1 minute, the flow was lowered to 2 µl/min. A make up liquid containing 10% acetonitrile and 0.1% formic acid was added to the effluent from the electrochemical cell at a flow rate of 50 µl/min to dilute the sample solution and to adjust the acetonitrile content to fit the start of the HPLC–gradient. 20 Minutes after the first injection, the diluted sample solution was injected with the second injection loop onto the HPLC column. The reductive potential over the electrochemical cell was kept at –0.5 V.

**5.3 Results and discussion**
N–0923 contains three major sites for oxidation, a tertiary amine, a thienyl group and a phenol function. The main metabolites reported from *in vitro* rat liver experiments are presented in Figure 5.2. The parent drug (1) (R=H) undergoes either hydroxylation or N–dealkylation to give the metabolites (2), (3), and (4), while metabolite (5) is the result of both hydroxylation and N–dealkylation. Glucuronidation and sulfation take place at the hydroxyl function of (1), (3), and (4). One of the hydroxyl groups in the metabolites (2) and (5) may also undergo glucuronidation and sulfation.
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Figure 5.2 The major metabolites of N-0923 observed in rat liver perfusion experiments. R=H, glucuronide or sulfate as explained in the Results and Discussion section.

Method 1, electrochemical oxidation

The products of electrochemical reactions of the parent drug are given in Figure 5.3. Voltammograms with detection of m/z values of protonated products are presented in Figure 5.4. As shown in Figures 5.3 and 5.4, the phenolic ring is oxidized to a catechol (or a p-hydroquinone) at about +0.2 V, but the products are immediately oxidized to the corresponding quinones. As the potential is increased to about +0.3 V, the tertiary amine function is also oxidized. Since the groups at the nitrogen are different, three N-dealkylation products are formed.
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Figure 5.3 N–0923, oxidation reaction pathways observed in the electrochemical cell. For simplicity, ortho–hydroxylation is shown, in analogy with the distribution observed in perfused rat liver experiments. para–Hydroxylation followed by oxidation to para–quinone can not be ruled out.

Figure 5.4 Voltammograms with detection of m/z values of protonated products from the electrochemical oxidation of N–0923
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Compared to the reactions that take place in the rat liver experiments, the N–dealkylation is mimicked by the electrochemical cell. The electrochemical oxidation of the phenol function does not give a complete simulation of enzymatic oxidation, since the products are further oxidized to quinones. Enzymatic oxidation is obviously more selective than electrochemical oxidation. Furthermore, in rat liver experiments glucuronidation and sulfation protect the hydroxyl function and therefore oxidation to the quinone structure does not take place. It also appears in the rat liver experiments that dealkylation can take place prior to hydroxylation of the aromatic ring. In the EC/MS experiments, oxidation of the phenol function occurs at a lower cell potential than dealkylation and metabolites (3) and (4) in Figure 5.2 can therefore not be formed in the absence of protection of the hydroxyl group.

**Electrochemical oxidation followed by reduction**

Upon electrochemical oxidation (method 1), the phenol function of N–0923 is oxidized at a lower potential than the tertiary amine function. By selecting the appropriate electrochemical conditions, dealkylated or non–dealkylated electrochemical products, can thus be produced selectively. Unlike the enzymatic oxidation, the electrochemical oxidation of the phenol function does not give catechols as final oxidation products, but the catechols are further oxidized to quinones. With an electrochemical system like the one described above, a change of the electrochemical conditions from oxidation to reduction is easily performed by switching the electrode potentials. By collecting the sample solution and injecting it at a reductive potential, the quinones are reduced back to catechols as described in Figure 5.5.
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![Figure 5.5](image)

**Figure 5.5** Electrochemical reduction of quinones

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+R1
O
\[\begin{array}{c}
\text{N} \\
\text{O}
\end{array}\]
\[\begin{array}{c}
\text{R2} \\
\text{O}
\end{array}\]
\[\begin{array}{c}
\text{R1} \\
\text{N}
\end{array}\]
\[\begin{array}{c}
\text{R2} \\
\text{OH}
\end{array}\]
\[\begin{array}{c}
\text{HO} \\
\text{R1}
\end{array}\]
\[\begin{array}{c}
\text{R2} \\
\text{OH}
\end{array}\]
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The oxidation products were collected after oxidation of 20 µM N–0923 at +1.0 V, a potential where both the phenol function and the tertiary amine are oxidized. Figure 5.6 shows the extracted ion voltammograms with detection of m/z values from the electrochemical reduction of the quinone product with mw 219 (Figure 5.3). As the reductive potential is increased, the [M+H]+ ion of the quinone is replaced by the [M+H]+ ion of the corresponding catechol (or p–hydroquinone). Metabolites (2) and (5) in Figure 5.2, can thus be generated in the EC/MS system.

**Method 2, the electrochemically assisted Fenton system**

The oxidation products of the EC–Fenton system were separated by gradient elution in an on–line LC/MS system. The mass chromatogram of the [M+H]+ ion of N–0923 is
presented in Figure 5.7a. The product ion spectra of the [M+H]+ ion of N–0923 is shown in Figure 5.7b with the suggested fragmentation given in Figure 5.8. The major fragment at m/z 147 corresponds to a loss of propyl–thienylethylamine, leaving the positive charge on the 5–hydroxytetralin structure. A gain of 16 Da to this fragment giving m/z 163 would thus suggest hydroxylation somewhere at the 5–hydroxytetralin structure. Following the same line of reasoning, the presence of a fragment at m/z 186, through a m/z 16 increase from m/z 170, would suggest that the oxygen is located somewhere at the propyl–thienylethylamine function. A fragment at m/z 127 instead of m/z 111 would then suggest hydroxylation on the thienylethyl group and a fragment at m/z 135 instead of m/z 119 would correspond to hydroxylation at either the phenolic ring or at the benzyl carbons 1 or 4.

Figure 5.7 a) Mass chromatogram of m/z 316, the [M+H]+ ion of N–0923. b) Product ion spectrum of the [M+H]+ ion of N–0923.
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![Diagram of N-0923 fragmentation](image)

**Figure 5.8** Suggested fragmentation of the [M+H]+ ion of N-0923.

When the EC–Fenton system was applied, at least five different products with a gain of oxygen were obtained. Figure 5.9a shows the mass chromatogram of m/z 332, corresponding to an m/z 16 increase from m/z 316, the [M+H]+ ion of N–0923. About 20% of the N–0923 was oxidized. The product ion spectra given in Figures 5.9b–5.9f correspond to the peaks in the mass chromatogram in Figure 5.9a.
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The spectra for the peaks at RT 6.9, 7.2 and 8.7 minutes (Figure 5.9b, 5.9c and 5.9e) contain the fragments 135 and 163, suggesting that the gain of oxygen is located at the
phenolic ring or at the benzyl carbons 1 or 4. Based on the product ion spectra alone, we were not able to fully characterize these products. These products are further discussed below.

The spectrum for the peak at RT 8.3 minutes in Figure 5.9d is similar to the spectrum of N–0923 with the exceptions that the fragment at m/z 170 has been replaced with m/z 186, and the fragment at m/z 111 has been replaced with m/z 127. This is consistent with a gain of oxygen on the thienylethyl group.

The spectrum for the peak with RT 9.1 minutes in Figure 5.9f contains fragments at m/z 127, 163 and 186, suggesting a gain of oxygen at the 5-hydroxytetralin structure as well as on the thienylethyl group. The absence of a fragment at m/z 135 suggests that the oxygen is not located at the phenolic ring or at the benzyl carbons. Since the product ion scan is performed on m/z 332, hydroxylation in two different positions is not possible. This could be a case of overlapping peaks, although extraction of the different fragment ions did not show any difference in retention times. In order to get fragments at both m/z 127 and 163 from a single molecule, the oxygen has to be located in such a position of the molecule that it can be transferred to either the 5-hydroxytetralin structure or to the thienylethyl function during the fragmentation. Following the discussion about the mechanism for the electrochemical dealkylation in chapter 2, hydroxylation at any of the positions next to the nitrogen (positions 2, 15 or 9) would have resulted in N-dealkylation. No N-dealkylation products were observed in the EC–Fenton experiments. The fragment at m/z 168 is only present in this spectrum and would be consistent with a gain of 16 Da to the propyl–thienylethylamine followed by a loss of water. An explanation for this fragmentation pattern would be the N-oxide. Some possible explanations for oxygen atom transfer during the fragmentation are given in Figure 5.10.
Comparison of the oxidation products from the EC–Fenton system with the 
electrochemically generated catechol (or p-hydroquinone) from the EC/MS system  

Interpretation of the product ion spectra for the peaks at RT 6.9, 7.2 and 8.7 minutes  
(Figures 5.9b, 5.9c and 5.9e) suggest that the gain of oxygen is located at the phenolic ring  
or at the benzyl carbons 1 or 4. Interpretation of the product ion spectra alone did not allow  
a more precise determination of the structures. The hydroxyl radical acts as an electrophile  
and reacts mainly by addition to unsaturated systems, but can also abstract a hydrogen  
atom. The main products would thus be expected to be hydroxylation on the phenolic  
ring. Hydroxylation at positions 1 and 4 is also possible by a hydrogen abstraction  
mechanism as described in chapter 4. In order to gain additional information about the  
products with RT 6.9, 7.2 and 8.7 minutes, a comparison of retention times and product ion  
spectra was made with the presumed catechol obtained from reduction of the quinone  
product with mw 329 (Method 1, Figure 5.3). Since the quinones can only be formed  
electrochemically if the initial hydroxylation occurs at ortho or para position, the reduced  
product with mw 331 is bound to be either the catechol or the para–hydroquinone. A  
comparison between the two experiments is presented in Figure 5.11. The retention times  
in Figure 5.11b differ from those given in Figure 5.9a because the experiments were  
performed on separate occasions and the split between the HPLC–column and the mass  
spectrometer was different.

Figure 5.10 Possible explanations for oxygen transfer during the fragmentation of N–0923 N–oxide.
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Figure 5.11 Mass chromatograms and product ion spectra of the oxidation products of N–0923. The signal intensities in counts/s are given in the upper right corner of each chromatogram and spectrum. The numbers above the peaks in chromatogram a and b represent retention times in minutes. a) Mass chromatogram of m/z 332, corresponding to a gain of oxygen to the [M+H]+ ion of N-0923, from reduction of the electrochemically generated quinone. b) Mass chromatogram of m/z 332 from the electrochemically assisted Fenton system. The retention times differ from Figure 5.9 because the experiments were performed on separate occasions. c) Product ion spectrum of the peak with RT 9.7 minutes in chromatogram a. d) Product ion spectrum of the peak with RT 9.7 minutes in chromatogram b.

Figures 5.11a and 5.11b show that the electrochemically generated catechol (or para–hydroquinone) elutes at the same retention time as the fourth peak from the EC–Fenton system (9.7 minutes in Figure 5.11b, 8.7 minutes in Figure 5.9a). The product ion spectra given in Figures 5.11c and 5.11d are also very similar. However, in the spectrum belonging to the product from the EC–Fenton system (5.11d), the fragment at m/z 145 has a much higher intensity than in the spectrum from the electrochemically generated catechol
(5.11c). This fragment results from a loss of water from the fragment at m/z 163. The MS/MS conditions were the same for both spectra and if the compounds were exactly the same, such a substantial difference would be highly unlikely between two injections of identical products. It is possible that the location of the hydroxyl group does not sufficiently change the polarity of the compound to allow separation of the isomers on the HPLC–column (C–18). In that case, the peak at RT 9.7 minutes could correspond to a mixture of N–0923 hydroxylated at ortho, meta and para positions. This would also mean, that the compound generated from the EC/MS system could consist of both the catechol and the p–hydroquinone.
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Figure 5.12 Possible fragmentation and stabilization pathways for the fragment at m/z 163 when the hydroxyl group is located at the a) para, b) ortho or c) meta positions

The tendency to lose a water molecule from the fragment at m/z 163 is thus the main difference between the products with RT 9.7 minutes from the two systems. Figure 5.12 describes the suggested continued fragmentation of the fragment at m/z 163 when the hydroxyl group is located at the ortho, meta or para positions. When the hydroxyl group is
located at the para position (Figure 5.12a) a proton can be transferred to the “para hydroxyl group” to make water a suitable leaving group. Hydrogen atom transfer from C3 to C2 provides a second route to the fragment at m/z 145.

When the hydroxyl group is located at the ortho or meta positions (Figures 5.12b and 5.12c) an initial hydrogen atom transfer is required to make the benzylic hydrogens at position 4 available for attack by the hydroxyl group. A hydrogen atom transfer can also lead to a stable structure of the ion at m/z 163 and thus prevent it from losing a water molecule.

Based on the proposed fragmentation of m/z 163 in Figure 5.12, we suggest that the product from reduction of the quinone in the EC/MS system is mainly the catechol (the hydroxyl group located at the ortho position) and therefore gives a low yield of the fragment at m/z 145. The product from the EC–Fenton system with the same retention time (9.7 minutes) could correspond to a mixture of N–0923 hydroxylated at the ortho, meta or para positions, with the para conformation responsible for the fragment at m/z 145.

If these assumptions are correct, the two compounds eluting at RT 8.2 and 8.5 minutes (RT 6.9 and 7.2 minutes in Figure 5.9a) would correspond to two compounds hydroxylated at position 1 or position 4.

In order to obtain further information about the ortho, meta, para distribution, the application of further techniques, such as NMR, is required. Because of the low flow rates (2 µl/min) required for good conversion efficiency in the EC–Fenton system, generation of amounts sufficient for analysis with proton NMR is a very time consuming process. At a flow rate of 2 µl/minute and 20 % yield of the desired oxidation product, it would take about 5 days (and nights) to synthesize 1 mg of a compound weighing 300 Da. The EC–Fenton system presented here is highly suitable for on–line generation of oxidation products in concentrations well above the required concentrations for analysis with electrospray mass spectrometry. For generation of large quantities of oxidation products, an off–line electrochemical system or a flow–through electrochemical cell with a larger surface area than the ESA Coulochem 5020 guard cell is needed.

**Summary**

The structures suggested for the final products from both systems are given in Figure 5.13, where a–c illustrates the compounds from the EC/MS system and d–f the products from the EC–Fenton system. Compared to the metabolites reported from rat liver experiments (Figure 5.2), the metabolites (2) and (5) can be generated in the EC/MS system by reduction of the quinone products. In the rat liver experiments, two dealkylation products (3) and (4) were detected, but only one dealkylation product of the catechol was observed (5). It is possible that compound b in Figure 5.9 was not formed in the liver experiment. On the other hand, it might have been formed in concentrations too low to be detected.
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Figure 5.13 Suggested oxidation products of the EC/MS system (a–c) and the EC-Fenton system (d–h). The compounds a–c are presented as catechols although the p–hydroquinone conformation can not be entirely excluded. The retention times in minutes given for compounds d–h are taken from the mass chromatogram in Figure 5.9a.
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Metabolite 2 in Figure 5.2 was also formed in the EC–Fenton system. None of the dealkylation products were observed in the EC–Fenton experiments. Four additional compounds were tentatively identified from the interpretation of the product ion scans: two compounds hydroxylated at positions 1 or 4, a compound with the oxygen located at the thienylethyl group, and the N–oxide. None of these products were reported from the rat liver experiments. Dealkylation is the main pathway for oxidation of tertiary amines by cytochrome P450 but N–oxides are also formed. Even though N–oxides of N–0923 were not formed in the rat liver experiment, the formation of N–oxides in the EC–Fenton system can be useful for the characterization of metabolites in general. Metabolites 3 and 4 can not be generated from any of the systems without the introduction of a protective group on the 5–hydroxyl group prior to the electrochemical oxidation.

5.4 Conclusions

The results presented here demonstrate that the two methods are well suited to complement each other. Even though the EC–Fenton system does not give much additional information in the case of N–0923, it is clear that some metabolites that can not be generated in the EC/MS system can be generated in the EC–Fenton system. In this case two compounds hydroxylated at the benzylic carbons, a presumed N–oxide, a compound with the oxygen located at the thienylethyl group and a compound hydroxylated at the phenolic ring without further oxidation to the quinone structure. Even though the mechanism of hydroxylation is different from the P450 mechanism, the large range of hydroxylated compounds usually obtained from the EC–Fenton system allows comparison of HPLC retention times with real metabolites and characterization with MS/MS.

The electrochemically assisted Fenton system described here makes use of the separation of the oxidation products by means of HPLC connected on–line with the mass spectrometer, a setup that was also used in this chapter for characterization of the products from the direct electrochemical oxidation. In a setup comprising an HPLC column the very informative electrochemical potential sweeps can not be performed, but a quick insight is obtained into the composition of a mixture of isomeric oxidation products.

If larger quantities are needed for further characterization with for example NMR, both methods can be scaled up by the use of a larger electrochemical cell.

5.5 References

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