European Journal of Pharmaceutical Sciences 65 (2014) 45–55

Contents lists available at ScienceDirect

European Journal of Pharmaceutical Sciences

journal homepage: www.elsevier.com/locate/ejps

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

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ARTICLE INFO

Article history:
Received 17 April 2014
Received in revised form 15 August 2014
Accepted 19 August 2014
Available online 9 September 2014

Keywords:
Titanium dioxide photocatalysis
Oxidation
Steroids
Biomimetics
Metabolism
Mass spectrometry

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.

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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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http://dx.doi.org/10.1016/j.ejps.2014.08.009
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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

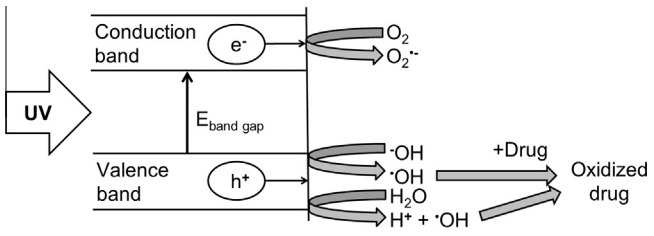

Fig. 1. The principle of TiO_2 photocatalysis. Modified from (Bhatkhande et al., 2002). See Fujishima et al. (2008) and Zhang et al. (2012) for more detailed descriptions of the TiO_2 photocatalysis mechanism.

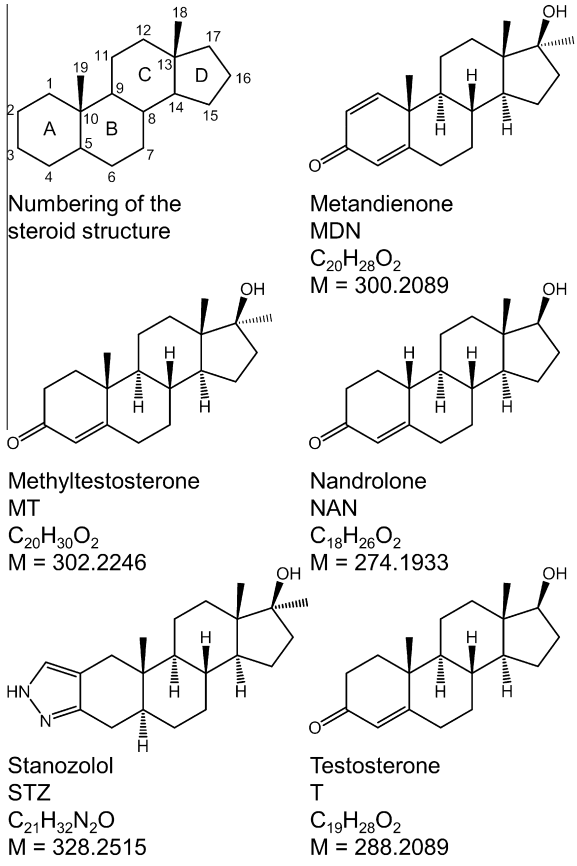

Fig. 2. Molecular structures, names, abbreviations, molecular formulas and monoisotopic masses of the test compounds used in this study. Top-left figure represents the numbering of the different carbon atoms in the steroid structure.

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