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Comparison of TiO₂ photocatalysis, electrochemically assisted Fenton reaction and direct electrochemistry for simulation of phase I metabolism reactions of drugs

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ABSTRACT

The feasibility of titanium dioxide (TiO₂) photocatalysis, electrochemically assisted Fenton reaction (EC-Fenton) and direct electrochemical oxidation (EC) for simulation of phase I metabolism of drugs was studied by comparing the reaction products of buspirone, promazine, testosterone and 7-ethoxycoumarin with phase I metabolites of the same compounds produced *in vitro* by human liver microsomes (HLM). Reaction products were analysed by UHPLC–MS. TiO₂ photocatalysis simulated the *in vitro* phase I metabolism in HLM more comprehensively than did EC-Fenton or EC. Even though TiO₂ photocatalysis, EC-Fenton and EC do not allow comprehensive prediction of phase I metabolism, all three methods produce several important metabolites without the need for demanding purification steps to remove the biological matrix. Importantly, TiO₂ photocatalysis produces aliphatic and aromatic hydroxylation products where direct EC fails. Furthermore, TiO₂ photocatalysis is an extremely rapid, simple and inexpensive way to generate oxidation products in a clean matrix and the reaction can be simply initiated and quenched by switching the UV lamp on/off.

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1. Introduction

Most drugs are metabolized in the body before excretion. Drug metabolism is therefore studied early in the drug discovery process to screen out drug candidates with unwanted metabolism. Before animal studies, drug metabolism is investigated *in vitro* using hepatocytes, recombinant enzymes, or subcellular fractions such as microsomes, cytosol, or S9 fraction. Drug metabolism is often divided into phase I and phase II. Phase I reactions are functionalization reactions, where a functional group is attached to or exposed in the drug molecule, whereas phase II reactions are conjugation reactions, that facilitate detoxification or excretion of the drug from the body. Phase I reactions may also give rise to toxic or reactive metabolites (Guengerich, 2001). The most important phase I metabolic pathways are oxidation reactions catalysed by cytochrome P450 (CYP450) isoenzymes (Guengerich, 2013). The mechanisms of CYP450-catalysed reactions, such as hydroxylation, dehydrogenation, and heteroatom oxidation and dealkylation, depend on the chemical structure of a particular substrate and the enzyme isoform involved to the metabolic reactions (For extensive reviews, see

Guengerich, 2001; Meunier et al., 2004). Even though the exact reaction mechanism may differ from the enzymatic route, many CYP450 reactions can be simulated by non-enzymatic oxidation methods, such as electrochemical oxidation (Johansson et al., 2007; Jurva et al., 2003), Fenton reaction (Liang et al., 2013; Van der Steen et al., 1973; Zbaida et al., 1986, 1987) and titanium dioxide (TiO₂) photocatalysis (Calza et al., 2004; Nissilä et al., 2011), which have been proposed as affordable and convenient alternatives to the traditional *in vitro* experiments in the early preclinical phase of metabolite screening.

Direct electrochemical oxidation (EC) is initiated by electron abstraction from the substrate by the electrode (Bussy and Boujtita, 2014). Even though, in principle, electrons can be abstracted from any bond, oxidation potentials of aliphatic hydrocarbons are typically higher than those of solvents (Nouri-Nigjeh et al., 2011), and the single electron transfer mechanism requires the presence of a nonbinding electron that can be initially abstracted (Bussy and Boujtita, 2014). Exceptionally, aliphatic hydroxylation has been achieved with use of a platinum electrode and 2 V potential (Baumann et al., 2009). The reaction is not direct EC but is likely mediated by water oxidation (Bussy and Boujtita, 2014). Direct EC is suitable for simulation of CYP450 reactions that can begin with single electron transfer mechanism, reactions such as dehydrogenation, S-oxidation, P-oxidation and N-dealkylation (Jurva et al., 2003). In addition, allylic (Baumann et al., 2009), benzylic (Jahn et al., 2011; Johansson et al., 2007) or aromatic hydroxylation (Madsen et al., 2008; van Leeuwen et al., 2005) as well as ipso-substitution (Bussy et al.,

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2013; Madsen et al., 2007) can occur in EC if there is an activating electron donating heteroatom substituent at a suitable position enabling initial electron transfer followed by electron delocalization to the above mentioned positions (Bussy and Boujtita, 2014). O-dealkylation was not achieved for 7-ethoxycoumarin (Jurva et al., 2003) but has been reported for verapamil (Jahn et al., 2011), metoprolol (Johansson et al., 2007) and toremifen (Lohmann and Karst, 2009). The mechanism was not discussed, however, and for verapamil and toremifen could actually be ipso-substitution. Electrochemical oxidation has resulted in product profiles very similar to those of in vivo and in vitro metabolism (Baumann et al., 2009), but also considerable differences have been reported (Jirasko et al., 2013; Johansson et al., 2007).

In the Fenton reaction, hydrogen peroxide oxidizes Fe^{2+} to Fe^{3+} and dissociates to hydroxyl radical and hydroxyl ion (Haber and Weiss, 1932). In electrochemically assisted Fenton reaction (EC-Fenton), Fe^{3+} is reduced at the working electrode back to active Fe^{2+} (Jurva et al., 2002). Hydroxyl radicals can react by addition to unsaturated bonds or abstract aliphatic hydrogen atoms, which should enable simulation of metabolism reactions initiated by hydrogen atom abstraction. Hydroxylation, dehydrogenation, and heteroatom oxidation and dealkylation reactions have been simulated by EC-Fenton (Johansson et al., 2007). Comparison of the Fenton reaction products with metabolites reported in the literature has shown reproduction of several metabolites of a number of compounds (Kugelmann et al., 2011; Liang et al., 2013; Zbaida et al., 1986, 1987; Zbaida and Kariv, 1990). Detailed experimental comparisons of metabolic and Fenton reaction product profiles are nevertheless scarce (Johansson et al., 2007).

In TiO_2 photocatalysis, ultraviolet (UV) light excites electrons from the valence band of TiO_2 to the conduction band (for a more detailed review on the fundamentals and mechanism of TiO_2 photocatalysis, see Fujishima et al., 2008). The formed electron-hole pair separates into a free electron and a free hole, which can diffuse to the surface of the TiO_2 particle. Surface-trapped holes can react with water to produce hydroxyl radicals or directly with organic compounds by electron transfer. Also, the reaction of surface-trapped electrons with oxygen can contribute to oxidation reactions, for example by producing superoxide anions. TiO_2 photocatalysis simulates several hydroxylation, dehydrogenation, and O- and N-dealkylation reactions of drugs (Calza et al., 2004; Nissilä et al., 2011; Ruokolainen et al., 2014). However, only a few studies have addressed the use of TiO_2 photocatalysis in simulation of drug metabolism (Calza et al., 2004; Nissilä et al., 2011; Raoof et al., 2013; Ruokolainen et al., 2014), and comparisons with other methods (such as EC and EC-Fenton) are completely lacking.

The aim of our study was to examine and compare how comprehensively TiO_2 photocatalysis, EC-Fenton reaction and direct electrochemical oxidation can simulate human phase I metabolism in HLM in vitro. The test compounds (buspirone, promazine, testosterone and 7-ethoxycoumarin) were chosen because they undergo several important phase I metabolism reactions, including aliphatic and aromatic hydroxylation, N-oxidation, S-oxidation, N-dealkylation and O-dealkylation. To compare the products formed in TiO_2 photocatalysis, EC-Fenton, EC and HLM incubations, we developed an ultra high performance liquid chromatography-mass spectrometry (UHPLC-MS) method, enabling separation of isomeric products, for analysis of the reaction products.

2. Materials and methods

2.1. Chemicals

Promazine hydrochloride was from Sigma-Aldrich (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and Orion (Orion Oy, Espoo, Finland). Buspirone hydrochloride, 7-ethoxycoumarin, testosterone, androstenedione, TiO_2 Degussa P-25, glacial acetic acid ($\geq 99.85\%$), formic acid (puriss. p.a. for mass spectrometry, app. 98%), ammonium acetate, NADPH, sodium dihydrogen phosphate, magnesium chloride hexahydrate, ethylenediaminetetraacetic acid disodium salt dehydrate

(EDTA, 99–101%), ferric chloride hexahydrate (98%), LC-MS grade acetonitrile and methanol were from Sigma-Aldrich. Acetonitrile (HPLC Supra-gradient) and methanol (absolute HPLC, min 99.9%) were from Biosolve BV (Valkenswaard, The Netherlands). Disodium hydrogen phosphate and sodium hydroxide were from Merck (Darmstadt, Germany). Water was purified with a Milli-Q Plus (Millipore, Molsheim, France) or Arium® 611 (Sartorius Stedim Biotech GmbH, Goettingen, Germany) purification system. Human liver microsomes were purchased from BD Gentest (BD Gentest New Jersey, USA). All reagents were of reagent grade or better.

2.2. HLM incubations

The phase I metabolism reactions in vitro were performed with human liver microsomes (HLM). The incubations were carried out in total volume of 100 μL , containing 0.5 mg mL^{-1} microsomal protein, 50 μM substrate, 5 mM MgCl_2 and 50 mM phosphate buffer (pH 7.4). Four different drugs were studied, with each drug incubated separately and incubations performed in duplicate. Buspirone and promazine were added to the incubation mixture in water, while testosterone and 7-ethoxycoumarin were added in water-acetonitrile solution. The final concentration of organic solvent during enzyme incubation was 0.5%. Reactions were initiated by the addition of NADPH (1 mM) and transfer of the tubes to a dry bath at 37 °C. After 2 h incubation, the reaction was terminated by protein precipitation with ice-cold acetonitrile (200 μL), and transfer of the tubes to –18 °C for 30 min. The precipitated HLM was removed by centrifugation for 10 min at 13,200 rpm.

2.3. Photocatalytic reactions

The photocatalytic reaction mixture (500 μL) contained 10 μM test compound and 1 g L^{-1} TiO_2 Degussa P25 particles. Photocatalytic reactions were performed in duplicate, and blanks were prepared without the substrate. The samples were magnetically stirred while exposed from above to a 5000-PC Series Dymax UV Curing Flood Lamp (Dymax Light Curing Systems, Torrington, CT, USA). The lamp was a metal halide UV lamp with nominal intensity of 225 mW cm^{-2} . After UV exposure, the TiO_2 particles were removed by 10 min centrifugation at 13,200 rpm. The first supernatant (470 μL) was centrifuged again for 10 min at 13,200 rpm. The second supernatant (450 μL) was collected for analysis without further treatment. For acquisition of the product ion spectra of the products of testosterone and 7-ethoxycoumarin, the 450 μL supernatant was evaporated to dryness under nitrogen in a 40 °C water bath. The dry residue was then reconstituted with 50 μL of eluent A (for 7-ethoxycoumarin) or eluent A:B 75:25 (for testosterone).

The effect of acetonitrile (1% or 50%) on the reaction routes, and the effect of UV exposure time were studied with buspirone. UV exposure times were 15–120 s in pure water, 0.5–12 min in the presence of 1% ACN and 1–30 min in the presence of 50% ACN. The comparison between TiO_2 photocatalysis and in vitro HLM metabolism was made with UV exposure times of 15 s (in pure water, buspirone and promazine) or 2 min (in 1% acetonitrile, testosterone and 7-ethoxycoumarin).

2.4. Electrochemically assisted Fenton reaction

The EC-Fenton setup was based on the methods previously reported (Johansson et al., 2007; Jurva et al., 2002). Each reaction mixture (550 μL) consisted of 50 μL 10 mM substrate (in water or acetonitrile), 50 μL 10 mM EDTA/ FeCl_3 in water, 125 μL or 75 μL acetonitrile, 275 μL or 325 μL water (depending on whether the sample was in water or acetonitrile, respectively) and 50 μL 0.1 M hydrogen peroxide (added just before the start of infusion through the EC cell). An ESA 5021A conditioning cell (ESA Inc., Bedford, MA, USA) with a working electrode of porous graphite was used for EC-Fenton and EC reactions. All reported cell potentials are versus a palladium reference electrode. The

electrochemical cell was controlled by an ESA Coulochem III potentiostat (ESA Inc.). Samples were infused through the electrochemical cell first at a flow rate of $50 \mu\text{L min}^{-1}$. After 1 min the flow rate was lowered to $2 \mu\text{L min}^{-1}$ and a potential of -500 mV was applied. Sample collection was started after 15 min. The samples were diluted 25 times with water before UHPLC–MS analysis.

2.5. Direct electrochemical oxidation

The test solution contained $10 \mu\text{M}$ test compound in $\text{ACN:H}_2\text{O}$ 50:50 + 0.1% HCOOH (EC-acidic). In the case of testosterone and 7-ethoxycoumarin, no products were observed at $10 \mu\text{M}$ concentration and the concentration was increased to $100 \mu\text{M}$. Also basic conditions (EC-basic) were tested for the oxidation of buspirone and promazine, with $\text{ACN:H}_2\text{O}$ 50:50 + 0.1% NH_4OH used as the solvent. The solution was infused at a flow rate of $50 \mu\text{L min}^{-1}$ through the electrochemical cell. The potential was ramped from 0 to 1500 mV with steps of $10 \text{ mV}/3.6 \text{ s}$. All the potential ramp experiments were made in duplicate. Online-ESI–MS was used to monitor the formed products. The delay between the electrochemical cell and the mass spectrometer was 30 s . Samples for UHPLC–MS analysis were collected at constant potentials chosen on the basis of the potential ramp experiments (Supplement, Table S1). The samples were diluted tenfold with water so that the amount of organic solvent would match better with the starting conditions of the gradient.

2.6. Liquid chromatography–mass spectrometry

A TSQ Quantum AM (Thermo Fisher Scientific, Waltham, MA, USA) triple quadrupole mass spectrometer was used for online monitoring of products during EC potential ramps. Spray voltage was 4000 V and skimmer offset 0 . Sheath gas pressure was 30 , auxiliary gas pressure 0 and capillary temperature 300°C . Tube lens voltage was optimized for each compound with direct infusion $5 \mu\text{L min}^{-1}$ (Supplement, Table S2). Scan time was 0.5 s and the scan range varied (Supplement, Table S2). Further analyses of the products from HLM, TiO_2 photocatalytic, EC-Fenton and direct EC reactions were performed with ACQUITY UPLC™ (Waters, Milford, MA, USA) and Xevo™ Q-TOF-MS (Waters, Manchester, UK) instruments. An ACQUITY UPLC BEH C-18 column ($100 \text{ mm} \times 2.1 \text{ mm i.d.}$, $1.7 \mu\text{m}$ particle size) was used for chromatographic separation of reaction products of each test compound. An in-line filter ($0.2 \mu\text{m}$) was used in front of the analytical column, and the flow was directed to waste for the first 0.5 – 0.7 min . Eluent A was 95:5 water/100 mM ammonium acetate and eluent B 95:5 MeOH/100 mM ammonium acetate. The pH of the ammonium acetate buffer was 6.0 (adjusted with acetic acid). The mobile phase flow rate was $200 \mu\text{L min}^{-1}$ in optimization of photocatalytic reaction conditions. In a more detailed analysis of the reaction products, the buffer pH was adjusted to 4.5 and the flow rate to $500 \mu\text{L min}^{-1}$. Gradients used for optimization of the UV exposure time and for the more detailed analysis of reaction products are listed in the Supplement, Tables S3 and S4, respectively. The injection volume was $5 \mu\text{L}$ with partial loop injection mode. The sample tray was kept at 8°C and the column at 40°C . MS and MS/MS spectra were collected in the positive ion mode with electrospray ionization (ESI). Nitrogen was used as the desolvation gas (1000 L h^{-1} , 500°C) and argon as the collision gas. The ion source temperature was set to 150°C and the capillary voltage was 3 kV . Extraction cone voltage was 3 V . Sampling cone voltage and collision energies used in MS/MS analysis of the reaction products were optimized separately for each test compound (Supplement, Table S5). MS data were acquired from m/z 30 to 560 with scan time of 0.1 s , centroided and corrected during acquisition using as external reference (Lockspray) leucine enkephalin (concentration $2 \text{ ng } \mu\text{L}^{-1}$, flow rate $20 \mu\text{L min}^{-1}$) at m/z 556.2771. Extracted ion chromatograms were generated with 0.05 Da mass window. Metabolynx XS V4.1

software (Waters, Milford, MA, USA) was used in the search for unexpected reaction products.

3. Results

3.1. Optimization of photocatalytic and electrochemical reaction conditions

In this study, organic solvent was added to increase the solubility of 7-ethoxycoumarin and testosterone. The effect of organic solvent (0, 1 or 50% acetonitrile in water) on the photocatalytic reaction product profile was examined with buspirone as test compound, as it was soluble in all solvents tested. In addition, UV exposure time was optimized for each solvent. Reaction products (Supplement, Table S6) were analysed by UHPLC–MS (Supplement, Table S3). The reaction products formed very fast in 100% water, and the intensity of the main products was highest at 15 s (Fig. 1a). At longer UV exposure times, the initial products underwent further reaction or degradation. Acetonitrile effectively slowed down the photocatalytic reactions even at 1% concentration, and the highest intensity of the main products was observed at 2 min (Fig. 1b). The effect was even more pronounced at 50% concentration, as the intensities of the products continuously increased within the time frame studied (Fig. 1c). Acetonitrile scavenges hydroxyl radicals (Li, 2013), which slows down the hydroxyl radical-

Fig. 1. Formation of an N,N -dealkylation product (m/z 360), an $M + O-2H$ product (m/z 400) and an $M-2H$ product (m/z 384) of buspirone in a) water, b) 1% ACN and c) 50% ACN.

mediated photocatalytic reaction. In addition to slow kinetics, addition of acetonitrile completely inhibited the formation of some of the products formed in 100% water, and completely new products not observed in 100% water were formed (Supplement, Table S6). These results are in line with our earlier observations on the effect of acetonitrile on the TiO₂ photocatalysis of anabolic steroids (Ruokolainen et al., 2014). On the basis of these preliminary findings, UV exposure times of 15 s and 2 min were chosen for TiO₂ photocatalysis in 100% water (buspirone and promazine) and 1% acetonitrile (testosterone and 7-ethoxycoumarin), respectively.

The electrochemical reaction conditions were studied with respect to EC potential using online-MS monitoring of reaction products during potential ramps (0 to 1500 mV). No reaction products of testosterone or 7-ethoxycoumarin were observed in acidic conditions at concentrations of 10 μ M and 100 μ M. This is in accordance with the literature: testosterone and 7-ethoxycoumarin are not electrochemically oxidized under either acidic or basic conditions (Johansson et al., 2007; Jurva et al., 2003). In contrast, several products of buspirone and promazine were observed in acidic conditions, and hence potential ramps were also performed in basic conditions. A few differences in products were observed in acidic and basic conditions, and samples for UHPLC–MS analysis were collected at a few potentials in both solvents (Supplement, Table S1).

3.2. Comparison of reaction products

A UHPLC–ESI/MS method using a Q-TOF mass spectrometer was developed for the analysis of reaction products. The high resolving power of the UHPLC (Fig. S1) allowed separation of nearly all isomeric compounds. The good repeatability of the retention times (RSD typically less than 0.5%), together with the mass spectrometric data, enabled reliable comparison of the products of different oxidation methods. All reaction products were observed as protonated molecules. The MS/MS spectra of the simulation reaction products and HLM metabolites with the same m/z of the protonated molecule (tolerance 30 ppm) and retention time (t_R , tolerance 0.05 min) were compared to confirm that these were, in fact, the same products (Fig. S1). In most cases, the MS/MS spectra correlated well, indicating that products and metabolites identified on the basis of m/z and retention time were indeed the same. In the few cases where the MS/MS spectra were different, products having the same retention time and precursor ion probably were different

isomers. To compare the reaction product profiles of different oxidation methods, we normalized the abundances of the reaction products relative to the most abundant reaction product obtained with the particular method. Products were classified as major (S), medium (M) or minor (W) according to their relative abundances (50–100%, 20–50% and 5–20%, respectively). The major metabolites and their simulation by compared non-enzymatic methods are presented in Figs. 2–5. The reaction products with over 5% relative abundance are listed in the Supplement (Tables S7–S10) and are briefly discussed, by compound, in the following.

3.2.1. Buspirone

Three major, six medium and nine minor HLM metabolites of buspirone (m/z 386) were observed (Fig. 6a, Table S7). In most cases, the modifications and location of modification could be identified based on the product ion spectra of the reaction products. The major HLM metabolites resulted from aliphatic hydroxylation at azaspirodecanedione moiety (R4, see Fig. 2), aromatic hydroxylation of pyrimidine (R1) and N-oxidation of piperazine (R2) (all M + O, m/z 402, Fig. 2). Both the major hydroxylation metabolites were produced by TiO₂ photocatalysis. In contrast, EC-Fenton and EC did not produce any of the major metabolites of buspirone.

The medium HLM metabolites of buspirone included three additional aliphatic hydroxylation products (M + O, m/z 402), an N-dealkylation product (m/z 165, 1-pyrimidinyl piperazine, M-R3–R4), an N-dealkylation–hydroxylation product (m/z 240, despyrimidinyl piperazine buspirone, M + O–R1–R2) and a dihydroxylation product (m/z 418, aromatic hydroxylation of pyrimidine and aliphatic hydroxylation of azaspirodecanedione). All the simulation methods produced the N-dealkylation product (m/z 165), but none produced the N-dealkylation–hydroxylation product (m/z 240). TiO₂ photocatalysis produced two of the hydroxylation products and EC-Fenton produced one.

Nine minor metabolites of buspirone were observed in HLM experiments. These comprised five M + 2O products, all of which had at least one hydroxyl group at azaspirodecanedione: one of the M + 2O products resulted from dihydroxylation of azaspirodecanedione, three from N-oxidation of piperazine and hydroxylation of azaspirodecanedione, and one of hydroxylation of pyrimidine and azaspirodecanedione. Only the last mentioned was formed in TiO₂ photocatalysis. In contrast, all the simulation methods reproduced the N,N-deethylenation metabolite (m/z 360, M-C₂H₂).

Fig. 2. The main metabolites of buspirone. S = 50–100% and W = 5–20% of the most abundant reaction product of the particular reaction system, — not observed.

Fig. 3. The main metabolites of promazine. S = 50–100%, M = 20–50% and W = 5–20% of the most abundant reaction product of the particular reaction system, – not observed.

Additionally, the M + O-2H metabolite (m/z 400) and the M-2H metabolite (m/z 384) were reproduced by TiO_2 photocatalysis and EC-Fenton. The N-dealkylation–dihydroxylation–dehydrogenation metabolite (m/z 254, M + 2O-2H-R1-R2) was not produced by any of the simulation methods.

All the simulation methods produced also other products than HLM metabolites (Fig. 6a, Table S7). TiO_2 photocatalysis produced 19 additional, non-HLM products: a product hydroxylated in azaspirone moiety (m/z 402), two products dihydroxylated in azaspirone moiety, an N-dealkylation product (m/z 308, loss of pyrimidine), three M + O-2H products, two M-2H products (m/z 384), a product resulting

from fragmentation of pyrimidine ring (M-36, m/z 350), an M-8 product (m/z 378), an M + O-CH₂ product (m/z 388), an M + O-4H product (m/z 398), an M + 2O product (m/z 406), two M + O-2H (m/z 416) products, an M + 3O product (m/z 434), and two M + 3O + 4H products (m/z 438). EC-Fenton produced 22 additional non-enzymatic products. These were mostly the same as in TiO_2 photocatalysis, except for m/z 398, 406 and 416, which were not produced by EC-Fenton, and the following products, which were not observed in TiO_2 photocatalysis: two more M + 2O products (m/z 418), an M + O-2H product (m/z 400), an M + 22 product (m/z 408), an M + 2O-4H product (m/z 414), an M + 3O-CH₂ product (m/z 420) and an M + 4O-CH₂ product (m/z 434). Photocatalytic and EC-Fenton products at m/z 378, m/z 406, m/z 408 and m/z 438 were produced by (multiple) hydroxylation(s) and opening or fragmentation of the pyrimidine ring. EC-acidic produced three additional products, namely: M-CH₂ (m/z 372), M + O-CH₂ (m/z 388) and M + 2O-2H (m/z 416), where the modifications are in piperazine. Three of five additional products in EC-basic were the same as in EC-acidic. As well, EC-basic produced an N,N-dealkylation product (m/z 235) and an M + O + NH₃-2H product (m/z 415), where ammonia in the basic solution participates in the reaction. Their late elution and MS/MS spectra suggest that the EC products involving addition of oxygen are N-oxides.

In summary, the HLM metabolism reactions of buspirone included aromatic hydroxylation, aliphatic hydroxylation of R3 and R4, with or without subsequent dehydrogenation, N-oxidation of R2, loss of the ethylene group from R2, N-dealkylation between R2 and R3,

Fig. 4. The main metabolites of testosterone. S = 50–100% and W = 5–20% of the most abundant reaction product of the particular reaction system, – not observed.

Fig. 5. 7-Ethoxycoumarin and its main metabolite. S = 50–100% and M = 20–50% of the most abundant reaction product of the particular reaction system, – not observed.

Fig. 6. Summary of simulation of HLM metabolites of a) buspirone, b) promazine, c) testosterone, d) 7-ethoxycoumarin by TiO₂ photocatalysis, EC-Fenton and EC. No reaction products of testosterone and 7-ethoxycoumarin were observed in EC-acidic.

and combinations of these (Table 1). All these reaction types except N-oxidation were observed in TiO₂ photocatalysis and EC-Fenton, but the combinations of reactions and isomers were often different from those in HLM metabolism. The oxidation reactions typically proceeded further in TiO₂ photocatalysis and EC-Fenton than in HLM; for example, dehydrogenation followed hydroxylation, and multiple hydroxylations of pyrimidine and opening or fragmentation of the pyrimidine ring were observed. EC was not capable of aromatic or aliphatic hydroxylation but was the only simulation method that produced N-oxides.

3.2.2. Promazine

The main HLM metabolites of promazine (*m/z* 285) resulted from N-demethylation (M-CH₂, *m/z* 271), S-oxidation and aromatic hydroxylation (M + O, *m/z* 301), most likely to 3-position based on the literature (Wojcikowski et al., 2003) (Table 1, Fig. 3). Only EC-basic simulated N-demethylation. All the simulation methods studied were capable of S-oxidation, and promazine sulfoxide was a major product in all of them except in EC-basic, which favoured N-demethylation. TiO₂ photocatalysis produced also the aromatic hydroxylation metabolite

Table 1

The main reaction types observed in HLM phase I metabolism, TiO₂ photocatalysis, EC-Fenton and EC reactions. n.a. = reaction not applicable for the compound.

	Aliphatic hydroxylation	Aromatic hydroxylation	Dehydrogenation	N-oxidation	S-oxidation	N-dealkylation	O-dealkylation	Hydrogenation	Addition of ammonia
Buspirone									
HLM	x	x	x	x	n.a.	x	n.a.		
TiO ₂	x	x	x		n.a.	x	n.a.	x	
EC-Fenton	x	x	x		n.a.	x	n.a.	x	
EC-acidic			x	x	n.a.	x	n.a.		
EC-basic			x	x	n.a.	x	n.a.		x
Promazine									
HLM		x	x	x	x	x	n.a.		
TiO ₂		x	x	x	x		n.a.		
EC-Fenton		x			x		n.a.		
EC-acidic			x		x	x	n.a.		
EC-basic			x		x	x	n.a.		x
Testosterone									
HLM	x	n.a.	x	n.a.	n.a.	n.a.	n.a.		
TiO ₂	x	n.a.	x	n.a.	n.a.	n.a.	n.a.		
EC-Fenton	x	n.a.	x	n.a.	n.a.	n.a.	n.a.		
EC-acidic		n.a.		n.a.	n.a.	n.a.	n.a.		
7-ethoxycoumarin									
HLM		Hydroxylation of ring structure							
TiO ₂	x	x	x	n.a.	n.a.	n.a.	x	x	
EC-Fenton	x	x	x	n.a.	n.a.	n.a.	x		
EC-acidic				n.a.	n.a.	n.a.			

as a minor product. None of the simulation methods produced the medium HLM metabolite promazine N-oxide (Table S8). Five minor HLM metabolites of promazine were observed: two M + O-CH₂ metabolites (*m/z* 287), two M + 2O metabolites (*m/z* 317) and an M + 2O-2H metabolite (*m/z* 315). The M + O-CH₂ metabolites (*m/z* 287) were N-demethylated S-oxidation and aromatic hydroxylation metabolites, and the former of these was reproduced by EC-acidic and EC-basic. TiO₂ photocatalysis and EC-Fenton both reproduced the M + 2O metabolite (*m/z* 317) resulting from S-oxidation and aromatic hydroxylation, whereas only TiO₂ photocatalysis reproduced the one resulting from S-oxidation or N-oxidation. The M + 2O-2H metabolite (*m/z* 315) was a minor product of EC-acidic.

In addition to HLM products, TiO₂ photocatalysis produced seven non-enzymatic minor products (Fig. 6b, Table S8). These consisted of three M + 2O products (*m/z* 317), an M + 2O-2H product (*m/z* 315), an M + 2O + 2H product (*m/z* 319), an M + 3O product (*m/z* 333) and an M + 3O + 2H product (*m/z* 335). EC-Fenton also produced an M + 2O product (*m/z* 317) and an M + 3O product (*m/z* 333). EC-acidic produced three additional products: an M + 2O product (*m/z* 317), an M + O-2CH₂ product (*m/z* 273) and an M + O-2H product (*m/z* 299). EC-basic produced six additional products: phenothiazine sulfoxide (*m/z* 216) by S-oxidation and N-dealkylation, an M-CH₄ product (*m/z* 269), the same M + O-2CH₂ (*m/z* 273) and M + O-2H (*m/z* 299) products as in EC-acidic, and two products where ammonia in the basic solution participated in the reaction, viz., M + O + NH₃-CH₄-2H (*m/z* 300) and M + O + NH₃-CH₄ (*m/z* 302).

In summary, HLM metabolism reactions of promazine included N-demethylation, S-oxidation, aromatic hydroxylation, N-oxidation, and dehydrogenation, alone or in different combinations (Table 1). TiO₂ photocatalysis and EC-Fenton mainly produced S-oxidation and aromatic hydroxylation products. EC also produced sulfoxides and was the only simulation method capable of N-dealkylation of promazine. Owing to the possibility of N-dealkylation and S-oxidation, EC was equal to TiO₂ photocatalysis and EC-Fenton with respect to the number of products duplicating HLM metabolites, even though the reaction product profiles were different.

3.2.3. Testosterone

The main reactions of testosterone (*m/z* 289) in phase I HLM metabolism, TiO₂ photocatalysis and EC-Fenton reactions were hydroxylation and dehydrogenation and combinations of these (Table 1), and several isomeric products of each type were formed (Table S9). The major metabolites of testosterone were a hydroxylation metabolite (M + O, *m/z* 305), most likely 6-hydroxytestosterone based on the literature (Choi et al., 2005; Krauser et al., 2004), and androstenedione (M-2H, *m/z* 287), which was confirmed through comparison with a standard by MS/MS (Fig. 4). Androstenedione was also the major reaction product of TiO₂ photocatalysis and EC-Fenton. Additionally, EC-Fenton produced the major hydroxymetabolite. Two of the three medium metabolites (M + O, *m/z* 305) observed in HLM were also produced by TiO₂ photocatalysis and EC-Fenton (Fig. 6c). Minor HLM metabolites of testosterone included three more M + O metabolites (*m/z* 305), an M-2H metabolite (*m/z* 287), four M + O-2H metabolites (*m/z* 303) and an M-4H metabolite (*m/z* 285). TiO₂ photocatalysis and EC-Fenton produced one of the minor M + O (*m/z* 305) metabolites and one of the M + O-2H (*m/z* 303) metabolites.

Furthermore, TiO₂ photocatalysis produced ten non-HLM products (Fig. 6c, Table S9): four M + O products (*m/z* 305), one M-2H product (*m/z* 287), four M + O-2H products (*m/z* 303) and an M + O-4H product (*m/z* 301), whereas EC-Fenton produced 13 products not observed in HLM: six M + O products (*m/z* 305), two M-2H products (*m/z* 287), four M + O-2H products (*m/z* 303) and an M + 3O-CH₂ product (*m/z* 323). The HLM metabolites and photocatalytic reaction products are in line with those found in our earlier study (Ruokolainen et al., 2014). Direct EC failed to oxidize testosterone at all.

3.2.4. 7-Ethoxycoumarin

The main HLM metabolism reaction of 7-ethoxycoumarin (*m/z* 191) was O-deethylation leading to 7-hydroxycoumarin (M-C₂H₄, *m/z* 163), which was also produced by TiO₂ photocatalysis and EC-Fenton as a medium and major product, respectively (Table 1, Figs. 5 and 6d). A medium metabolite M + O + 4H-CO (*m/z* 183) and two minor metabolites M + O-C₂H₄ and M + O-CH₂ + 4H (*m/z* 179 and 197, respectively) were also observed in HLM (Table S10). On the basis of its MS/MS spectrum, the M + O-C₂H₄ metabolite (*m/z* 179) was concluded to result from deethylation and hydroxylation of the ring structure, where 3-hydroxylation (see Fig. 5 for numbering) is most likely to occur (Fisher et al., 2002; Jung et al., 1985). A major product of TiO₂ photocatalysis and EC-Fenton was M + O-2H (*m/z* 205), where the modifications are in the ethyl group. In addition, TiO₂ photocatalysis produced one and EC-Fenton two ring hydroxylated M + O products (*m/z* 207). Direct EC failed to oxidize 7-ethoxycoumarin at all.

4. Discussion

Fig. 6 presents an overall comparison of the feasibility of TiO₂ photocatalysis, EC-Fenton and EC for simulation of HLM phase I metabolism. Reaction types observed with the test compounds in HLM metabolism and the simulation reactions are listed in Table 1. N-Dealkylation, dehydrogenation and S-oxidation reactions were observed with all three simulation methods. The reaction types observed in HLM metabolism and TiO₂ photocatalysis were almost the same (Table 1). Despite the possibility for the same reaction types, products of the compared oxidation methods often differed, owing to the possibility for combinations of reactions and the possible formation of different isomeric products.

TiO₂ photocatalysis was able to reproduce almost half of the HLM reaction products; the coverage was considerably poorer for EC-Fenton, and direct EC failed to produce most of the HLM reaction products. Direct EC is known to be limited to reactions initiated by single electron transfer. Reactions initiated by hydrogen atom abstraction have higher oxidation potential, which means that oxidation of the solvent is more likely than oxidation of the drug compound (Jurva et al., 2003). In contrast, TiO₂ and EC-Fenton were capable of both aliphatic and aromatic hydroxylation and O-dealkylation because hydroxyl radicals can abstract hydrogen atoms. However, the hydroxylation reaction is not equally selective and may produce different isomers from those produced in vitro in HLM. Also, TiO₂ photocatalysis and EC-Fenton easily overoxidize the compounds (e.g., hydroxylation followed by dehydrogenation) as compared with in vitro metabolism. TiO₂ photocatalytic reaction can begin by hydrogen atom abstraction by hydroxyl radicals or by single electron transfer to an electron hole on the TiO₂ surface, both of which are important from the mechanistic point of view when simulating in vitro metabolism.

In view of the many trace products, reaction products of less than 5% relative abundance were excluded from the reaction product profiles. Thus, some of the HLM products were actually found in trace amounts in TiO₂ photocatalysis and EC-Fenton, and, reversely, some of the additional products of the simulation reactions were also found in trace amounts in the HLM reactions. Most of these trace products were isomeric products of buspirone, promazine and testosterone produced by reaction mechanisms including hydroxylation. Thus, synthesis of more HLM metabolites in useful amounts by TiO₂ photocatalysis and EC-Fenton methods might become possible by increasing the concentration of the starting compound. However, in both TiO₂ photocatalysis and EC-Fenton, also non-enzymatic products were found below the 5% abundance limit, which means that the correlation of overall product profiles would not necessarily improve with increasing concentration of starting compound. Further, it bears notice that some of the simulation reaction products not found in HLM could be of clinical importance, as not all enzymes responsible for drug metabolism are present in HLM, and other non-biological oxidation

products could form, for example through incorrect storage of the medicinal preparation.

The value of simulation methods might be particularly in investigating reactive metabolites, which are difficult to detect in biological matrices owing to their fast reactions with proteins and DNA and other biological molecules. Rapid online methods based on microchips (Nissilä et al., 2011; Van den Brink et al., 2015) would be ideal for this purpose since immediate analysis best enables the observation of intact reactive products. The feasibility of TiO_2 photocatalysis for studying reactive intermediates is an interesting area for future research. We also observed, in this and our earlier study (Ruokolainen et al., 2014), that acetonitrile can be used to manipulate the formation and degradation rates of the photocatalytic products and direct the photocatalytic reactions. These results suggest that organic solvents may provide a way to tune the TiO_2 photocatalytic reaction. Another often suggested use of simulation methods is the synthesis of metabolite standards in pure matrix, obviating the need to develop multistep synthetic strategies or to purify compounds present in complex biological matrices. This approach would be especially valuable where standards are not easily produced by other methods. Importantly, TiO_2 photocatalysis can provide an extremely rapid and inexpensive way to produce aliphatic and aromatic hydroxylation products that direct EC is not capable of. Only 15 s UV exposure was needed for production of reaction products of the water soluble drugs. Especially in comparison to EC-Fenton, which also allowed hydroxylation reactions, TiO_2 photocatalysis is much faster. Furthermore, the reaction can be easily controlled by UV exposure and no other specific instrumentation is needed. Finally, the one of the most interesting uses of radical-based methods, especially TiO_2 photocatalysis, in the drug discovery process could be rapid production of multiple hydroxylated derivatives of bioactive compounds for activity testing.

5. Conclusions

In this study, TiO_2 photocatalysis performed better in HLM phase I metabolism simulation than did EC-Fenton or direct EC. The advantage of TiO_2 photocatalysis over EC-Fenton and EC is that the reaction can proceed via hydroxyl radical attack or single electron transfer at the TiO_2 surface, enabling the reproduction of more phase I HLM metabolism reactions than was possible with EC-Fenton or EC. In no case, however, was the correlation between metabolites and products produced by the simulation methods good enough for exact prediction of metabolites, either because the simulation methods do not reproduce certain reactions or the enzymatic reactions are more selective. Simulation methods often produce different isomers than enzymes do, and analysis of these isomers requires proper chromatographic separation to avoid false matches. Nevertheless, TiO_2 photocatalysis provides a remarkably rapid and simple way of generating oxidation products in a very clean matrix. Moreover, the reaction is easily controlled by turning the UV lamp on/off. Therefore, TiO_2 photocatalysis is a useful addition to the tool box of simulation methods, offering a feasible approach for rapid synthesis of several metabolites and more hydrophilic derivatives of lead compounds.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.ejps.2015.12.012>.

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