Electrochemical Behavior of Triflusal, Aspirin and their Metabolites at Glassy Carbon and Boron Doped Diamond Electrodes

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Abstract: The electrochemical behavior of triflusal (TRF) and aspirin (ASA), before and after hydrolysis in water and in alkaline medium using two different electrode surfaces, glassy carbon and boron doped diamond, was studied by differential pulse voltammetry over a wide pH range. The hydrolysis products were 2-(hydroxyl)-4-(trifluoromethyl)-benzoic acid (HTB) for triflusal and salicylic acid (SA) for aspirin, which in vivo represent their main metabolites. The hydrolysis processes were also followed by spectrophotometry. The UV results showed complete hydrolysis after one hour for TRF and after two hours for ASA in alkaline solution. The glassy carbon electrode enables only indirect determination of TRF and ASA through the electrochemical detection of their hydrolysis products HTB and SA, respectively. The oxidation processes of HTB and SA are pH dependent and involve different numbers of electrons and protons. Moreover, the difference between the oxidation peak potential of SA and HTB was equal to 100 mV in the studied pH range from 1 to 8 due to the CF₃ of the aromatic ring of HTB molecule. Due to its wider oxidation potential range, the boron doped diamond electrode was used to study the direct oxidation of TRF and ASA, as well as of their respective metabolites HTB and SA.

Keywords: Triflusal, aspirin, HTB, salicylic acid, differential pulse voltammetry.

INTRODUCTION

Aspirin (ASA), [acetylsalicylic acid], was patented by Bayer in 1899, and has been used for more than 100 years as analgesic, anti-inflammatory and antipyretic drug. Triflusal (TRF), [2-(acetyloxy)-4-(trifluoromethyl) benzooic acid] (C₉H₇F₃O₄) (Scheme 1), a fluorinated salicylate derivative, is a white crystal with a melting point of 110-112 °C and molar mass 248.16 g mol⁻¹. The pharmacological properties of TRF are similar to those of ASA, since both prevent formation of blood clots, inhibit the enzyme cyclooxygenase, reduce production of thromboxane A₂, and are a stimulator of platelet aggregation, which interferes with the formation of thrombi, thereby reducing the risk of strokes, heart attacks or other serious circulatory problems [1-6]. In humans, TRF and ASA are quickly metabolized in the liver into their major metabolites, 2-(hydroxy)-4-(trifluoromethyl)-benzoic acid (HTB) and salicylic acid (SA), respectively (Scheme 1).

Evidence of the efficacy and safety of TRF is derived from clinical trials performed in patients with unstable angina, acute myocardial infarction, stroke, aortocoronary by-pass, atrial fibrillation, valve replacement, and asthmatic patients intolerant to aspirin and/or non-steroidal anti-inflammatory drugs. Moreover, TRF has shown clinical advantages when compared with ASA in the secondary prevention of vascular events [2-7]. Recently, it has been observed that TRF and its metabolite HTB also display anti-aggregate activity [8].

Scheme 1. Chemical structures of triflusal (TRF), HTB, aspirin (ASA) and salicylic acid (SA).

The chemical structure of TRF and HTB has the substituent trifluoromethyl group in position four of the aromatic ring of TRF molecule [1], and this group does not exist in ASA and SA (Scheme 1), and both HTB and SA metabolites are formed by hydrolysis of their corresponding precursors. The introduction of fluorine atoms produces changes in the pharmacokinetic and pharmacodynamic properties of some drugs. The hydrolysis of ASA and TRF is dependent on the solution pH and temperature. They are hydrolyzed in the pH range 11.0 - 12.0, but their hydrolysis rate is slow in the pH range 4.0 - 8.0 and maximum stability is attained at low pH [9].
investigated directly and indirectly, In the present study the oxidation process of TRF was method has been reported. Although several methods have been proposed in the literature for the determination of TRF, no electrochemical analysis laboratories. Therefore, to support pharmacokinetic studies, suitable analytical methods are required. Whereas the analysis and determination of ASA was reported in various studies [9-20], for TRF, only a very few spectrophotometric, high-performance liquid chromatography, LC-MS-MS and LC-MS-MS with electrospray ionization methods [9, 20-22] have been applied for its determination in bulk, human plasma and pharmaceutical preparations. Most of these methods are sophisticated and require expensive instrumentation that is not always available in routine analysis laboratories.

In the present study the oxidation process of TRF was investigated directly and indirectly, via electrochemical determination of its metabolite HTB, and the results were compared with the electrochemical behavior of ASA and SA in order to understand the electrochemical oxidation mechanism for TRF and HTB.

The indirect electrochemical determination of ASA at the glassy carbon (GC) electrode is only possible via the voltammetric detection of hydrolyzed product SA [16], and of TRF via the voltammetric detection of the hydrolyzed product HTB.

The use of thin films of boron-doped diamond (BDD) as an electrode substrate is now well established, mainly due to its attractive properties. Although hydroxyl radicals are electrochemically generated in situ on a BDD electrode surface [23], BDD is stable to corrosion in very aggressive media, presents a very low and stable background current, an extreme electrochemical stability in both alkaline and acidic media, a high analyte sensitivity, low sensitivity to dissolved oxygen and, a very wide working potential window, which can be larger than 3.5 V [24-27].

The direct electrochemical determination of ASA and TRF using the BDD electrode is now possible. The purpose of this work was to investigate the electrochemical behavior of TRF and ASA before and after their hydrolysis in water and in alkaline medium at both GC and BDD electrodes by differential pulse voltammetry over a wide pH and potential range, from 0 V to + 1.4 V for the GC electrode and from 0.0 V to + 2.50 V for the BDD electrode.

**EXPERIMENTAL**

**Materials and Reagents**

Aspirin (ASA), salicylic acid (SA) were obtained from Sigma, and triflusal (TRF) and 2-hydroxy-(trifloromethyl) benzoic acid (HTB) from Apin Chemicals Limited, UK, and were used without further purification. A stock solution of 10 mM SA was prepared in deionized water and was stored at 4ºC. All supporting electrolyte solutions (Table 1), were prepared using analytical grade reagents and purified water from a Millipore Milli-Q system (conductivity ≤ 0.1 μS cm−1).

Microvolumes were measured using EP-10 and EP-100 Plus Motorized Microliter Pippettes (Rainin Instrument Co. Inc., Woburn, USA). The pH measurements were carried out with a Crison micropH 2001 pH-meter with an Ingold combined glass electrode. All experiments were carried out at room temperature (25 ± 1ºC).

<table>
<thead>
<tr>
<th>pH</th>
<th>Composition</th>
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<tbody>
<tr>
<td>1.0</td>
<td>H₂SO₄</td>
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<tr>
<td>2.0</td>
<td>HCl+ KCl</td>
</tr>
<tr>
<td>3.3</td>
<td>HAcO + NaAcO</td>
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<tr>
<td>4.1</td>
<td>HAcO + NaAcO</td>
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<td>8.0</td>
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In aqueous medium, the hydrolysis of TRF gives HTB and the hydrolysis of ASA gives SA. For this reason new stock solutions were always prepared before each experiment. The experiments were repeated after 24 h using the same stock solution which was stored at 4ºC. However, the hydrolysis of TRF and ASA is slow in neutral and acid media and for the acceleration of the hydrolysis reaction a new set of experiments was performed, in which the stock solutions of TRF and ASA were prepared in 20 mM NaOH solution.

The following designations are used: ASA 0h and TRF 0h for the fresh solutions prepared before each experiment, and ASA 24h and TRF 24h for the solutions used after 24h. Between the preparation of ASA 0h or TRF 0h and the experiments, there was a time interval of ~ 10 min.

**UV Measurements**

Absorption spectra were recorded using the UV-Vis spectrophotometer SPECORD S100 from Carl Zeiss Technology with Win-Aspect software. The experimental conditions for absorption spectra were integration time of 25 ms and an accumulation of 1000 points. All UV-Vis spectra were measured from 190 nm to 350 nm.

**Voltammetric Parameters and Electrochemical Cells**

Voltammetric experiments were performed using a μAutolab running with GPES 4.9 software, Eco-Chemie, The Netherlands.

Measurements were carried out using a glassy carbon electrode (GCE) (d = 2.0 mm) as working electrode (Metrohm 6.1204.110), a Pt foil counter electrode (Metrohm 6.0305.100), and a Ag/AgCl (3 M KCl) as reference (Metrohm 6.0733.100), in a 20 mL one-compartment electrochemical cell. Differential pulse (DP) voltammetry conditions were pulse amplitude 50 mV, pulse width 70 ms, scan rate 5 mV s⁻¹. The GCE was polished using diamond spray (particle size 6 μm) before each experiment. After polishing, the electrode was rinsed thoroughly with Milli-Q water for 30s; then it was sonicated for 1 minute in an ultrasonic bath and again rinsed with water. After this mechanical treatment, the GCE was placed in buffer.
electrolyte and various voltammograms were recorded until a steady state baseline voltammogram was obtained. This procedure ensured very reproducible experimental results.

The boron-doped diamond (BDD) films were prepared at the Centre Suisse de Electronique et de Microtechnique SA (CSEM), Neuchatel, Switzerland, on silicon wafers using the hot filament chemical vapour deposition (HF-CVD) technique with a filament temperature in the range 2440-2560°C and a gaseous mixture containing methane, H₂ and trimethylboron. This HF-CVD process gives a columnar, randomly textured polycrystalline BDD film with the surface dominated by {111} facets. The final boron content was of the order of 8000 ppm, 5.7x 6.1 mm² surface area, ~ 1 µm thickness [24].

Prior to use the BDD electrode was washed with ethanol and Milli-Q water, and several different electrochemical procedures were investigated for the “surface activation”. The first procedure consisted of an anodic pre-treatment applying a potential of + 3.0 V for 20 s in nitric acid or sulphuric acid. The other procedure consisted of a cathodic pre-treatment applying the potential of -2.5 V for 20 s in nitric acid or sulphuric acid. The best results were obtained by cycling the potential in the supporting electrolyte solution between the potential limits of $E_1 = 0.0$ V and $E_2 = +2.5$ V, until a stable signal was detected (15-20 cycles at a potential scan rate of 150 mV s⁻¹). For this reason, the last pretreatment procedure was chosen for all electrochemical experiments to be described. The morphology of the BDD electrode used was characterized by SEM and AFM [23].

Acquisition and Presentation of Voltammetric Data

The differential pulse voltammograms presented were background-subtracted and baseline-corrected using the moving average application with a step window of 5 mV included in GPES version 4.9 software. This mathematical treatment improves the visualization and identification of peaks over the baseline without introducing any artefact, although the peak intensity is, in some cases, reduced (<10%) relative to that of the untreated curve. Nevertheless, this mathematical treatment of the original voltammograms was used in the presentation of all experimental voltammograms for a better and clearer identification of the peaks. The values of peak current presented in all plots were determined from the original untreated voltammograms after subtraction of the baseline.

RESULTS AND DISCUSSION

Spectrophotometric Study of ASA and TRF

Prior to the voltammetric determination, a spectrophotometric study of TRF and ASA hydrolysis in water, in 20 mM NaOH and 10 mM H₂SO₄ solutions, was performed.

The results in water (Fig. 1A, B), show that TRF was hydrolyzed faster, whereas ASA is hydrolyzed very slowly. In freshly prepared solutions the maximum absorbance for TRF and ASA is at the same wavelength, $\lambda = 269.5$ nm, and for HTB is at $\lambda = 308$ nm and SA is at $\lambda = 296.5$ nm (Fig. 2A), and in [9]. After 24 h, the UV spectrum of TRF solution presented two well defined peaks at 269.5 nm, attributed to TRF, and at 308 nm, attributed to HTB, whereas the ASA solution only exhibits a small shoulder after 24 h corresponding to the wavelength of maximum absorbance of SA and another peak at 269.5 nm corresponding to ASA which was not hydrolyzed (Fig. 2B).

The results in a 20 mM NaOH solution, Fig. (1C, D), show that the hydrolysis of TRF and ASA is much faster. For fresh solutions of TRF 0h and ASA 0h, no maximum absorbance at 269.5 nm was observed. Since the measurement of the fresh sample was approximately 10 min after the preparation of stock solution, this indicates that the hydrolysis of ASA or TRF in NaOH solution was very fast. A maximum absorbance was obtained for TRF after 1 h and for ASA after 2 h, indicating that hydrolysis of TRF to HTB, $\lambda = 308$ nm, and ASA to SA, $\lambda = 296.5$ nm, reached equilibrium (Fig. 1C, D). The spectra for ASA 2h and ASA 24h and also for SA 0h, SA 1h, SA 2h and SA 24h were the same.

The results in a 10 mM H₂SO₄ solution (Fig. 1E, F), show that the hydrolysis of ASA and TRF is much slower. The UV spectra of ASA and TRF only presented a small shoulder after 24 h, corresponding to HTB, $\lambda = 308$ nm, and SA, $\lambda = 296.5$ nm.

Differential Pulse Voltammetry at a Glassy Carbon Electrode

The electrochemical oxidation behaviour of TRF, HTB, ASA and SA on the GC electrode was investigated using DP voltammetry over a wide pH range between 1 and 8, and stock solutions prepared in water and in 20 mM NaOH solution. All DP voltammograms were recorded in solutions of 1 mM ASA, 1 mM TRF, 0.1 mM SA and 0.1 mM HTB, and in different pH electrolyte solutions of 0.2 M ionic strength (Table 1).

In a fresh solution of ASA 0h, no oxidation peak was observed even for high concentrations, showing that the ASA is not oxidizable in these conditions (results not shown). The experiments were repeated for ASA 24h and a small peak, $P_{SA}$, corresponding to the SA oxidation appeared (Figs. 2A, 3). These results show that, although ASA is not electroactive at the CGE and direct determination is not possible, indirect electrochemical determination of ASA can be performed after ASA total hydrolysis to SA.

In a fresh solution of TRF 0h a small peak was observed and the peak current increased for TRF 24h (Fig. 2B). Hydrolysis of TRF in water gives rise to HTB. So, the oxidation of HTB was investigated at the GC electrode surface, by DP voltammetry (Fig. 2B). The HTB oxidation peak, $P_{HTB}$, occurred at the same potential as the small oxidation peak TRF 0h and TRF 24h. Since TRF is not electroactive in the potential range studied, $P_{HTB}$ is attributed to HTB, the hydrolysis product of TRF. Moreover, at all pHs a small oxidation peak occurred for TRF 0h and not for ASA 0h, which is in agreement with UV results (Fig. 1A, B).
Fig. (1). UV spectra: (A-B) in water 1 mM: (A) (---) TRF 0h, (•••) ASA 0h, (-----) SA 0h and (-----) HTB 0h and (B) (---) TRF 24h, (•••) ASA 24h, (-----) SA 24h and (-----) HTB 24h. (C-D) in 20 mM NaOH 200 μM: (C) (---) ASA 0h, (•••) ASA 2h and (-----) SA and (D) (---) TRF 0h, (•••) TRF 1h, (-----) SA and (-----) HTB. (E-F) in 10 mM H₂SO₄ 200 μM: (E) (---) ASA 0h, (•••) ASA 24h and (-----) SA and (F) (---) TRF 0h, (•••) TRF 2h, (-----) TRF 24h, (---) HTB 0h, (•••) HTB 2h, and (-----) HTB 24h.
The slope of 61 mV per pH unit indicates that the oxidation of SA involves the same number of electrons and protons. In addition, in these electrolytes, the width at half height of the SA oxidation peak, \( P_{\text{SA}} \), was \( W_{1/2} \approx 60 \text{ mV} \), close to the theoretical value corresponding to an electrochemical reaction involving the transfer of two electrons [28]. Thus, it can be concluded that the oxidation reaction of SA at a glassy carbon surface occurs with the transfer of two electrons and two protons for pH lower than 3.0.

For \( 3.0 < \text{pH} < 7.0 \), the oxidation peak potential of SA, \( P_{\text{SA}} \), depends on the electrolyte pH but the slope is 29 mV per pH unit, indicating a mechanism involving two electrons and only one proton, since for \( \text{pH} > 3.0 \) the SA oxidation product undergoes chemical deprotonation (Figs. 2A, 3).

For \( \text{pH} > 7.0 \), the oxidation peak potential of SA, \( P_{\text{SA}} \), was independent of electrolyte pH.

In a 0.2 M sulfuric acid solution the SA oxidation peak, \( P_{\text{SA}} \), occurred at \( E_{\text{pa}} = +1.24 \text{V} \) (not shown) and the oxidation potential was displaced to less positive values with increasing pH (Figs. 2A, 3). In Fig. 3 three linear portions can be observed: the first for \( \text{pH} < 3.0 \), the second for \( 3.0 < \text{pH} < 7.0 \), and the third for \( \text{pH} > 7.0 \).

For \( 1.0 \leq \text{pH} < 3.0 \) the dependence is linear and follows the relationship \( E_{\text{pa}} \) (V) = 1.24 - 0.061 pH (Fig. 3). The break point around \( \text{pH} 3.0 \) corresponds to the first \( pK_a \) for SA [17].
The oxidation of HTB as a function of pH at the glassy carbon electrode follows a similar behaviour to SA (Figs. 2B, 3). In the pH range of 1.0 < pH < 3.0 the slope observed was 61 mV per pH unit, for 3.0 < pH < 7.0 the slope observed was 29 mV per pH unit, and for pH > 7.0 was pH independent.

However, the oxidation peak potential of HTB is at more positive values then the oxidation peak potential of SA. The difference between peak potentials, $P_{HTB}$ and $P_{SA}$, is ~ 100 mV due to the CF$_3$ group present in the HTB molecule.

The DP voltammograms of stock solutions of ASA and TRF prepared in 20 mM NaOH solution (Fig. 4), show well defined oxidation peaks of the hydrolysis products, $P_{HTB}$ and $P_{SA}$, for both fresh solutions of ASA 0h and TRF 0h and, as expected, the peak currents increased with time. For ASA, after 2 h, the oxidation peak current was almost the oxidation peak current for SA (Fig. 4A), confirming that the hydrolysis product, SA, is the electroactive specie, as discussed in the spectrophotometric studies (Fig. 1). For TRF, 1h was enough for its total hydrolysis (Figs. 1, 4B), to its main metabolite, the electroactive HTB, and this was confirmed by the HTB voltammogram (Fig. 4B).

**Differential Pulse Voltammetry at a Boron Doped Diamond Electrode**

The electrochemical oxidation behaviour of ASA, TRF, SA and HTB on the BDD electrode was investigated using DP voltammetry over a wide pH range between 1.0 and 7.0 using 0.2 M ionic strength supporting electrolyte solutions. Two different stock solutions, prepared in water (Fig. 5), and in 20 mM NaOH (Fig. 6), were used.

However, using the BDD electrode an oxidation peak associated with water oxidation and electrochemical generation of hydroxyl radicals corresponding to the transfer of one electron and one proton was reported [23], and the electrogenerated hydroxyl radicals can interact and enhance the electro-oxidation of other compounds in solution.

DP voltammograms of a fresh solution of ASA 0h prepared in water, Fig. (5A) show a well defined pH independent oxidation peak in all electrolyte solutions at $E_{pa} = +1.9$ V.

The DP voltammograms of SA on the BDD electrode show one main oxidation peak, $P^1_{SA}$, at $E_{pa} = +1.35$ V.
Fig. (6). DP voltammograms at BDD: (A) (—) 0.2 M H$_2$SO$_4$ (s.e.), and 200 μM: (•••) ASA 0h, (•••) ASA 2h and (•••) SA and (B) (—) 0.2 M H$_2$SO$_4$ (s.e.), and 200 μM: (•••) TRF 0h, (•••) TRF 1h, (•••) SA and (•••) HTB. The stock solutions were prepared in 20 mM NaOH and the experiments recorded in 0.2 M H$_2$SO$_4$.

(Figs. 5B, 6). The chemical hydroxylation of SA occurs via hydroxyl radicals generated on the BDD electrode surface [23] leading to the formation of 2,3-DHBA (2,3-dihydroxylated benzoic acid) and 2,5-DHBA (2,5-dihydroxylated benzoic acid) [18] and two small oxidation peaks were observed, before and after the main peak, P$_{SA}^1$ and P$_{SA}^2$, corresponding to the electrochemical oxidation of 2,3-DHBA and 2,5-DHBA at the BDD electrode [19]. The proposed reactions between SA and the OH radicals electrogenerated on the BDD electrode surface are presented in Scheme 2.

All oxidation peak potentials of SA change linearly with increasing pH, the slope observed was 20 mV per pH unit, and the oxidation peak current of ASA and SA decreased with increasing pH.

Scheme 2. Proposed scheme of electrochemically induced hydroxylation of salicylic acid (SA) on the BDD electrode surface and formation of 2,3-dihydroxylated benzoic acid (2,3-DHBA) and 2,5-dihydroxylated benzoic acid (2,5-DHBA).

The behavior of TRF at the BDD electrode surface is similar to that observed for ASA, but the oxidation peak potential of TRF, at $E_{pa} = +2.1$V, is shifted by ~200 mV to a more positive value than that of ASA (Fig. 5C). After 24h, the DP voltammograms of TRF are still very similar to the voltammograms in a fresh solution (not shown). Additionally, the TRF oxidation peak currents decreased with increasing pH faster than the ASA oxidation peak currents, and in alkaline media, pH > 8.0, TRF is not oxidized. Moreover, as can be seen in Fig. (5D), for the same concentration, the oxidation peak current of HTB, the TRF metabolite, is smaller than the oxidation peak current of SA, the ASA metabolite, and the HTB oxidation potential was displaced by ~ 200 mV to a more positive value when compared with the SA oxidation potential.

The DP voltammograms were recorded in 0.2 M sulfuric acid for the 200 μM ASA (Fig. 6A), and 200 μM TRF (Fig. 6B), solutions prepared in 20 mM NaOH, in order to accelerate the hydrolysis process and the formation of SA and HTB, respectively.

CONCLUSIONS

The electrochemical behavior of ASA and TRF before and after hydrolysis in water and in alkaline medium on two different electrode surfaces, GC and BDD, was investigated by differential pulse voltammetry over a wide pH range and spectrophotometric studies were also performed, which showed that in 20 mM NaOH solution the hydrolysis of ASA was completed in two hours and of TRF in one hour.

The maximum absorbances for ASA and TRF were at the same wavelength, 269.5 nm, whereas for SA and HTB they were at 296.5 nm and 308 nm.

At the GC electrode surface only indirect determination of ASA or TRF was possible, through the electrooxidation of
their hydrolysis products, SA and HTB. The oxidation occurs for SA and HTB, at pH < 3.0 with the transfer of two electrons and two protons, whereas, for 3.0 < pH < 7.0 oxidation follows a mechanism involving two electrons and only one proton.

Since the hydrolysis reaction in sodium hydroxide medium is faster than hydrolysis in water, the DP voltammograms for ASA 0h prepared in 20 mM NaOH showed two peaks attributed to the oxidation of ASA and SA, while for ASA 2h only the oxidation peak for SA due to complete hydrolysis of ASA was observed, which was already verified in the spectrophotometric study (Fig. 1C-D).

The oxidation mechanism for TRF and HTB, the hydrolysis product, is similar to ASA and SA, respectively, as can be observed in Fig. (6B). However, for all pHs, the oxidation peak potentials of TRF and HTB were always shifted by ~ 200 mV to more positive potential values than the oxidation peak potentials of ASA and SA.

At the BDD electrode surface the direct determination of ASA or TRF was possible and the TRF and its main metabolite, HTB, is oxidised at higher potentials than ASA and SA due to the trifluoromethyl group on the aromatic ring.

ACKNOWLEDGEMENTS

Financial support from Fundação para a Ciência e Tecnologia (FCT), Ph.D. Grant SFRH/BD/37231/2007 (T.A. Enache), projects PTDC/QUI/65255/2006, PTDC/QUI/65732/2006, PTDC/QUI/098562/2008, POCI (co-financed by the European Community Fund FEDER), and CEMUC-R (Research Unit 285), and CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) Post-Doctoral Scholarship 4383-07-9 (O. Fatibello-Filho), is gratefully acknowledged.

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