Simulating the Phase II Metabolism of Raloxifene on a Screen-Printed Electrode

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Abstract

Raloxifene (RLX) is a selective estrogen receptor modulator widely used for the treatment of osteoporosis in post-menopause women. Toxicological in vitro studies suggested the reactivity of RLX through phase I metabolism. Herein, we describe a simple and inexpensive method for monitoring the reactive metabolism and detoxification of RLX by electrochemistry (EC) and mass spectrometry (MS). The phase I metabolite was synthesized electrochemically on a screen-printed electrode (SPE) and subsequently reacted with glutathione (GSH). The resulted GSH-adducts and GSH disulfides were characterized off-line by electrospray ionization (ESI) /MS.

1. Introduction

Raloxifene (RLX) has been approved by FDA for the treatment and prevention of osteoporosis [1]. The drug belongs to a class of molecules known as selective estrogen receptor modulators (SERMs) and contains a benzothiophene heterocyclic moiety [2, 3], figure 1. During metabolism RLX undergoes a direct P450-mediated two-electron oxidation to form RLX di-quinone methide. During phase II metabolism the highly reactive and unstable intermediate reacts rapidly with glutathione (GSH) via Michael addition and forms RLX-GSH adducts [4]. RLX di-quinone methide as an electrophile has the potential to induce toxicity via the covalent modification of DNA and proteins [5]. Current methodologies for monitoring reactive metabolism are tedious and expensive [6] involving mainly cellular organelles [7], subcellular fractions [8], recombinant enzymes [9], synthetic metalloporphyrins [10] and animal models [11].

However, electrochemical methods can provide a cheaper and faster alternative for simulating metabolic redox reactions [12]. Screen-printed electrodes (SPEs) are inexpensive sensors widely used in electroanalytical chemistry. Major applications include forensics [13], drug formulations [14], metabolism [15] and microbiology [16]. Ideal for research and routine analysis offering disposability, portability, low cost and low volume of reagent [14, 15]. SPEs are fabricated from inks printed into ceramic or plastic substrates [17,18,19]. The Inks are composed with polymeric binders, particles and additives to improve the printing process. However, the exact ink formulation in commercially available sensors is unknown to the users. The three-electrode configuration provides a fully functional cell in a miniaturized format for both detection and synthesis [15, 19, 20]. In the present study, we describe the mimicry of RLX metabolism on a bare

SPE. The reactive intermediate RLX di-quinone methide was generated electrochemically and reacted subsequently with GSH.

2. Materials and Methods

2.1 Reagents

RLX (>98%, purity) and reduced GSH (>98%, purity) were purchased from Sigma Aldrich (UK). Considering the low solubility of RLX in aqueous media, a series of stock solutions ($2.5x10^{-4}$, $2.5X10^{-3}$) in methanol (Fisher, UK) were prepared and dissolved in 0.1 M phosphate buffer, 0.1 M ammonium acetate (Fisher, UK) and 0.1 M ammonium bicarbonate (Fisher, UK). The phosphate buffers were prepared by potassium phosphate monobasic, 99.5% (Sigma, UK) and sodium phosphate dibasic, 99.999%, (Sigma, UK), adjusted accordingly on the required pH with 0.1 M sodium hydroxide or phosphoric acid (Fisher, UK). Voltametric experiments were recorded at $2.5x10^{-5}$ M of RLX and control potential electrolysis (CPE) at 2.5 $x10^{-4}$ M, $2.5x10^{-5}$ M of RLX and $5x10^{-4}$ M, $5x10^{-5}$ M of GSH. All reagents were of analytical grade and solutions were prepared using water that was deionised in laboratory using the Elgastat prima 3 reverse osmosis unit (Elga Ltd, High Wycombe, UK).

2.2 Apparatus

The electrochemical measurements were performed in disposable SPEs (DS-150) purchased from DropSens (Spain). Each SPE contained a carbon working electrode, a silver reference electrode and a platinum counter electrode. An edge connector interface DRP-DSC

(DropSenses, Spain) was used to couple the sensor with an SP-30 (Bio-logic, France) potentiostat. The metabolites were characterized by ESI/MS using an ion trap thermo scientific mass spectrometer (UK), an Esquire HCT ultra II ion trap (Bruker, UK) was also used. The *m/z* ranged from 150 to 2000, spray voltage was set at 4.05 KV, capillary temperature at 200.30 ^{O}C and spray current at 0.37 mA.

2.3 Electrochemical synthesis

Sample solution (50 µL) was loaded into the surface of SPE and the electrochemical signal was recorded. Subsequently the electrogenerated solution was analyzed by ESI/MS. The experiments were performed in triplicate and each time a new sensor was used to avoid memory effects.

3. Results and Discussion

3.1 Electrochemical response

RLX in 0.1 M phosphate buffer (pH 6) presented a well-defined redox pair, figure 2a. Scanning in the positive direction from 0 V to 0.6 V at 0.155 Vs^{-1} scan rate, an anode potential was generated at 0.26 V. On the reverse scan a cathode potential was obtained at 0.16 V. The separation peak potential was found to be 0.1 V indicating a quasi–reversible system [21]. However, further cyclic voltammograms were recorded separately to investigate in detail the slow electron kinetics. No signal was observed in the absence of RLX, which confirms the electrochemical reaction between RLX and carbon working electrode, figure 2b.

3.2 Participation of protons

Cyclic voltammograms were performed in several pH values (1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.4, and 8.8) for determining the participation of protons, at a scan rate of 0.1 Vs^{-1}. The anode potential shifted with the change of pH indicating the involvement of protons during the electron transfer [22]. As can be seen in figure 3a, the anode potential shifted to less positive values from 0.35 V (pH 1) to 0.15 V (pH 8.8) with the increase of pH, suggesting a pH dependence.

3.3 Stability

The stability of phase I metabolite was determined by the effect of peak current ratio vs pH. Ratios closed to unity at a given pH suggest product stability [22], since further interactions with molecules in solution are not obtained and currents have same magnitudes. The generated intermediate had the greatest stability at pH 6 with a peak current ratio of 1.08, figure 3b.

3.4 Kinetics and mass transport

RLX in phosphate buffer (pH 6) was investigated in various scan rates from 0.010-0.155 Vs^{-1} to determine the electron transfer kinetics and mass transport, figure 4a. The electron transfer was quasi-reversible since a linear relationship (R^2 = 0.97) was obtained from the calculated slope between the anode current vs square root (SQRT) of scan rate figure 4b.

Additionally, the separation peak potential [23] was increasing with the increase of scan rate figure 4c. At slower scan rates (0.01 Vs^{-1} and 0.05 Vs^{-1}) separation peak potentials were close to the Nernestian reversible slope 0.059 V. A behavior which is well explained in practice since at slow scan rates the current responds faster to the applied potential and consequently smaller separation peak potentials can be obtained [24].

The transport mode was determined by the plot of Log anode current Vs Log scan rate. Slopes closed to 1 imply complete adsorption, below 0.5 diffusion and in between 0.5 to 1 a mixture of both adsorption and diffusion [23]. The obtained slope was found to be 0.76 confirming a mixture of both adsorption and diffusion, figure 4d. kinetics and mass transport were both in agreement with previous studies in conventional electrode [25], suggesting the dehydrogenation of RLX into the reactive RLX di-quinone methide.

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3.5 Cyclic voltammetry in the presence of GSH

RLX was studied in the presence of various GSH concentrations from $5x10^{-5}$ M to $2.5x10^{-4}$ M, figure 5. Voltammograms were performed at a scan rate of 0.1 Vs^{1} in ammonium acetate buffer pH 7.4. The gradual addition of GSH caused shifts in currents and potentials implying the formation of an electroactive phase II metabolite. The anode current increased considerably with the increase of GSH whereas the cathode current decreased, suggesting the depletion of RLX di-quinone through GSH interaction.

3.6 Electrochemical synthesis

In order to generate sufficient amounts for detection CPE was performed at various potentials. RLX ($2.5x10^{-5}$ M) in the presence of GSH ($5x10^{-5}$ M) was electrolyzed for 0.5 min from 0.35 V to 0.70 V, with increasing steps of 0.05 V. The potential range was selected from voltammetry studies in figure 5 and operated over a mass transport limit. Indications for RLX di-quinone methide formation were obtained at 0.55 V through the formation of RLX-GSH adduct. By increasing the concentrations of RLX ($2.5x10^{-4}$ M) and GSH ($5x10^{-4}$ M) and extending the time of electrolysis to 30 min, improved signals were obtained as shown in figure 6. The corresponding electrogenerated metabolites were RLX-GSH adduct (*m/z* 779.09), GSH disulfide (GSSG, *m/z* 612) and di-GSH adduct *(m/z* 1084). The parent drug (*m/z* 474) was regenerated and GSH (*m/z* 308) depleted suggesting two parallel pathways, conjugation and catalysis.

3.7 Reaction mechanism

Figure 7 represents the proposed reaction mechanism of RLX. In slightly alkaline conditions (pH 7.4), RLX was electrolyzed into the corresponding RLX di-quinone methide via the transfer of two electrons and two protons from the hydroxyl moieties. Subsequently in the presence of GSH, RLX di-quinone methide conjugated with GSH leading to RLX-GSH adducts and di-GSH adducts. Furthermore, the reactive intermediate reacted catalytically with GSH forming GSSG and causing the regeneration of parent drug.

4. Conclusions

SPEs are inexpensive disposable sensors with a wide range of applications in analytical chemistry. The metabolic reactions of RLX (phase I and phase II) were mimicked successfully on bare SPEs. The reactive phase I metabolite RLX di-quinone methide was generated electrochemically via the transfer of two electrons and two protons. Subsequently, in the presence of GSH stable phase II metabolites were formed via conjugation and catalysis. SPEs can provide an alternative approach over the traditional in vitro techniques for mimicking drug metabolism.

References

1) Messali, E.M.; Scaffa, C. *Int. J. Women Health*. **2009,** 1,11.
2) Pandey, J.; Kulshreshtha, A. *EC. Chemistry*. **2015,** 2, 92.
3) Muchmore D.B. *Oncologist.* **2000,** 5, 388.
4) Moore, C.D.; Relly, C.A.; Yost G.S. Biochem. J. **2010,** 49, 4466.
5) Attia, S.M. *Oxid. Med. Cell. Longev*. **2010,** 3, 238.
6) Liu, X.; Jia, L. *Curr. Drug. Metab*. **2007,** 8, 822.
7) Zhang, D.; Luo, G.; Ding, X.; Lu, C. *Acta. Pharm. Sinic*. B. **2012,** 2**,** 549.
8) Brandon, E.F.A.; Raap, C.D.; Meijerman, I.; Beijnen, J.H.; Schellens, J.H.M. *Toxicol. App. Pharmacol.* **2003,** 189, 233.
9) Stringer, R.A.; Strain-Damerell, C.; Nicklin, P.; Houston, J.; P. *Drug. Metabolism and Dispos*. **2009**, 37,1025.
10) Schulz, S.; Wong, R.J.; Vreman, H.J.; Stevenson, D.K. *Front. Pharmacol.* **2012,** *4*.
11) Long, R.E. In *Pharmacokinetics in drug development: Regulatory and development paradigm;* American association of pharmaceutical scientists, USA, 2004; p.94.
12) Lohmann, W.; Baumann, A.; Karst U. *Lc Gc Europe.* **2010,** 23,1.
13) Smith, J.P.; Metters, J.P.; Kampouris, D.K.; Liedo-Fernandez, C.; Sutcliffe, O.B.; Banks. C.E. *Analyst.* **2013,** *138*, 6185.
14) Radi, A.E.; Khafagy, A.; El-shobagy, A.; El-mezeyen, H. *JPA.* **2013**, 3, 132.
15) Kauffmann, J.M.; Antwerpen, P.V.; Sakarkbi, A.; Feier, B.; Tarik, S.; Aydogmus, Z. *Electroanal*. **2011**, 23, 264.
16) Laczka, O.; Skillman, L.; Ditcham, W.G.; Hamdorf, B.; Wong, D.K.Y.; Berquist, P.; Sunna, A. *J. Microbiol. Methods*. **2013,** 95,182.

17) Miserere, S.; Ledru, S.; Ruille, N.; Criveau, S.; Boujtita, M.; Bedioui, F. *Electrochem Commun*. **2006,** 8, 238.
18) Mistry, K.K.; Layek, K.; Mahapatra, A.; RoyChaudhurib, C.; Sahab, H.A. *Analyst*. **2014,** 139, 2289.
19) Khaled, E.; Hassan, H.N.A.; Habib, I.H.I.; Metelka, R. *Int. J. Electrochem. Sci*. **2010,** *5*,158.
20) Li, M.; Li, Y.T.; Li, D.W.; Long, Y.T. *Anal. Chim. Acta*. **2012,** 734, 31.
21) Wang, Z.L.; Liu, Y.; Zhang Z. *Handbook of nanophase and nanostructure materials;* Kluwer, New York, 2003.
22) Nematollahi, D.; Sgayani-Jam, H.; Alimoradi, M.; Niroomand, S. *Electrochim. Acta*. **2009**, 54,7407.
23) Ndangli, P.M.; Jijana, A.N.; Olowu, R.N.; Mailu, S.N.; Ngece, F.R.; Williams, A.T.; Waryo, T.; Baker, P.J.L. *Int. J. Electrochem*. **2011,** 6 ,1438.
24) Zhang, S.; Yuan, XZ.; Wang, H. In *PEM fuel cell diagnostic tools;* CRC press, USA, 2012, p.73.
25) Bagheri, A.; Hosseini H. Bioelectrochemistry. **2012,** 88, 164.

Figure captions

Figure 1. Chemical structure of RLX.

Figure 2. Electrochemical behavior of RLX. a) In the presence of 2.5 x10^{-5} M RLX. b) Phosphate buffer pH 6.

Figure 3. Effect of pH. a) Anode potential. b) Peak current ratio.

Figure 4. Effect of scan rate. a) Scan rate range. b) Anode current. c) Separation peak potential. d)Transport mode.

Figure 5. Effect of GSH concentration. a) 5x10^{-5} M. b) 1.2 x10^{-4} M. c) 2.5x10^{-4} M.

Figure 6. Mass spectrum a) 0.55 V. b) Prior electrolysis.

Figure 7. Reaction mechanism of RLX.