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Imitation of phase I oxidative metabolism of anabolic steroids by titanium dioxide photocatalysis

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ABSTRACT

The aim of this study was to investigate the feasibility of titanium dioxide (TiO_2) photocatalysis for oxidation of anabolic steroids and for imitation of their phase I metabolism. The photocatalytic reaction products of five anabolic steroids were compared to their phase I *in vitro* metabolites produced by human liver microsomes (HLM). The same main reaction types – hydroxylation, dehydrogenation and combination of these two – were observed both in TiO_2 photocatalysis and in microsomal incubations. Several isomers of each product type were formed in both systems. Based on the same mass, retention time and similarity of the product ion spectra, many of the products observed in HLM reactions were also formed in TiO_2 photocatalytic reactions. However, products characteristic to only either one of the systems were also formed. In conclusion, TiO_2 photocatalysis is a rapid, simple and inexpensive method for imitation of phase I metabolism of anabolic steroids and production of metabolite standards.

1. Introduction

The metabolism of a drug candidate *in vitro* is usually studied using hepatocytes, microsomes, or recombinant enzymes. Although drug metabolism is in general a safe and natural way to facilitate the elimination of drugs from the body, phase I metabolism can transform drugs into pharmacologically active or toxic compounds, and metabolism is also an important cause for drug candidate failure. Thus, metabolism studies have shifted to earlier stages of the drug discovery process reflecting the 'fail early – fail cheaply' paradigm (Ekins et al., 2000). The increasing number of compounds to be tested has provoked a need for faster, cheaper, or more convenient alternatives to the traditional *in vitro* enzymatic metabolism experiments. As the most important phase I metabolic reactions are oxidation reactions catalyzed by cytochrome P450 (CYP) isoenzymes, various nonenzymatic oxidation methods, such as metalloporphyrins (Bernadou and Meunier, 2004), Fenton reaction (Liang et al., 2013; Van der Steen et al., 1973; Zbaida et al., 1986), and electrochemical reactions (Johansson et al., 2007; Jurva et al., 2003), have been studied for mimicking phase I metabolism reactions.

Metalloporphyrins acting as surrogates for the active centers of CYP enzymes have been shown to be capable of imitating all types of phase I metabolism reactions (Bernadou and Meunier, 2004; Meunier, 1992). However, yields were low for some reactions and additional reactions, which are not observed in CYP mediated metabolism reactions, can also occur due to lack of selectivity of metalloporphyrins (Bernadou and Meunier, 2004). In addition, metalloporphyrins are strong oxidizing agents and easily oxidizable drugs can be overoxidized to stable products, thus bypassing possible reactive intermediates formed in phase I metabolism reactions. Therefore, the production of the desired product in sufficient amounts requires careful selection of suitable metalloporphyrin, oxygen donor, and reaction conditions.

Electrochemistry (EC) can mimic reactions which can be initiated with single electron transfer step, such as N-dealkylation, S- and P-oxidation, alcohol oxidation and dehydrogenation (Johansson et al., 2007; Jurva et al., 2003). In contrast, reactions initiated by hydrogen atom abstraction, such as O-dealkylation, aliphatic hydroxylation, or hydroxylation of non-substituted aromatic rings, have higher oxidation potential than water, and thus, these are less likely reactions as solvent is oxidized instead of the organic compound (Nouri-Nigjeh et al., 2011). However, overoxidation is common in hydroxylation of substituted aromatic rings, as the hydroxylation products may be oxidized further at lower potentials than the starting compound (Jurva et al., 2003).

Fenton reaction involves hydrogen peroxide (H_2O_2) and a ferrous salt (Fenton, 1894). Fe^{2+} is oxidized to Fe^{3+}, while H_2O_2 is dissociated to a hydroxyl ion and a hydroxyl radical (Haber and Weiss, 1932), which can react with various organic compounds

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by addition to unsaturated bonds or hydrogen abstraction from aliphatic carbons. Active Fe^{2+} can be regenerated from Fe^{3+} by using a chemical reductive agent, such as ascorbic acid, or electrochemical reduction at the working electrode (Jurva et al., 2002). Electrochemically assisted Fenton (EC-Fenton) reaction has been shown to be capable of imitating hydroxylations, dealkylations and heteroatom oxidations (Johansson et al., 2007). However, Fenton reaction is relatively non-selective (Barry et al., 2012) because of its hydroxyl radical based mechanism (Barry et al., 2012; Mile, 2000).

Titanium dioxide (TiO_2) photocatalysis has been widely applied for degradation of organic pollutants (Bhatkhande et al., 2002; Fujishima et al., 2000). TiO_2 can catalyze both oxidation and reduction reactions when exposed to ultraviolet (UV) light of high enough energy to excite the electrons from the valence band to the conduction band of TiO_2 leaving holes on valence band (Fig. 1). The holes and electrons can either recombine, or migrate to the surface of a TiO_2 particle, where they can react with water or directly with organic compounds. Reactive hydroxyl radicals ($^{\cdot}OH$) and superoxide anions ($O_2^{\cdot-}$) are formed in aqueous solution. Few studies have described the application of TiO_2 photocatalysis for imitation of phase I metabolism reactions (Calza et al., 2004; Medana et al., 2011, 2013; Nissilä et al., 2011; Raoof et al., 2013), but on the basis of these recent reports, TiO_2 photocatalysis seems to be a promising alternative to imitation of drug metabolism, since several biologically important reactions are possible. Hydroxylation, dehydrogenation, N- and O-dealkylation reactions have been observed in TiO_2 photocatalysis (Nissilä et al., 2011) and TiO_2 photocatalysis has been shown to produce products, which are similar to the metabolites formed *in vivo* and *in vitro* (Calza et al., 2004; Medana et al., 2011, 2013; Nissilä et al., 2011; Raoof et al., 2013).

Steroids are lipophilic, important endogenous compounds, which play a number of important physiological roles. Steroids are synthesized from cholesterol via numerous enzymatic reactions resulting in variety of biologically active forms. Since steroids regulate many cellular and physiological actions, the metabolism of steroids has been largely studied by using *in vivo* and *in vitro* experiments. Poor water solubility of lipophilic steroids can limit usefulness of biological *in vitro* metabolism assays. The solubility of steroids can be increased by adding organic solvent to aqueous solvents. However, even low concentrations of organic solvents (2–5%) can significantly reduce enzyme activity (Busby et al., 1999; Chauret et al., 1998; Gonzalez-Perez et al., 2012; Hickman et al., 1998; Li, 2009).

Although biomimetic oxidation techniques can circumvent the limitations presented by the use of organic solvents in enzymatic metabolism assays (Bernadou and Meunier, 2004; Johansson et al., 2007) they have seldom been used for mimicking the metabolism reactions of steroids and other poorly soluble compounds. Metalloporphyrins have been shown to catalyze hydroxylation of some steroid substrates regioselectively (Breslow et al., 1997; Fang and Breslow, 2006; Stuk et al., 1991; Yang and Breslow, 2000), however the addition of binding groups to the steroid substrate is necessary to control the orientation of the steroid respective to the metalloporphyrin. Jurva et al. showed that two different aliphatic hydroxylation products of testosterone were formed in EC-Fenton and metalloporphyrin catalyzed reactions, as well as in experiments made with liver microsomes (Johansson et al., 2007). Direct EC reactions did not, however, produce aliphatic hydroxylation reaction products with testosterone, suggesting that direct EC is not the method of choice for mimicking phase I metabolism reactions of steroids. Several aliphatic hydroxylation products and further oxidation products were observed in TiO_2 photocatalytic reactions of dexamethasone (Calza et al., 2001). The hydroxyl radical based mechanism enables aliphatic hydroxylation, which makes TiO_2 photocatalysis a potential method for the imitation of steroid metabolism. In these biomimetic oxidation studies, comparisons with enzymatic reactions, using for example human liver microsomes (HLM), are unfortunately very limited and therefore solid conclusions on the usefulness of the mimetic oxidation methods for predicting phase I reactions of steroids cannot be made.

In this work we study the feasibility of TiO_2 photocatalysis for oxidizing and imitation of phase I metabolism of five anabolic steroids (Fig. 2). The reaction conditions, with respect to reaction time and solvent composition, are studied. The products from TiO_2 photocatalytic oxidizing reactions are compared to those produced by *in vitro* phase I metabolic reactions using human liver microsomes. An ultra high performance liquid chromatography–high resolution mass spectrometric (UHPLC–HRMS) method is developed for the analysis of reaction products. The method provides high chromatographic resolution for the separation and analysis of possible

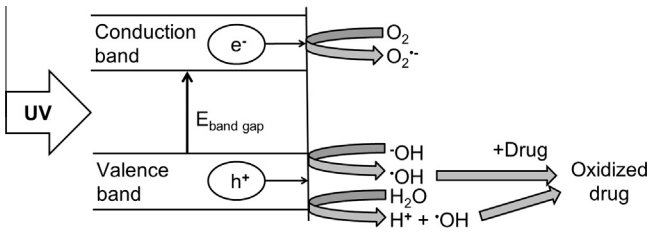

Fig. 1. The principle of TiO_2 photocatalysis. Modified from (Bhatkhande et al., 2002). See Fujishima et al. (2008) and Zhang et al. (2012) for more detailed descriptions of the TiO_2 photocatalysis mechanism.

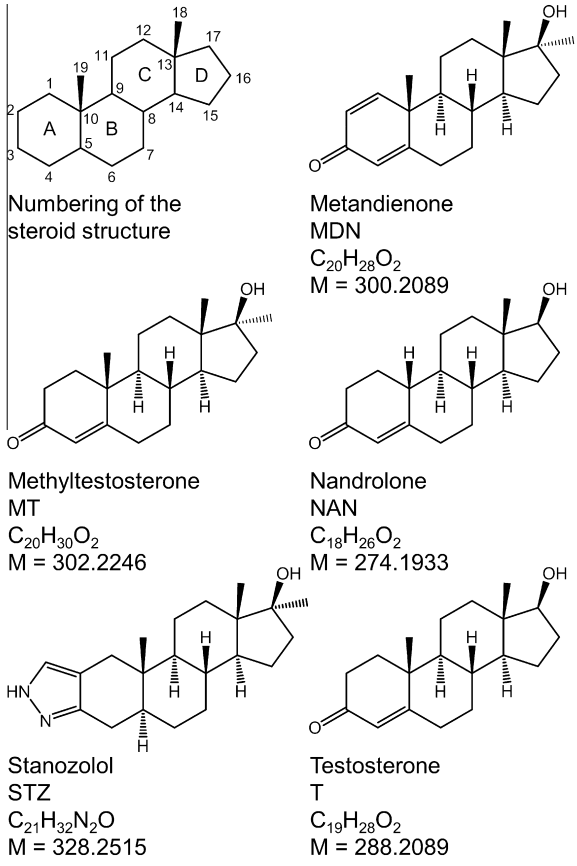

Fig. 2. Molecular structures, names, abbreviations, molecular formulas and monoisotopic masses of the test compounds used in this study. Top-left figure represents the numbering of the different carbon atoms in the steroid structure.

structural isomers. Separation of isomeric products is particularly important in order to examine, if the oxidation site is the same in phase I metabolism and TiO_2 photocatalytic reactions.

2. Materials and methods

2.1. Chemicals

Methyltestosterone (MT) and nandrolone (NAN) were from Diosynth (Oss, the Netherlands), metandienone (MDN) and androstenedione were from Steraloids (Newport, RI, US), and stanozolol (STZ) was from Sterling-Winthorp (New York, NY, US). Testosterone (T), TiO_2 Degussa P-25, glacial acetic acid ($\geqslant$99.85%), ammonium acetate, β-nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate (NADPH), sodium dihydrogen phosphate, acetonitrile (ACN) and methanol were from Sigma–Aldrich (Steinheim, Germany). Magnesium chloride, perchloric acid (70–72%), disodium hydrogen phosphate and sodium hydroxide were from Merck (Darmstadt, Germany). Water was purified with a Milli-Q Plus purification system (Molsheim, France). Human liver microsomes were purchased from BD Gentest (New Jersey, USA). The solvents were LC–MS grade and all the reagents at least reagent grade.

2.2. Photocatalytic reactions

Photocatalytic reactions were performed in duplicate, and blank samples were prepared without a steroid. The photocatalytic reaction mixture (500 μL) contained 10 μM steroid and 1 g L^{-1} TiO_2 Degussa P25 particles. The effect of acetonitrile content was studied with testosterone with different percentages of acetonitrile in the reaction mixture. With water–acetonitrile 99:1, the UV exposure times used were 0, 1, 2, 3, 4, 6, and 12 min, and with water–acetonitrile 50:50 and 5:95, 0, 1, 5, 10, 15, 20, and 30 min. The UV exposure times used for comparison of photocatalytic reaction products to phase I *in vitro* metabolites were 2 min for testosterone, methyltestosterone, metandienone, and nandrolone in water–acetonitrile 99:1, and 15 min for stanozolol in water–acetonitrile 50:50. The samples were magnetically stirred while UV exposed from above with 5000-PC Series Dymax UV Curing Flood Lamp (Dymax Light Curing Systems, Torrington, CT, USA). The nominal intensity of the metal halide UV lamp was 225 mW $(cm^2)^{-1}$. After UV exposure, the TiO_2 particles were removed by centrifugation at 13,200 rpm for 10 min. The first supernatant (470 μL) was centrifuged again at 13,200 rpm for 10 min. The second supernatant (450 μL) was collected for analysis without further pretreatment when the effect of acetonitrile content was studied, and was pretreated as described in 2.4, when used for comparison to metabolism samples.

2.3. HLM incubations

Phase I metabolism reactions were studied *in vitro* using HLM. The incubations were carried out in a total volume of 100 μL containing 50 μM substrate, 5 mM $MgCl_2$, 50 mM phosphate buffer (pH 7.4), and 0.5 mg mL^{-1} total protein. Each steroid was incubated separately and added into the incubation mixture in acetonitrile (testosterone, methyltestosterone, metandienone, and nandrolone) or methanol (stanozolol) solution. The final concentration of organic solvent was 1%. The reaction was initiated by addition of 5 μL NADPH (20 mM) and transferring the tubes on a dry bath of 37 °C. The final concentration of NADPH in the incubation mixture was 1 mM. 5 μL of 20 mM NADPH was added again after 6 h when the total incubation time was 12, 18, or 24 h. The reaction was terminated by protein precipitation with 4 M perchloric acid (10 μL) and transferring the tubes into an ice bath. The precipitated HLM were removed by centrifugation at 13,200 rpm for 10 min. The supernatants were pooled from 1, 3, 6, 12, 18, and 24 h incubations. Blank incubations were carried out similarly without the substrate. Control experiments were also made to ensure, that perchloric acid does not oxidize the steroids.

2.4. Solid phase extraction

The second supernatant (450 μL) of photocatalytic reaction and the pooled supernatant (270 μL) from HLM incubations were diluted each with 1 mL of 100 mM ammonium acetate buffer (pH 6.0) for solid phase extraction. Waters Oasis 30 mg 1 mL cartridges were conditioned with 1 mL of methanol and 1 mL of purified water. The sample was loaded to the cartridge, which was then washed with 1 mL of water–methanol 95:5 (v:v) and 1 mL of water–methanol 95:5 (v:v) +2% acetic acid. Steroids and the reaction products were eluted from the cartridge with 1.5 mL acetonitrile (testosterone, methyltestosterone, metandienone, and nandrolone) or 1.5 mL methanol (stanozolol). The samples were evaporated to dryness in a water bath under nitrogen. Dry residues were reconstituted in 100 μL (HLM samples) or 50 μL (photocatalysis samples) of the LC mobile phase A:B 75:25 (v:v). The eluent A was 95:5 water–100 mM ammonium acetate (pH 6.0, adjusted with acetic acid) and eluent B was 95:5 methanol–100 mM ammonium acetate (pH 6.0). Photocatalysis samples used for studying the effect of the organic solvent content and the duration of the UV exposure were not purified nor enriched with solid phase extraction.

2.5. Liquid chromatography–mass spectrometry

Samples were analyzed with ACQUITY UPLC™ (Waters, Milford, MA, USA) and Xevo™ Q-TOF-MS (Waters, Manchester, UK) instruments. An Acquity UPLC BEH C-18 column (100 mm × 2.1 mm i.d., 1.7 μm particle size) was used for chromatographic separation of reaction products of each steroid. A Vanguard BEH C18 (5 mm × 2.1 mm i.d., 1.7 μm particle size) precolumn was used in front of the analytical column. A fast gradient (Table A.1) was used in the optimization of photocatalytic reaction conditions, after which the gradients were optimized separately for the reaction products of each steroid (Tables A.2–A.6). The flow rate of the mobile phase was 300 μL min^{-1}. Injection volume was 5 μL 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 using electrospray ionization (ESI). Nitrogen and argon were used as the desolvation (800 L h^{-1}, 450 °C) and collision gas, respectively. The source temperature was 120 °C and capillary voltage was 3 kV. The cone voltage was 25 V and extraction cone voltage 3 V. Data were acquired with resolution of 10,000 from 30 to 560 Da with a scan time of 0.1 s and the centroid mass data was corrected during acquisition using an external reference (Lockspray) leucine enkephalin (concentration 2 ng μL^{-1}, flow rate 20 μL min^{-1}) at m/z 556.2771. Extracted ion chromatograms were generated with 20 ppm mass window. Two collision energies were used in the MS/MS analysis of each steroid (Table A.7). There were up to 5 simultaneous MS/MS scans in addition to the MS scan. Metabolynx XS V4.1 software (Waters, Milford, MA, USA) was used to search for unexpected reaction products. If a reaction product with the same m/z and retention time (t_R) as in HLM experiments was found also in TiO_2 photocatalytic reactions, its product ion spectrum was compared to the product ion spectrum of the corresponding metabolite.

3. Results and discussion

3.1. Optimization of reaction and measurement conditions

In order to study the feasibility of TiO_2 photocatalysis in mimicking phase I metabolism reactions of anabolic steroids, a UHPLC–ESI/HRMS method using a q-tof mass spectrometer was developed for the analysis of reaction products. As several isomeric oxidation products of the selected anabolic steroids were formed in the reactions, the chromatographic conditions were optimized (Tables A.2–A.6) in order to maximize the separation efficiency for isomeric reaction products. Good chromatographic performance was recognized as narrow and non-tailing peaks that allowed separation of several isomeric oxidation products of the anabolic steroids formed in the reactions (Fig. 3). The relative standard deviations of retention times were typically less than 0.25% indicating good chromatographic repeatability that is required in order to allow for comparison of the products formed in TiO_2 photocatalysis and HLM reactions. ESI mass spectra of the anabolic steroids and their oxidation products showed an abundant protonated molecule with few fragment ions typically formed by the loss of one or two water molecules.

In TiO_2 photocatalysis, addition of organic solvent to water was necessary in order to increase the solubility of anabolic steroids. Acetonitrile was selected as organic solvent because it has weaker radical scavenging activity than methanol (Li, 2013). Thus, it was expected, that acetonitrile allows the photocatalytic reaction but slows down the reaction rate by reducing hydroxyl radicals available for oxidation reactions. The effect of acetonitrile on the oxidation reaction rates was studied with three different acetonitrile concentrations: water–acetonitrile 99:1, 50:50 and 5:95 using testosterone as a model compound.

Degradation of testosterone was rapid and the photocatalytic reaction products formed fast in water–acetonitrile 99:1 (Fig. 4). The highest abundance of reaction products was achieved in 2 min after which their degradation was faster than formation. Testosterone and the oxidation reaction products were degraded completely in 12 min. As expected, increasing acetonitrile concentration inhibited the reaction: The degradation of testosterone and the formation of reaction products were slower in water–acetonitrile 50:50 and 5:95 than in water–acetonitrile 99:1. These results show that the addition of acetonitrile to the reaction mixture provides a simple way to control the TiO_2 photocatalyzed oxidation reactions. In water–acetonitrile 50:50, the abundance of reaction products increased with increasing UV exposure time during the 30 min time frame studied, but in water–acetonitrile 5:95, the formation of the products became slower than their degradation by the end of the 30 min time frame studied.

Acetonitrile was found not only to inhibit but also to direct the photocatalytic reactions. In water–acetonitrile 99:1 and 50:50, the same products were observed, although their relative intensities were different (Table 1). In water–acetonitrile 50:50 and water–acetonitrile 5:95, the formation of a dehydrogenation product (M−2H, *m/z* 287.20, t_R 3.34 min) was clearly favored over formation of hydroxylation (M+O, *m/z* 305.21, t_R 2.99 min) and hydroxylation + dehydrogenation (M+O−2H, *m/z* 303.19, t_R 2.67 min) products, whereas these three product types were formed with relatively similar abundances in water–acetonitrile 99:1 (Fig. 4). The addition of acetonitrile, having electron scavenging capability, inhibits formation of reactive oxygen species (Fig. 1) and thus formation of hydroxylation products of the steroids. Therefore, at higher acetonitrile concentrations steroids will rather react directly with the photogenerated holes on the TiO_2 surface via

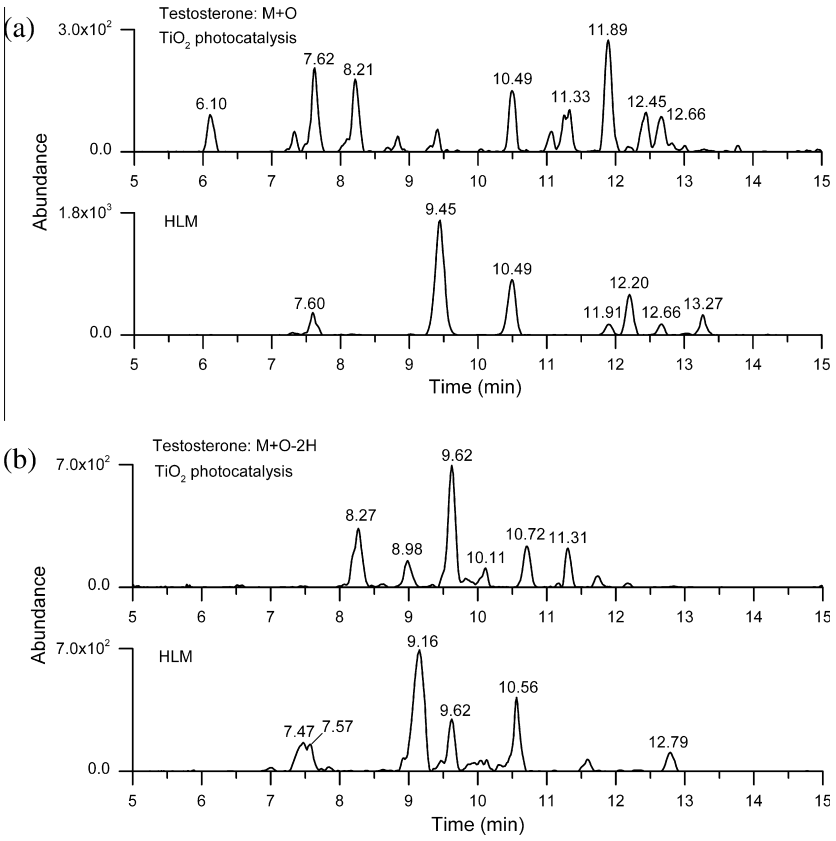

Fig. 3. Extracted ion chromatograms of (a) *m/z* 305.2117 and (b) *m/z* 303.1960 corresponding to M+O and M+O−2H reaction products of testosterone, respectively. Peaks observed at the same retention time and with matching MS/MS spectra in TiO_2 photocatalysis and HLM reactions were considered identical compounds.

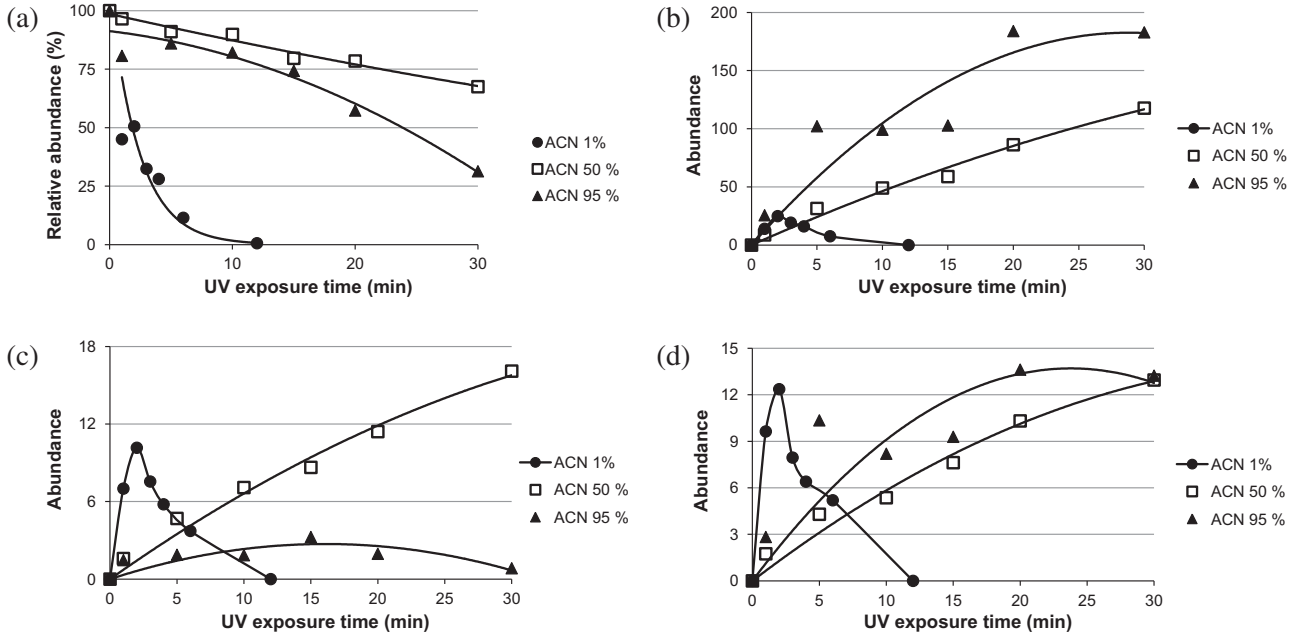

Fig. 4. Effect of acetonitrile concentration on degradation of testosterone and formation of selected photocatalytic reaction products of testosterone. (a) Degradation of testosterone (*m/z* 289.22, t_R 3.55 min), (b) formation of an M−2H product (*m/z* 287.20, t_R 3.34 min), (c) formation of an M+O product (*m/z* 305.21, t_R 2.99 min), and (d) formation of an M+O−2H reaction product (*m/z* 303.20, t_R 2.67 min) of testosterone.

Table 1
Reaction products observed in TiO_2 photocatalytic oxidation of testosterone in different acetonitrile concentrations. Peaks observed at the same retention time were considered identical compounds.

Reaction	*m/z*	t_R (min)	ACN 1%	ACN 50%	ACN 95%
Dehydrogenation M−2H	287.20	3.34	x	x	x
Hydroxylation M+O	305.21	2.63	x	x	
		2.89	x	x	
		2.99	x	x	x
		3.16			x
Hydroxylation + dehydrogenation M+O−2H	303.20	2.67	x	x	x
		2.79	x	x	x
		2.88	x	x	
		2.99	x	x	x
Dihydroxylation M+2O	321.21	2.80	x	x	
		3.07			x
		3.17			x
Dihydroxylation + dehydrogenation M+2O−2H	319.19	2.23	x	x	x

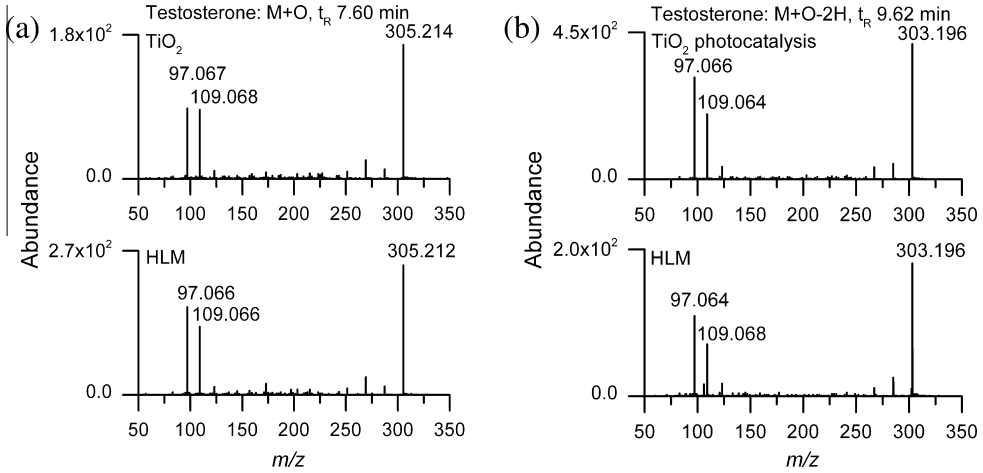

Fig. 5. Examples of correlation of MS/MS spectra (CE 20 V) in TiO_2 photocatalysis and HLM reactions. (a) MS/MS spectra of M+O (*m/z* 305.2117, t_R 7.60 min) and (b) MS/MS spectra of M+O−2H (*m/z* 303.1960, t_R 9.62 min) reaction product of testosterone.

Table 2
Coverage of HLM reactions by TiO_2 photocatalysis calculated using Eq. (1).

Reaction/steroid	MT (%)	NAN (%)	T (%)	STZ (%)	MDN (%)
Dehydrogenation M−2H	50	100	50	0	0
Hydroxylation M+O	63	75	43	67	17
Hydroxylation + dehydrogenation M+O−2H	40	13	33	33	40

one electron oxidation followed by deprotonation producing dehydrogenation products of the steroids.

Generally the formation of M+O and M+O−2H products was less abundant and slower in water–acetonitrile 5:95 than in water–acetonitrile 50:50, and many of these products were not formed at all in water–acetonitrile 5:95, while some products were only formed in water–acetonitrile 5:95 (Table 1). In most cases, however, a greater abundance of products could be achieved in water–acetonitrile 50:50, than in water–acetonitrile 99:1, with longer UV exposure time. This is probably due to the slower degradation of formed products when more radical scavenging acetonitrile is present.

Higher acetonitrile percentages with longer UV exposure times produce greater abundance of oxidation products, which may be useful in photocatalytic synthesis of metabolite standards. However, water–acetonitrile 99:1 and a UV exposure time of 2 min were chosen for photocatalytic reactions for comparison to phase I *in vitro* HLM metabolism reactions, because these conditions are closer to physiological conditions. Furthermore, the reactions are inhibited less, and are thus much faster and more convenient than with higher acetonitrile concentrations. However, stanozolol was not soluble enough in water–acetonitrile 99:1, and thus water–acetonitrile 50:50 and UV exposure time of 15 min were used for stanozolol.

3.2. Comparison of reaction products from TiO_2 photocatalysis and phase I metabolism in HLM

The main reaction types, hydroxylation and dehydrogenation and/or hydroxylation + dehydrogenation, were mostly the same in TiO_2 photocatalysis and in the phase I HLM metabolism reactions. Several same isomeric oxidation products were formed in HLM metabolism and TiO_2 photocatalytic reactions based on the ion chromatograms of protonated molecules recorded with 20 ppm mass window and 0.5% retention time window (Fig. 3,

Table A.1
Gradient for reaction products of testosterone used in optimization of photocatalytic reaction conditions. Eluent A was 95:5 water–100 mM ammonium acetate (pH 6.0, adjusted with acetic acid) and eluent B was 95:5 methanol–100 mM ammonium acetate (pH 6.0).

Time (min)	B (%)
0.00–2.50	50 → 100
2.50–4.00	100
4.00–4.10	100 → 50
4.10–6.50	50

Table A.2
Gradient for reaction products of methyltestosterone.

Time (min)	B (%)
0.00–9.00	30 → 45
9.00–15.00	45 → 50
15.00–19.00	50 → 100
19.00–21.00	100
21.00–21.10	100 → 30
21.10–23.20	30

Table A.3
Gradient for reaction products of nandrolone.

Time (min)	B (%)
0.00–10.00	25 → 40
10.00–20.00	40 → 100
20.00–22.00	100
22.00–22.10	100 → 25
22.10–24.50	25

Table A.4
Gradient for reaction products of testosterone.

Time (min)	B (%)
0.00–13.00	33 → 50
13.00–18.00	50 → 100
18.00–20.00	100
20.00–20.10	100 → 33
20.10–22.50	33

Table A.5
Gradient for reaction products of metandienone.

Time (min)	B (%)
0.00–7.50	30 → 40
7.50–15.00	40 → 45
15.00–20.00	45 → 100
20.00–22.00	100
22.00–22.10	100 → 30
22.10–24.50	30

Table A.6
Gradient for reaction products of stanozolol.

Time (min)	B (%)
0.00–4.00	30 → 58
4.00–11.00	58 → 64
11.00–15.00	64 → 100
15.00–17.00	100
17.00–17.10	100 → 30
17.10–19.50	30

Table A.7
Collision energies (CE) used in acquiring product ion spectra of each steroid and its reaction products.

Steroid	CE 1 (V)	CE 2 (V)
T	20	30
MT	20	25
MDN	15	20
NAN	20	25
STZ	42	47

Table A.8
Reaction products of methyltestosterone found in HLM or TiO_2 photocatalysis samples classified according to their signal intensity: s = strong, m = medium, w = weak.

Reaction	$[M+H]^+$	t_R (min)	HLM	TiO_2 photocatalysis
M	303.2324	17.85	s	s
M−2H dehydrogenation	301.2168	16.60	w	
		17.36		w
		17.46		m
		17.66	s	w
M+O hydroxylation	319.2273	7.71		m
		8.18		m
		8.39	w	w
		9.46	w	m
		10.53	s	w
		11.36	s	m
		12.12		m
		12.49		m
		12.65	m	
		13.56		w
		13.67	m	
		13.82	s	
		14.68	w	w
M+O−2H hydroxylation + dehydrogenation	317.2117	9.32	w	
		9.84	m	s
		10.67		m
		10.93	m	w
		12.44		w
		13.31	m	
		14.22	m	
M+2O dihydroxylation	335.2222	5.44	w	
		7.71	m	
		8.36	m	
M+2O−2H dihydroxylation + dehydrogenation	333.2066	5.50		m

Tables A.8–A.12). The identifications of matching products were confirmed by MS/MS. Most of the MS/MS spectra correlated well (Fig. 5), but five products (denoted with [a] in Tables A.9–A.12) with matching mass and retention time were found to be different isomers in HLM metabolism and TiO_2 photocatalytic reactions.

Approximately half of the M−2H, M+O, and M+O−2H HLM reaction products of all the steroids studied could be imitated by TiO_2 photocatalysis (Eq. (1), Table 2). However, there were also isomeric products only observed in either of the systems (Tables A.8–A.12), which can be attributed to the non-selectivity of the hydroxyl radical attack in TiO_2 photocatalysis. As a whole, the main phase I HLM metabolism reactions of methyltestosterone, nandrolone, testosterone, and stanozolol were imitated considerably better than those of metandienone.

$$\text{Coverage} = \frac{\text{number of the same reaction products in } TiO_2 \text{ photocatalysis and HLM reactions}}{\text{total number of metabolites produced by HLM}} \times 100\% \quad (1)$$

M−2H metabolites of structurally similar methyltestosterone, nandrolone, and testosterone were imitated well by TiO_2 photocatalysis. The main M−2H products of testosterone and nandrolone were the same in TiO_2 photocatalysis and HLM metabolism reactions. The main M−2H product of testosterone was identified as androstenedione by using a standard. Correspondingly, the main M−2H product of nandrolone is most likely formed by oxidation of 17β-hydroxyl to ketone. 17-keto metabolites are the main excreted metabolites of anabolic steroids having a secondary 17β-hydroxy group, such as testosterone and nandrolone (Schänzer, 1996). Obviously the 17α-methyl group of methyltestosterone, metandienone, and stanozolol inhibits their 17β-oxidation. An intense M−2H metabolite was observed in HLM metabolism reactions of methyltestosterone, metandienone, and stanozolol, that is probably due to formation of a double bond in the steroid skeleton instead of 17β-oxidation. The most obvious site of dehydrogenation is the C6–C7 bond, since in this case the increased degree of conjugation of double bonds stabilizes the product via π-electron delocalization. Dehydrogenation of the C6–C7 bond has also been observed after oral administration of methyltestosterone (Pozo et al., 2009b) and metandienone (Schänzer, 1996), but it has been suggested to form via a labile conjugate. A few M−2H products were formed in TiO_2 photocatalysis of methyltestosterone, metandienone, and stanozolol, which were not formed in HLM reactions.

The M+O metabolites of all the other steroids studied, except metandienone, could be imitated relatively well by TiO_2 photocatalysis. Enzymatic hydroxylation of the steroids is possible at several sites (Bullock et al., 1995; Choi et al., 2005; Masse et al., 1989; Parr et al., 2012; Pozo et al., 2009a; Schänzer et al., 1991). Seven hydroxylation products were observed in HLM metabolism of testosterone (Fig. 3). These include most likely the 1β-, 2β-,

Table A.9
Reaction products of nandrolone found in HLM or TiO_2 photocatalysis samples classified according to their signal intensity: s = strong, m = medium, w = weak.

Reaction	$[M+H]^+$	t_R (min)	HLM	TiO_2 photocatalysis
M	275.2011	15.00	s	s
M−2H	273.1855	14.00	s	s
dehydrogenation		14.51		m
		14.58		m
		14.74	s	w
M+O	291.1960	6.78		w
hydroxylation		7.79	m	w
		8.44	s	m
		9.39	s	w
		9.69	s	w
		10.56	s	
		10.99		w
		11.35		w
		11.78		w
		12.07	w	m
		12.22	s	w
		12.74	s	w[a]
M+O−2H	289.1804	7.34	m	
hydroxylation + dehydrogenation		7.92	m	
		8.44	w	
		8.92		m
		9.08	s	
		9.20		m
		9.44	m	
		9.77	s	s
		10.11	s	
		10.56	s	
		11.64		m
M+2O	307.1909	3.43	w	
dihydroxylation		3.81	w	
		3.91	w	
		4.64	w	
		4.87	w	
		6.20	m	
		6.75	w	

[a] The MS/MS spectra do not correlate well enough and the products are most likely different isomers in HLM and TiO_2 photocatalytic reactions.

6β-, 11β-, 15β-, and 16β-hydroxytestosterones previously found in HLM reactions, of which the 6β-hydroxylation product is the dominant metabolite (Choi et al., 2005; Krauser et al., 2004; Yamazaki and Shimada, 1997). Also minor 1α-, 2α-, 6α-, 7α-, 16α-, and 18/12α-hydroxylation products of testosterone have been found in HLM incubations (Bullock et al., 1995). The metabolism of the other steroids used in this study has mainly been examined using human subjects (Masse et al., 1989; Poelmans et al., 2002; Pozo et al., 2009a), various animal species (Biddle et al., 2009; Blokland et al., 2005; Roig et al., 2007), or *in vitro* using liver preparations from different animals (Bullock et al., 1995; Scarth et al., 2010). Because of species differences in metabolism and differences between excreted metabolites and other metabolites formed in the body, these former studies cannot be directly compared to our in-house HLM results.

Several M+O−2H metabolites were formed both in HLM and TiO_2 reactions. The M+O−2H metabolites of metandienone, methyltestosterone, stanozolol, and testosterone were imitated better by TiO_2 photocatalysis than those of nandrolone (Table 2). Generally, the M+O−2H metabolites were not imitated as well as M−2H and M+O metabolites. The combination of two reactions can produce more possible isomers and the probability that the same isomers are formed in both systems is lower. In addition to M−2H, M+O, and M+O−2H products, dihydroxylation products (M+2O) were formed mainly in HLM reactions and products formed by dihydroxylation + dehydrogenation (M+2O−2H) were favored by TiO_2 photocatalytic reactions. This implies that the oxidation proceeds further in TiO_2 photocatalysis. In addition, a few other minor oxidation products were observed in TiO_2 photocatalysis (Table A.12).

4. Conclusions

TiO_2 photocatalysis is a fast, simple, and inexpensive oxidation method. Here, we have shown that TiO_2 photocatalysis is a suitable method for oxidation of relatively non-polar compounds, and acetonitrile can be used as solvent for hydrophobic compounds and to slow down the degradation of formed products. Radical reaction enables aliphatic hydroxylation in several positions, which is an essential reaction for imitation of phase I metabolism of anabolic steroids, and a number of important metabolism reaction products can be produced by TiO_2 photocatalysis. Therefore, TiO_2 photocatalysis appears to be a possible method for rapid and low-cost production of metabolite standards, if the reaction is scaled-up and the conditions optimized for better yield.

Even though the main reaction types were the same in TiO_2 photocatalysis and in HLM phase I metabolism, isomeric products can be different and products characteristic to only either one of the systems are also formed. Thus, TiO_2 photocatalysis cannot fully imitate phase I metabolism, which is understandable when comparing a relatively non-selective oxidation method with inherently highly selective enzymatic reactions. Nonetheless, in earlier studies, adequate imitation of the whole range of phase I oxidation reactions has not been achieved with metalloporphyrins, Fenton reaction or electrochemical methods either, and the performance of TiO_2 photocatalysis for imitation of phase I metabolism is comparable to these methods. Most importantly, TiO_2 photocatalysis provides a straightforward method for imitation of phase I metabolism reactions under standard laboratory conditions without need of special equipment, such as electrochemical cells. This study also highlights the importance of optimization of chromatographic

Table A.10
Reaction products of testosterone found in HLM or TiO_2 photocatalysis samples classified according to their signal intensity: s = strong, m = medium, w = weak.

Reaction	$[M+H]^+$	t_R (min)	HLM	TiO_2 photocatalysis
M	289.2168	16.03	s	s
M−2H dehydrogenation	287.2011	15.05	s	s
		15.59		w
		15.75	m	
M+O hydroxylation	305.2117	6.10		w
		7.60	m	m
		8.21		m
		9.45	s	
		10.49	m	w
		11.33		m
		11.91	w	m[a]
		12.20	m	
		12.45		w
		12.66	w	w
		13.27	m	
M+O−2H hydroxylation + dehydrogenation	303.1960	7.47	w	
		7.57	w	
		8.27		m
		8.98		w
		9.16	m	
		9.62	m	m
		10.11		w
		10.56	m	
		10.72		m
		11.31		m
		12.79	w	
M+2O−2H dihydroxylation + dehydrogenation	319.1909	4.29		m
M−4H 2x dehydrogenation	285.1855	14.48	m	

[a] The MS/MS spectra do not correlate well enough and the products are most likely different isomers in HLM and TiO_2 photocatalytic reactions.

Table A.11
Reaction products of metandienone found in HLM or TiO_2 photocatalysis samples classified according to their signal intensity: s = strong, m = medium, w = weak.

Reaction	$[M+H]^+$	t_R (min)	HLM	TiO_2 photocatalysis
M	301.2168	17.86	s	s
M−2H dehydrogenation	299.2011	17.16		w
		17.30		w
		17.87	s	
M+O hydroxylation	317.2117	6.89		m
		7.05	w	
		7.72	m	m
		9.10	s	
		9.82	s	
		10.64		w
		11.92		m
		12.15	s	
		13.55	w	
		16.58		w
M+O−2H hydroxylation + dehydrogenation	315.1960	8.25	m	m
		8.77		s
		8.94	w	
		9.89	w	m
		10.99	w	w[a]
		11.25		w
		12.45	m	
M+2O dihydroxylation	333.2066	3.95	w	
		4.64	w	
		6.60	m	
M+2O−2H dihydroxylation + dehydrogenation	331.1909	5.57		w

[a] The MS/MS spectra do not correlate well enough and the products are most likely different isomers in HLM and TiO_2 photocatalytic reactions.

Table A.12
Reaction products of stanozolol found in HLM or TiO_2 photocatalysis samples classified according to their signal intensity: s = strong, m = medium, w = weak.

Reaction	$[M+H]^+$	t_R (min)	HLM	TiO_2 photocatalysis
M	329.2593	13.66	s	s
M−2H dehydrogenation	327.2436	12.72		m
		13.11		m
		13.45	m	
M+O hydroxylation	345.2542	5.55		s
		5.67	m	s
		5.79	m	s
		6.20		s
		6.81		m
		6.89	w	s
		7.03		m
		7.33	w	s
		7.48	s	
		8.45	s	s
		8.75		w
		9.11	s	
		9.42		m
		10.30	s	
		10.50	s	w
M+O−2H hydroxylation + dehydrogenation	343.2386	5.80		s
		6.28		m
		6.40		m
		6.85		m
		7.03		s
		7.17		s
		7.58	s	s
		7.90	s	
		8.55		s
		8.94	m	s[a]
M+2O dihydroxylation	361.2491	3.40	m	
		3.61	w	
		4.04		w
		4.65	m	
		4.75		w
		4.91	s	
		5.33		w
		5.46	s	
		5.94		w
		6.12		w
		6.18	s	
		6.32	m	
		6.77	m	
		7.03	m	
		7.46		w
M+2O−2H dihydroxylation + dehydrogenation	359.2335	4.17	m	
		4.40		m
		4.70	s	s[a]
		4.86	w	
		4.90		w
		5.10		m
		5.15	m	
		5.41	w	w
		5.59	m	
		5.79	s	
		6.30	m	
		7.73	w	
M+3O−2H trihydroxylation + dehydrogenation	375.2284	3.39		m
		3.60		m
M−CH_4 demethylation	313.2280	11.07		m
M+O−CH_4 hydroxylation + demethylation	329.2229	5.77		m
		6.14		m
		6.85		w

[a] The MS/MS spectra do not correlate well enough and the products are most likely different isomers in HLM and TiO_2 photocatalytic reactions.

conditions for separation of isomeric oxidation products in order to get more reliable information on the feasibility of oxidative chemical reactions for mimicking metabolic reactions.

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Appendix A

See Tables A.1–A.12.

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