Photocatalysis Combined with Chromatographic Methods as a New Promising Tool in Drug Metabolism Studies – A Review

M. Gawlik, R. Skibiński*, J. Trawiński and Ł. Komsta

Department of Medicinal Chemistry, Pharmaceutical Faculty, Medical University of Lublin, Jaczewskiego 4, 20-090 Lublin, Poland

Received: 15 December 2016; accepted: 16 December 2016

The number of new drugs launched to the market is constantly increasing; however, the metabolism of many of them is still not fully established. The knowledge of drug metabolism pathways is crucial for the efficacy and safety of therapies and, in classical approach, requires the use of animals as well as human volunteers, but this kind of research is expensive and time-consuming. Therefore, nowadays, more and more biological and chemical in vitro methods are developed for the drug metabolism study. This review is focused on the photocatalytic degradation of chemicals and the application of this process in chromatographic methods of drug metabolism research. A theoretical background of photocatalysis and all its applications in a drug metabolism study were reviewed, and other in vitro methods that are actually used were summarized and discussed. Other analytical methods used in this area were also discussed and compared.

Keywords: Titanium dioxide, Photocatalysis, Photooxidation, Drug metabolites, LC–MS

Introduction

The medical progress nowadays allows to treat previously incurable diseases and also to use advanced therapies concerning previously known diseases. The measure of this development is, among many others, a growing number of new drugs entering the market. The research on new medicines is expensive, time-consuming, and a laborious process, which require significant human resources. In this area, seeking to develop more effective methods of pharmacotherapy makes it necessary to obtain detailed information about the fate of the substance, occurring when it is introduced into the human body, as a xenobiotic (a molecule foreign to it). This multistep process is performed to determine the pharmacokinetic LADME parameters (liberation, absorption, distribution, metabolism, and excretion). One of the many important issues associated with the creation of a new molecular entity is to investigate the pathways of the metabolism. The vast majority of drugs are metabolized commonly in the liver with the participation of P450 cytochrome isoenzymes. Biochemical conversions related to metabolism are divided into two major types of reactions, which are largely responsible for the formation of potentially toxic intermediates. The phase I biotransformations are decomposition processes based also on the introduction of polar groups (such as hydroxyl group) into the drug structure. Besides the redox processes, other reactions (as substitution, hydrolysis, and elimination) also take place. The phase I reactions may lead to the formation of free radicals, which, due to an unpaired electron, are highly reactive and can form irreversible connections with tissue nucleophiles. This process is especially dangerous because of the possible oxidization of cell membranes, which can lead to, e.g., cancerous process, as a result of cell genome damage. During the second phase, the intermediates obtained earlier undergo coupling with endogenous cofactors, such as sulfuric, glucuronic, or amino acids. Despite the fact that the majority of the toxic metabolites are formed in the first transition phase, there are also toxicity cases after the second phase, e.g., when coupled intermediates hydrolyze in tissue-specific pH to give local toxic effects. A combination of compounds formed is usually ionized at physiological pH, which facilitates the further elimination from the organism: this process is sometimes called "Phase III." The presence of toxic intermediates became the reason for which, during the drug discovery process, the metabolism of each drug candidate must be evaluated. Therefore, one of the most important things before introducing the drug candidate into market is to check, in the early stages of research, whether the drug forms possible toxic metabolites. The formation of reactive intermediates is the reason of adverse reactions and repeatedly was the reason to withdraw the drugs from the market (e.g., ticrynafen, buformin) [1–6].

One of the most widely reported cases of occurrence of toxic metabolites, based on biotransformation with cytochrome P450 enzymes, is acetaminophen (Figure 1). It is well known that the metabolism of this drug produces *N*-acetyl-*p*-benzoquinone imine (NAPQI), a toxic metabolite, which is a very reactive electrophile, inactivated with conjugation with glutathione (GSH). Depletion of glutathione reserves causes exhaustion of metabolic pathways and is the main reason for appearance of strong, life-threatening poisoning [7, 8].

A lot of studies with diclofenac revealed link between drug metabolism and toxicity to hepatocytes. This drug has two major biotransformation pathways catalyzed by cytochrome P450 enzymes, leading to the formation of the 4-hydroxydiclofenac and 5-hydroxydiclofenac. Both metabolites are further oxidized to *p*-benzoquinone imines, which are reactive electrophiles (Figure 2). Quinone imines are implicated in causing oxidative stress and in affecting of proteins functions due to covalent binding with their sulfhydryl groups. These metabolites play significant role in hepatotoxic activity of diclofenac [9, 10].

Another drug, the toxicity of which is caused by radicals, is valproic acid. This substance is used in epilepsy therapy and requires special precautions for use in young children. Hepatotoxicity (due to mitochondrial damage and impairment of fatty acid β-oxidation) is a consequence of 2-propyl-4-pentenoic

* Author for correspondence: robert.skibinski@umlub.pl

DOI: 10.1556/1326.2016.00202

First published online: 08 March 2017

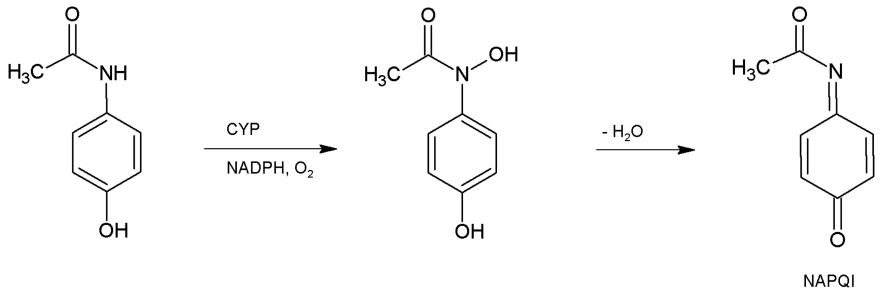

Figure 1. Biotransformation pathway of acetaminophen (paracetamol) [8]

acid formation. This unsaturated derivative is a strong reactive entity (Figure 3) [11].

Dangerous hepatotoxicity occurs also as a succession of halothane metabolism. Despite the fact that 60–80% of the drug is excreted unchanged in 24 h, in some conditions of low oxygenation, the reductive pathway of biotransformation is becoming preferred. That results in 1-chloro-2,2,2-trifluoroethyl radical creation, which has a strong affinity to biological membranes and may cause dangerous hepatocytes necrosis (Figure 4) [12].

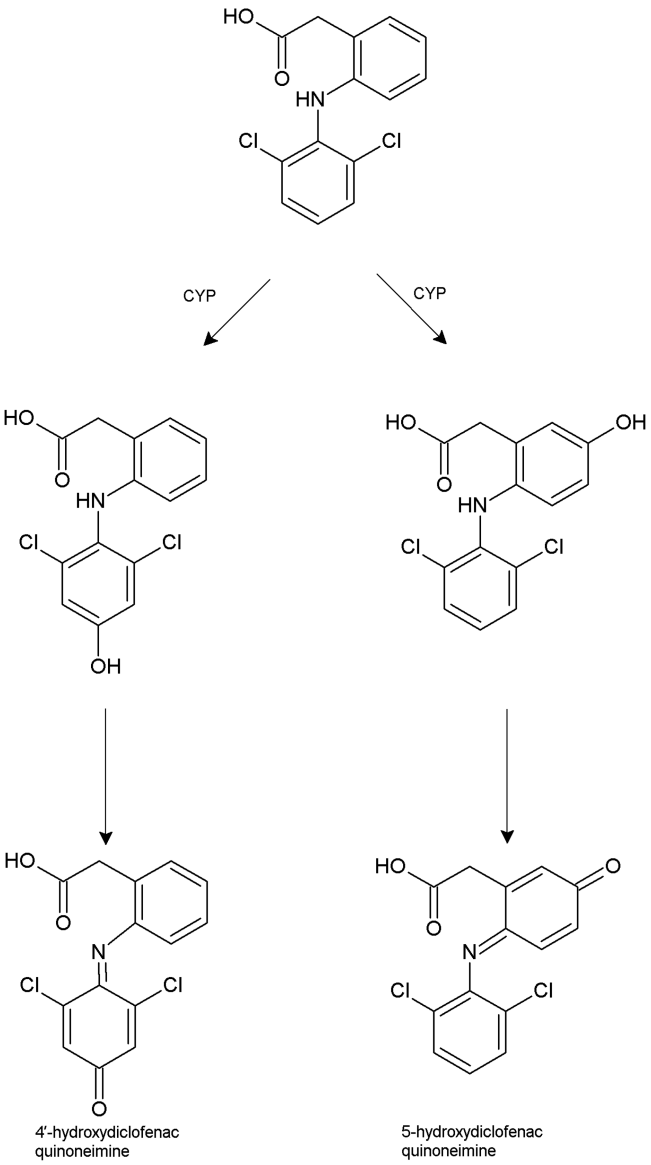

Figure 2. Biotransformation pathway of diclofenac [9]

Additionally, some prodrugs acquire medicinal properties after biochemical conversions occurring in the liver, and toxic compounds may also be formed during these processes. Cyclophosphamide, a drug used in cancer treatment, is being metabolized to active compounds and the acrolein, which is responsible for the urotoxicity of this drug (Figure 5). Ifosfamide, which is a structural isomer of cyclophosphamide, is also a prodrug and requires biotransformation to become cytotoxic. Acrolein is formed similarly during the biotransformation, as in the case of cyclophosphamide [13, 14].

As it was mentioned earlier, drugs form toxic metabolites usually in phase I of metabolism. From this point of view, it is very important to dispose methods allowing quick and easy analysis of the metabolism of a test substance. In this context, the in vitro methods, such as electrochemistry (EC), metalloporphyrins, or human liver microsomes (HLM), appear particularly promising. One of the newest in vitro method mentioned in the literature for this kind of research is the induction of photocatalytic transformation of drugs with the use of titanium dioxide (TiO_2). The achieved results indicate that identified degradation products are the same as obtained in in vivo metabolism.

Among aforementioned methods of organic compounds degradation, application of the TiO_2 is especially popular in the environmental analysis. The achieved results indicate the presence of the same degradation products as obtained in vivo [15].

This review focuses on the challenges associated with the study of drug metabolism with particular reference to the method based on photocatalytic degradation of drugs.

Methods Used in Drug Metabolism Studies

Studies of drug metabolism in vivo are one of the most important LADME elements. However, they consist of a plurality of phases and a number of factors must be taken into

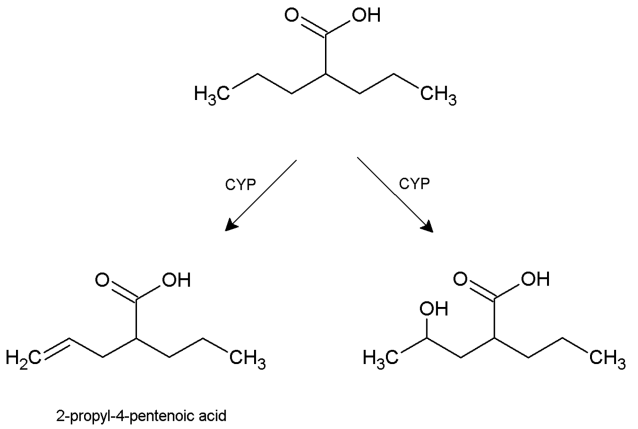

Figure 3. Biotransformation of valproic acid to 2-propyl-4-pentenoic acid [11]

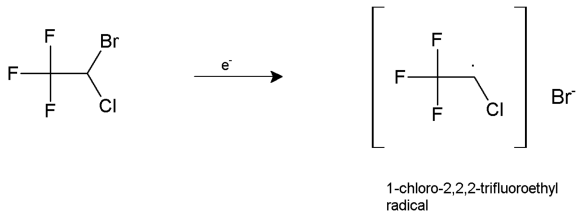

Figure 4. Reductive pathway of halothane [11]

account. Pharmaceutical clinical trials usually comprise of four stages. During the first three steps, a basic information about possible toxic reactions and drug dosage is evaluated. After the final confirmation of the efficacy of the tested substance, a drug can be registered and marketed. The phase IV research is also currently performed to verify the results obtained in the previous stages and the possible detection of new therapeutic indications, as well as side effects.

Due to the fact that human studies are time-consuming, expensive, and intensive, an effort was made to develop methods mimicking the metabolic phase I. These aspirations resulted in the acquisition of several in vitro methods.

As has been mentioned previously, the biotransformation of drugs, in general, depends on cytochrome P450 activity. Isoenzymes belong to the heme proteins superfamily, which are present in almost all mammalian cell types. They are found mainly in the microsomes of the endoplasmic reticulum and mitochondria membrane structures. A particularly large number of cytochrome P450 isoenzymes were found in cells of kidneys, lungs, heart, and skin, but the greatest number of cells is present in the liver. Among many functions performed by the P450s, one of the most important is the drug biotransformation ability. Numerous studies have shown that the most active is CYP3A4 isoenzyme of P450 cytochrome, which metabolizes approximately 50% of all drugs [16].

These discoveries led to several in vitro human liver models, such as supersomes, human liver cytosole, S9 fractions, cell lines, hepatocytes, liver slices, and isolated perfused liver, but the human liver microsomes (HLM) have become the most frequently used model in drug metabolism studies. These subcellular fragments are obtained from centrifugation of homogenizated liver and are used in phase I metabolism studies with NADP as a cofactor. The results obtained using pooled HLM are more reliable than using hepatocytes. This is due to the use of hepatocytes from different donors, which contributes to decrease of the reproducibility of test results. Correlation of metabolism of the drug with gender can also be evaluated with gender-specific human liver microsomal pools. This simple, low-cost (and easy to use) method had also some weakness, e.g., influence of pH of reaction mixture and effect of organic solvent that can disrupt the process. It can mimic biotransformation reactions such as hydroxylation, hydroxylation with demethylation, *N*-demethylation, and sulfoxidation [17–24]. The results achieved in HLM tests closely correspond to the results obtained by in vivo methods, which is the reason of a frequent use of the method in the context of newly introduced substances for the pharmaceutical market [25].

Rat liver microsomes (RLMs) are also used in drug metabolism studies. The methodology and schedule of research are exactly the same as in the case at HLM's. In some cases, it was shown that the RLMs give similar metabolite profiles and it becomes a suitable method to mimic the phase I; but on the contrary, some results indicate the presence of additional metabolites unachievable using these microsomes [25, 26].

Further exploration of suitable methods for drug metabolism studies resulted in the use of metalloporphyrins. These compounds are able to mimic the action of the active centers of cytochrome. Metalloporphyrins are a group of heterocyclic macrocycle organic compounds, conjugated with metal ligand usually on the second or third stage of oxidation. First, compounds used in this method were chemically unstable and allowed to perform only a few catalyzed reaction. Obtaining metalloporphyrins more resistant to disintegration allowed to improve the efficiency of the catalytic process significantly. Iron and manganese complexes are the most commonly used compounds in this method [27]. The Fe/meso-tetrakis(2-nitrophenyl)-octachloroporphyrin dianion/iodosylbenzene system used with albendazol degradation resulted strictly in the same metabolites, as those obtained in in vivo studies [28]. The

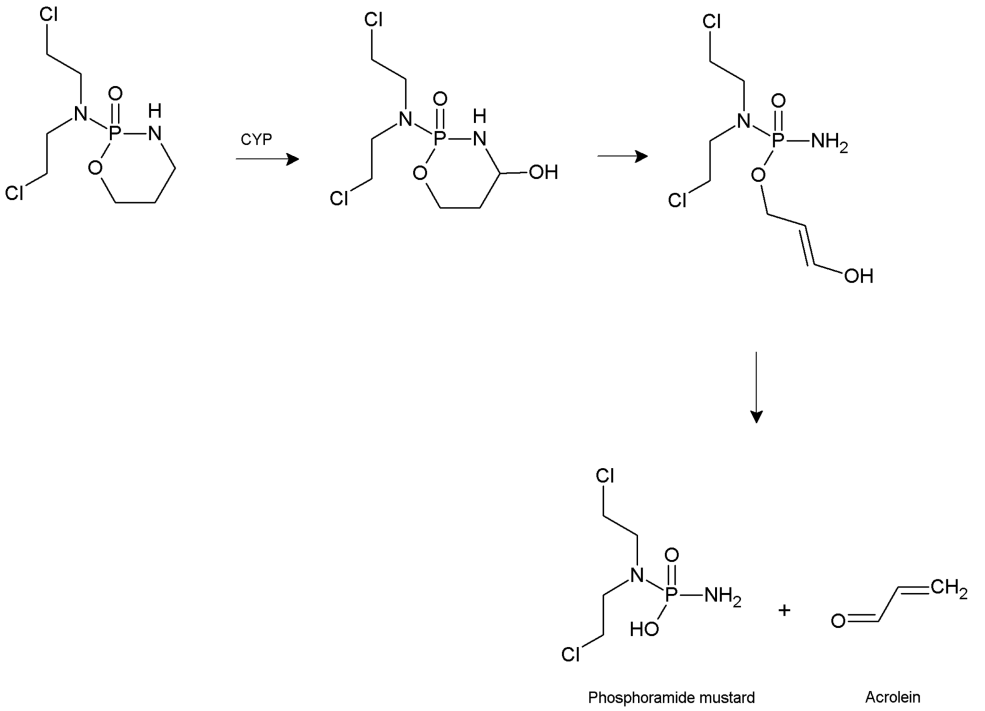

Figure 5. Biotransformations of cyclophosphamide [13]

interesting fact is that the water-soluble Mn/meso-tetrakis(4-*N*-methylpyridinumyl)porphyrin tetracation complex was able to mimic acetaminophen degradation to quinone-imines (NAPQI) in the presence of potassium monopersulfate ($KHSO_5$). Another widely used drug, diclofenac, was examined with Mn or Fe/meso-tetrakis(2,6-dichlorophenyl)porphyrin dianion system with the presence of hydrogen peroxide (H_2O_2) or tert-butyl hydroperoxide (*t*-BuOOH) as a oxygen surrogate. Obtained results indicated a 5-hydroxylated metabolite, whereas the confirmed cytochrome P450 (CYP2C9) human metabolism with releases specific 5-hydroxylated products [29].

This method has significant disadvantages including possible overoxidation of the tested substance. It also requires the selection of a suitable metalloporphyrin (an oxygen donor) which depends on the chemical properties of drug. It should also be noted that the metalloporphyrins could catalyze hydroxylation of some compounds regioselectively [30–32].

The next method, widely used in vitro to the study of drug metabolism, is the electrochemical method (EC). Its main aim is mimicking biochemical processes of drugs decomposition in a controlled manner with the use of various types of electrochemical cells: coulometric flow-through, amperometric thin-layer, or in-source electrochemical cells. It makes it possible to imitate the chemical reactions initiated by the removal of a single electron from a substrate, such as dehydrogenation, *N*-dealkylation, *S*- and *P*-oxidation, and oxidation of alcohols to aldehydes, and allows mimicking only oxidation pathways of metabolism processes. Electrochemistry helps also to predict the group which is most susceptible to oxidation in the test compound, and this corresponds closely to the results obtained in vivo [33]. The electrochemical oxidation is most often performed on a glassy carbon (GC) boron doped diamond, gold, or platinum working electrode supported with potentiostat, reference, and counter electrode [34, 35].

Direct coupling of electrochemical set with mass spectrometry (MS) is one of the most often used technique in this type of studies. The results are obtained in the form of mass voltammograms, providing only basic information about oxidative labile sites of a drug. Unfortunately, the method fails when isomeric degradation products of the test drug are formed. Then, it is necessary to obtain the separation of metabolites using liquid chromatography [35–37]. On the other hand, the method allows the direct detection of reactive metabolites when coupled with liquid chromatography/electrospray ionization–mass spectrometry (LC/ESI–MS). Moreover, it also enables direct phase II studies, where glucuronic or sulfuric adducts are formed. It was shown that this methodology was able to detect reactive *o*-quinone imide metabolite products of troglitazone electrochemical oxidation, which resulted in its withdrawal from the market [38]. Aside from some drawbacks, electrochemistry method seems to be an adequate technique to study drug metabolism on a small, laboratory scale.

Another method to mimic the phase I of drug metabolism is Fenton reaction (1–4). This method utilizes the oxidation reaction in an aqueous solution of iron salt (Fe^{2+}) in the presence of the hydrogen peroxide (H_2O_2), leading to oxidation of the iron ions to the third stage of oxidation (Fe^{3+}) and the formation of the hydroxyl radical ($^{\bullet}OH$), which has strong oxidizing properties because of the unpaired electron. The active iron ions (Fe^{2+}) can be regenerated by using a chemical reductive compounds or electrochemically with working electrode, EC-Fenton [39, 40]. This method was able to simulate some metabolic reactions during process, e.g., aliphatic and aromatic hydroxylation; *S*-, *O*-, and *N*-dealkylation; and hydrogenation. The main disadvantage is the need to maintain a pH below 3, preventing precipitation of iron hydroxides. It should be also noted that Fenton reaction is nonselective, so the results may not correspond with the results obtained in vivo [41].

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\cdot} \quad (1)$$

$$Fe^{3+} + H_2O_2 \leftrightarrow \left[Fe^{3+}(HO_2)\right]^{2+} + H \quad (2)$$

$$\left[Fe^{3+}(HO_2)\right]^{2+} \rightarrow HO_2^{\cdot} + Fe^{2+} \quad (3)$$

$$Fe^{3+} + HO_2^{\cdot} \rightarrow Fe^{2+} + O_2 + H^+ \quad (4)$$

Completely different procedures that allow early prediction of metabolic threats are computer calculation methods. From economical point of view, earlier detection of abnormalities lowers investment costs, thus, predicting that the possible toxicity and stability of metabolites begin at the stage of designing molecules and require the cooperation of expert teams in various fields. Due to constantly increasing computing power, the available computers become an integral, suitable tool for setting up early experimental screens to evaluate metabolic pathways of drugs. These methods, called in silico, give hope to the preliminary assessment of metabolism before the creation of new molecular entity. The fact that the toxicity of the molecule is the result of its functional groups and determines its chemical nature became the basis for toxic compounds databases establishment [42]. The data are mathematical relationships between chemical structure and activity with metabolic properties in a quantitative manner (QSAR). Various commercially available software resources have been also applied for predicting the metabolic fate of xenobiotics, e.g., METEOR, MetabolExpert, or MEXAlert.

Ekins et al. compared the results of aprepitant, trovofloxacin, 4-hydroxytamoxifen, and artemisinin metabolism obtained with the use of MetaDrug-MetaCore software with the results obtained in vivo previously described in the literature. This software has been set with approximately 70 human metabolic reaction rules, and as it was shown, it has proved as a suitable tool to give similar decomposition pathways to literature metabolic information for humans [43].

Computational methods are also used in prevention of drugs interactions, where one drug has strong inhibitory activity of CYP enzymes. It was shown that concomitant use of statins and drugs demonstrating inhibition of cytochrome enzymes causes rhabdomyolysis [44]. Obtained information about the metabolites can be also achieved and made available on the network, such as METLIN database.

Photocatalytic Degradation of Drugs

Because of the several disadvantages of the abovementioned methods, continuous efforts were made in the search for new in vitro methods to simulate the phase I of metabolism reactions. Due to the ability of the semiconductor materials to the formation of active oxygen species (AOS) in the heterogeneous catalysis process, they have been applied to simulate the metabolic processes. The selected compound should have several attributes, of which the most important are chemical inertness, photostability, and photoreactivity. Substance should be also easy to prepare and inexpensive. A chemical compound with all of those qualities and used in the studies of drug metabolism is titanium dioxide (TiO_2), which

is an n-type semiconductor [45–49]. TiO_2 has several crystal structures, including anatase and rutile.

A significant feature of semiconductor is the energy gap. This is the range of energy values prohibited for electrons in the crystal lattice of a solid. This parameter is essential for conductive properties of the substance. According to the Planck equation, energy is inversely proportional to the wavelength:

$$E = h\nu = h\lambda^{-1}c, \tag{5}$$

which means that the excitation of the semiconductor with a smaller band gap requires an electromagnetic radiation with higher wavelength.

In the case of TiO_2, the energy gap between the valence band and conduction band lies at about 3.2 eV for anatase and 3.0 eV for rutile, which correspond directly to 385 and 410 nm wavelength [50]. Semiconductor property plays crucial role in its photocatalytic abilities. When TiO_2 absorbs adequate photon energy from ultraviolet radiation (<400 nm), the electrons are excited from its valence band to the conduction band leaving holes on valence band which allows titanium dioxide to participate in chemical reactions as a catalyst (Figure 6) [51]. Generated electrons and holes can recombine, or they can migrate to the surface. In this excited state, titanium dioxide acts as catalyst for many reactions, and in aqueous solutions, reactive radicals are formed (6–10).

$$TiO_2 + h\nu(\mathrm{UV}) \rightarrow TiO_2\ (e_{CB}^- + h_{VB}^+) \tag{6}$$

$$TiO_2\ (h_{VB}^+) + H_2O \rightarrow TiO_2 + H^+ + OH^{\cdot} \tag{7}$$

$$TiO_2\ (h_{VB}^+) + OH^- \rightarrow TiO_2 + OH^{\cdot} \tag{8}$$

$$TiO_2\ (e_{CB}^-) + O_2 \rightarrow TiO_2 + O_2^{\cdot -} \tag{9}$$

$$O_2^{\cdot -} + H^+ \rightarrow HO_2^{\cdot} \tag{10}$$

Hydroxyl radical ($^{\bullet}OH$), the oxidation product of main photocatalytic process, is formed with positively charged holes. Photoproduced electrons can recombine with oxygen from reaction environment and produce superoxide anion radical ($O_2^{\bullet -}$). Hydroxyl radical is an oxidizing agent, and its unpaired electron determines its strong reactivity. Hydrogen peroxide, molecular hydrogen, and singlet oxygen are also formed in process. It shows that TiO_2 can catalyze both oxidation and reduction reactions. Degradation products are formed in the presence of radicals or directly with organic compounds by electron transfer [52, 53]. McLintock and Ritchie's [54] study is one of the first to prove that the photocatalytic process with TiO_2 could oxidize organic compounds completely to CO_2 and H_2O. It was demonstrated that TiO_2 Degussa P25 is the most photoreactive comparison to TiO_2 catalysts due to its crystal composition consisting of 75% anatase and 25% rutile and with particle size of 20 nm and surface area of 50 $m^2\ g^{-1}$. Inhibition of the recombination process is the result of rutile presence: its crystal structure absorbs photons, while electrons promoted to the conduction band are transferred to the anatase crystal structure. In comparison to the Degussa P25, other catalysts were characterized by lower catalytic activity, which was probably the reason of 100% anatase structure [55, 56]. However, the brookite structure has also been studied as a photocatalyst and it has shown practically the same activity as compared with Degussa P25 [57].

It should be noticed also that the TiO_2 is an often used photocatalytic agent in environmental research due to its ability to remove organic contaminants and pesticides [58–60].

Imitation of Phase I Metabolism by the Use of Photocatalysis

Nowadays, photocatalysis using TiO_2 becomes more and more willing method for testing drug metabolism in vitro, which is reflected in the increasing number of scientific papers taking this topic.

In one of the first publications describing this method, the feasibility of TiO_2 photocatalysis to degradate of dexamethasone was tested. Calza et al. [61] applied Degussa P25 TiO_2 and aqueous/methanol medium, using irradiation with ultraviolet A (UVA) source. The use of two different solvents had an effect on the kinetics of dexamethasone, which proceeded much faster in pure water. The addition of methanol significantly reduced the rate of reaction, which is linked with hydroxyl radical scavenging activity of methanol. The authors created the assumption that the transformations of dexamethasone proceed

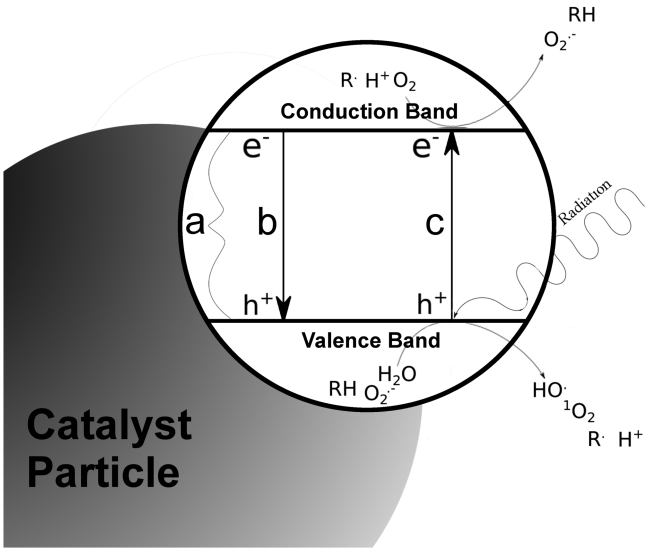

Figure 6. Mechanism of photocatalysis. Band gap (a); electron–hole pair recombination (b); electron excitation (c)

in two paths during the single oxidation step with one hydroxyl radical and a dual attack of hydroxyl radicals directed to quinoid moiety of the drug. Despite the fact that TiO_2 can catalyze both oxidative and reductive reactions, only the oxidated derivatives were obtained.

The objective of the study was to compare the metabolites generated artificially by imitating the conditions of the drug transformations (taking place in living organisms) with the results really present and described in the literature. From the content of the publication, one can find out that the goal of the study was achieved. The main metabolite, already found in liver studies earlier, 6-hydroxydexamethasone, was identified during experiments. The authors also payed attention on the dominant role in the hydroxylation process during the photocatalytic process and suggest the ability to detect other hydroxylated derivatives in future studies. The whole study was performed on liquid chromatography system coupled with ion trap (IT) MS.

Calza et al. [45] also studied the feasibility of TiO_2 to generate artificial metabolites of buspirone. The same catalyst, Degussa P25 TiO_2, was used; however, irradiation was performed with the use of xenon lamp coupled with 340 nm cutoff filter. After 30 min of irradiation, the sample was analyzed by an HPLC–IT mass spectrometer and an MS^2 experiments were performed. The obtained results were compared with several metabolic studies of buspirone, performed earlier in vivo on rats and horses. As it was shown, four hydroxyl derivates of buspirone and two dihydroxy derivates, as well as 1-pirymidinyl piperazine and despirymidinyl buspirone, both in in vivo and photocatalytic studies, were detected. The results coincided with the in vivo studies in rats in eight cases, while in the case of in vivo on horses, only three metabolites were the same. This notices also that some transformation products of buspirone, as despyrimidinyl buspirone-amidine, despyrimidinyl-hydroxy-buspirone-amidine, and oxo-buspirone derivate, were only found by the use of photocatalytic method.

Medana et al. [47] also attempted to compare synephrine transformations in vivo with results obtained by simulated metabolism by TiO_2 photocatalysis. It should be also noticed that the octopamine, which is the *N*-demethylated derivative of synephrine, is a prohibited substance in antidoping control. It turns out that many supplements for athletes contain synephrine; therefore, it is important to investigate the metabolism pathways of that substance since the presence of metabolites are a proof of prohibited chemical compound administration. For this purpose, human volunteers and rats were administered with synephrine dose. Photocatalytic metabolism simulation was performed in ultrapure water with the use of UVA irradiation source (60 min). All analyses were performed by HPLC–MS high-resolution mass spectrometry (Orbitrap) system.

In this study, seven new transformation products of synephrine and three products of octopamine were identified. The main pathways of synephrine that occurred during the process were: hydroxylation with aromatic ring opening, hydroxylation with phenolic group oxidation, aromatic ring loss with hydroxylation and, especially relevant for the potential risk of octopamine forming, *N*-demethylation. Similar metabolic pathways (aromatic ring loss with hydroxylation, hydroxylation with phenolic group oxidation, and simple hydroxylation) were observed for octopamine. The results obtained in TiO_2 photocatalytic process correspond also to the tests described previously in the literature, which mention only about hydroxy derivatives and the phase II conjugates of synephrine.

The same study also made an attempt to identify metabolic structures of sildenafil in vivo and with the photocatalytic process utilizing TiO_2. The authors pointed out that the detection of metabolites in samples of blood or urine of horses is a sign of the use of prohibited by the antidoping control compounds and, thus, emphasizes the importance of the knowledge about the biotransformation pathways of xenobiotics in living organisms. For this purpose, urine and plasma samples from horses after administration of sildenafil were analyzed. Photocatalytic degradation of sildenafil in ultrapure water (with the use of Degussa P25 TiO_2) was used as a reference method. The irradiation was performed using UVA during 10 min. A similar analytical system (HPLC-Orbitrap MS), which has been described in previous study, was used here. In this research, nine intermediates products of sildenafil were identified, four of which were already detected in previous studies. During the photocatalytic transformation, the main routes of metabolism reactions were proven, including hydroxylation, demethylation, de-ethylation, and combination of those, which allowed explanation of the metabolites structures [46].

A slightly different approach with the use of TiO_2 was proposed by team of Nissilä et al. [48]. They noticed the need for a new, rapid, and inexpensive method of testing the toxicity of metabolites of drugs entering the market. For this purpose, a new microchip method, made of silicon wafer with a cover of anatase-phase TiO_2 nanolayer coupled with electrospray ionization (ESI) tip and external UV lamp to induce photocatalytic process was proposed. The developed microchip method combined with MS analysis was used to study photocatalytic transformations of verapamil, propranolol, metoprolol, lidocaine, *S*-methylthiopurine, and 2-acetamidofluorene. As a reference method, HLM, rat hepatocytes, and CYP450 enzymes were used. It was shown that the metabolic reactions of the main phase I, such a hydroxylation, oxidation, dehydrogenation and *N*- and *O*-dealkylation, were mostly the same with the use of microchip. The authors point to an 80% cover of results obtained by microchip with TiO_2 nanolayer with HLM incubation method, especially in the case of dehydrogenated and dealkylated derivatives. However, the results obtained with developed technique did not fit completely with those described in the literature data in vivo experiments on humans. This method has also other disadvantages, because there is no possibility to identify the regioisomeric products of photocatalytic process and no chromatographic separation is provided before MS analysis.

Ruokolainen et al. [62] compared two methods of in vitro drug metabolism studies: photocatalysis with the use of TiO_2 and HLM incubation, on five steroids: metandienone, methyltestosterone, nandrolone, stanozolol, and testosterone. Photocatalytic reactions were performed with the use of Degussa P25 particles and UVA irradiation source (15 min). All studies were performed by an ultra-performance liquid chromatography–quadrupole time-of-flight mass spectrometry (UPLC–Q-TOF MS) system. The authors point the fact that the approximately 50% of dehydrogenated and hydroxylated derivatives of all steroids could be obtained by the TiO_2 photocatalysis. It should be also noticed that the metabolism imitation of the phase I with the HLM was worst in the case of metandienone. However, it was proven in this study that the main reaction type, for instance, hydroxylation, dehydrogenation, and combination of them, was strictly the same as in the phase I simulation with HLM and TiO_2 photocatalysis, but some isomeric products could be different and some characteristic metabolites for each method also were formed. The highest coverage of HLM reaction by TiO_2 photocatalysis was in the case of dehydrogenated derivatives of nandrolone. High convergence results were also obtained for hydroxylated derivatives of nandrolone and methyltestosterone. On the other hand, no coverage was noted for dehydrogenated derivatives of metandienone and stanozolol.

The same authors also made efforts to check degradation profiles of another organic compounds during photocatalytic process with the use of TiO_2. They compared the mentioned technique with the electrochemically assisted Fenton reaction and the direct electrochemistry method, as well as the HLMs. The authors studied four different molecules: promazine, buspirone, testosterone, and 7-ethoxycoumarin. The photocatalytic reactions were performed with Degussa P25 particles coupled with UVA radiation source, and exposure time was set to 15 s for the first two compounds and 2 min for the others. All analyses were performed by MS system used previously. The study showed that the HLM method gave 18 buspirone derivatives and the main degradation reactions were the hydroxylation as well as *N*-oxidation. In this context, photocatalytic process, which produced 19 additional non-HLM metabolites, seems to be particularly interesting. In the case of promazine, the photocatalysis produced seven additional metabolites, while photodegradation of testosterone became a source of another 10 metabolites with respect to HLM method. Metabolism pathway of 7-ethoxycoumarin was practically the same as with all simulation methods, excluding electrochemistry, which failed to oxidize that compound at all. The authors suggest that, in spite of similar observed metabolic reactions, the formed metabolites may be different due to various combinations of them. Photocatalytic process with the use of TiO_2 provided the best coverage of HLM reaction, enabling almost half of HLM products. [63]

Raoof et al. [64] used the photocatalytic method to determine the metabolic pathways of acetaminophen and cocaine. The study allowed to obtain the phase II results with conjugation of the formed products with glutathione (GSH). The photocatalysis was performed in a cylindrical vessel, and the drug-containing suspension was irradiated in the presence of TiO_2 with UVA source during 160 and 120 min period. The incubation with rat liver microsomes was applied as a reference method. The study was performed by HPLC–MS, with the use of multiple reaction monitoring mode (MRM). It was shown that the reaction between acetaminophen and GSH does not occur spontaneously, but the presence of TiO_2, together with adequate UVA irradiation, was the main cause of the formation of NAPQI–GSH complex. The obtained results correspond with the literature data, where NAPQI appears as a main toxic metabolite of acetaminophen and the conjugation with GSH is one of the detoxification mechanisms in human liver. In the case of metabolism of cocaine, the authors described six photodegradation products, achieved in the reaction conditions similar to acetaminophen studies. On the contrary, incubation with liver microsomes allowed only to receive three metabolites. The authors suggest that the photocatalytic process might be even more efficient than with the use of microsomes.

The summary of the literature data concerning the use of photocatalysis in drug metabolism study is presented in Table 1.

Analytical Methods Used in Photocatalytic Metabolism Study

The choice of an analytical method for the analysis of prepared samples is one of the main tasks of the research team. Only the correct methodology allows the right interpretation of the results. Due to the physicochemical properties of test substances, the method of choice is liquid chromatography (LC), especially high-performance liquid chromatography (HPLC) coupled with mass spectrometry. HPLC allows the fraction separation, and the efficiency of the equipment depends on its performance. For this reason, high pressure pumps and columns packed with <2 μm particle size are being increasingly used. The high separation power is an important property of ultrahigh-performance liquid chromatography (UHPLC), because of the ability to resolve isomeric compounds [66, 67]. Additionally, the use of LC coupled with MS system allows the structural identification of the analyzed

Table 1. Application of photocatalysis in drug metabolism study

Compound	Irradiation source	Photocatalysis conditions/irradiation time	Medium	Analysis method	Comparison method of drug metabolism	Ref.
Dexamethasone	Xenon lamp 1500 W (>340 nm)	Degussa P25 TiO_2, 15 min	H_2O	HPLC–APCI/IT MS	Literature data	[61]
Synephrine octopamine	UVA lamp (40 W/m^2)	Degussa P25 TiO_2 60 min – synephrine 30 min – octopamine	H_2O	HPLC–ESI/Orbitrap MS	In vivo method (human and rats)	[47]
Sildenafil	UVA lamp (40 W/m^2)	Degussa P25 TiO_2 10 min	H_2O	HPLC–ESI/Orbitrap MS	In vivo method (2 horses)	[46]
Buspirone	Xenon lamp 1500 W (>340 nm)	Degussa P25 TiO_2 30 min	H_2O	HPLC–ESI/IT MS	Literature data	[45]
Promazine Buspirone Testosterone 7-Ethoxycoumarin	UVA lamp (225 mW/cm^2)	Degussa P25 TiO_2 15 s – buspirone, promazine 2 min – testosterone, 7-ethoxycoumarine	H_2O–ACN (99:1)	UPLC–ESI/Q-TOF MS	HLM (2 h incubation) and EC method	[63]
Nandrolone Methyltestosterone Metandienone Testosterone Stanozolol	UVA Lamp (225 mW/cm^2)	Degussa P25 TiO_2 2 min - nandrolone Methyltestosterone Metandienone Testosterone 15 min – stanozolol	H_2O–ACN (99:1) H_2O–ACN (50:50)	UPLC–ESI/Q-TOF MS	HLM (24 h incubation)	[62]
Verapamil Metoprolol Propranolol Lidocaine 2-Acetemidofluorene *S*-methylthiopurine	UVA lamp (100 mW/cm^2)	Nanoreactor microchip system with anatase TiO_2 nanolayer coating (TiO_2-μPESI microchip) 15 min	H_2O	UPLC–ESI/Q-TOF MS	HLM (48 h incubation)	[48]
Paracetamol Cocaine	UVA lamp (6x8W)	Degussa TiO_2 160 min – paracetamol 120 min – cocaine	H_2O–ACN (50:50)	HPLC–ESI/IT MS	HLM (1 h incubation)	[64]

metabolites. It should be noticed that, in MS method, the electrospray ionization (ESI) was preferred as a ion source [45–48, 62–64]. This is probably justified because ESI ionization is more efficient technique than atmospheric pressure chemical ionization (APCI) in photodegradation studies of selected drugs [65].

It can be also observed that high-resolution mass spectrometry (HR-MS) is the most often used method in this kind of research. Quadrupole time of flight (Q-TOF) [48, 62, 63] and modified ion trap (Orbitrap) [46, 47] analyzers are most often used in this case. HR spectrum allows to determine the exact mass of the analyzed metabolites, and additional MS/MS experiments allow elucidation of their accurate structure. However, a typical ion trap IT-MS (low resolution) mass spectrometry has been also used to photocatalytic drug metabolism study, due to the ability to perform MS^n experiments [45, 61, 64].

In summary, the UHPLC–HR-MS system allows the most accurate analysis of the tested samples and it becomes the most popular approach in the drug metabolism studies; however, one can expect that more specific spectroscopic method (NMR, FTIR) will be used for this kind of research in the future.

Conclusions

Nowadays, in vitro biological methods are very often used in drug metabolism studies; however, a significant increase of the use of chemical and electrochemical methods is also observed. An especially promising tool in this research seems to be the process of photocatalytic degradation of drugs because this strategy can be very useful in the study of the phase I biotransformation of the active compounds. The main aim of this method is mimicking the biochemical processes of drug decomposition in a controlled manner and what is important with the use of easy and inexpensive methodology and simple equipment. Additionally, in photocatalytic conditions, more metabolites can be found in comparison with traditional biological or electrochemical in vitro methods.

Ultra high-performance liquid chromatography, coupled with the high-resolution hybrid mass spectrometry system turns out to be the most powerful analytical tool in the in vitro drug metabolism studies.

References

1. DiMasi, J. A.; Hansen R. W.; Grabowski H. G. *J. Health. Econ.* **2003**, *22*, 151.
2. Lynch, T.; Price, A. *Am. Fam. Physician.* **2007**, *76*, 391–396.
3. Manier, J. W.; Chang, W. W. L.; Kirchner, J. P.; Beltaos, E. *Am. J. Gastroenterol.* **1982**, *77*, 6.
4. Scheen, A. J.; Lefèbvre, P. *J Drug-Safety* **1995**, *12*, 32.
5. Changjiang, X.; Yong-Tao, L. C.; Kong, Ah-Ng. T. *Arch. Pharm. Res.* **2005**, *28*, 249.
6. Park, K.; Williams, D. P.; Naisbitt, D. J.; Kitteringham, N. R.; Pirmohamed, M. *Toxicol. Appl. Pharm.* **2005**, *207*, 425.
7. Jan, Y. H.; Heck, D. E.; Dragomir, A. C.; Gardner, C. R.; Laskin, D. L.; Laskin, J. D. *Chem. Res. Toxicol.* **2014**, *27*, 882.
8. Bessems, J. G. M.; Vermeulen, N. P. E. *Crit. Rev. Toxicol.* **2001**, *31*, 55.
9. Boelsterli, U. A. *Toxicol. Appl. Pharm.* **2003**, *192*, 307.
10. Bort, R.; Ponsoda, X.; Jover, R.; Gomez-Lechon, M. J.; Castell, J. V. *J. Pharmacol. Exp. Ther.* **1999**, *288*, 65.
11. Baillie, T. A. *Chem. Res. Toxicol.* **1988**, *1*, 195.
12. Anders, M. W.; Pohl, L. R. *Halogenated alkanes. In Bioactivation of Foreign Compounds*; Academic Press, Orlando, 1985.
13. McDonald, G. B.; Slattery, J. T.; Bouvier, M. E.; Ren, S.; Batchelder, A. L.; Kalhorn, T. F.; Schoch, H. G.; Anasetti, C.; Gooley, T. *Blood* **2003**, *101*, 2043.
14. Boddy, A. V.; Proctor, M.; Simmonds, D.; Lind, M. J.; Idle, J. R.; *Eur. J. Cancer* **1995**, *31*, 69.
15. Salaeh, S.; Juretic, Perisic D.; Biosic, M.; Kusic, H.; Babic, S.; Stangar, U. L.; Dionysiou, D. D.; Bozic, A. L. *Chem. Eng. J.* **2016**, *304*, 289.
16. Purnapatre, K.; Khattar, S. K.; Singh, Saini K. *Cancer Lett.* **2008**, *259*, 1.
17. Li, A. P. *Drug Discov. Today Technol.* **2005**, *2*, 179.
18. Pelkonen, O.; Kaltiala, E. H.; Larmi, T. K.; Karki, N. T. *Chem. Biol. Interact.* **1974**, *9*, 205.
19. Brandon, E. F. A.; Raap, C. D.; Meijerman, I.; Beijnen, J. H.; Schellens, J. H. M. *Toxicol. Appl. Pharm.* **2003**, *189*, 233.
20. Johnson, J. A.; Herring, V. L.; Wolfe, M. S.; Relling, M. V. *J. Pharmacol. Exp. Ther.* **2000**, *294*, 1099.
21. Hamman, M. A.; Thompson, G. A.; Hall, S. D. *Biochem. Pharmacol.* **1997**, *54*, 33.
22. Olesen, O. V.; Linnet, K. *Drug Metab. Dispos.* **1997**, *25*, 740.
23. Venkatakrishnan, K.; von Moltke, L. L.; Duan, S. X.; Fleishaker, J. C.; Shader, R. I.; Greenblatt, D. J. *J. Pharm. Pharmacol.* **1998**, *50*, 265.
24. Wojcikowski, J.; Pichard-Garcia, L.; Maurel, P.; Daniel, W. A. *Br J Clin Pharmacol* **2003**, *138*, 1465.
25. Lang, D.; Freudenberger, C.; Weinz, C. *Drug Metab. Dispos.* **2009**, *37*, 1046.
26. Mielczarek, P.; Smolucha, M.; Kotlinska, J. H.; Labuzd, K.; Gotszalke, T.; Babije, M.; Sudera, P.; Silberringa, J. *J. Chromatogr. A* **2015**, *1389*, 96.
27. Bernadou, J.; Meunier, B. *Adv. Synth. Catal.* **2004**, *346*, 171.
28. Maurin, A. J. M.; Iamamoto, Y.; Lopes, N. P.; Lindsay-Smith, J. R.; Bonato, P. S. *J. Braz. Chem. Soc.* **2003**, *14*, 322.
29. Othman, S.; Mansuy-Mouries, V.; Bensoussan, C.; Battioni, P.; Mansuy, D.; *C. R. Acad. Sci. Paris* **2000**, *3*, 751.
30. Breslow, R.; Zhang, X.; Huang, Y. *J. Am. Chem. Soc.* **1997**, *119*, 4535.
31. Fang, Z.; Breslow, R. *Org. Lett.* **2006**, *8*, 251.
32. Yang, J.; Breslow, R.; *Chem. Int. Ed. Engl.* **2000**, *39*, 2692.
33. Baumann, A.; Karst, U. *Drug Metab. Toxicol.* **2010**, *6*, 715.
34. Baumann, A.; Lohmann, W.; Schubert, B.; Oberacher, H.; Karst, U. *J. Chromatogr. A* **2009**, *1216*, 3192.
35. Baumann, A.; Lohmann, W.; Jahn, S.; Karst, U. *Electroanalysis* **2010**, *22*, 286.
36. Lohmann, W.; Meermann, B.; Möller, I.; Scheffer, A.; Karst, U. *Anal. Chem.* **2009**, *80*, 9769.
37. Lohmann, W.; Doetzer, R.; Gütter, G.; Van Leeuwen, S. M.; Karst, U. *J. Am. Soc. Mass Spectrom.* **2009**, *20*, 138.
38. Madsen, K.; Grönberg, G.; Skonberg, C.; Jurva, U.; Hansen, S. H.; Olsen, J. *Chem. Res. Toxicol.* **2008**, *21*, 2035.
39. Haber, V. F.; Weiss, J. *Naturwissenschaften* **1932**, *20*, 948.
40. Jurva, U.; Wikstrom, H. V.; Bruins, A. P. *Rapid Commun. Mass Spectrom.* **2002**, *16*, 1934.
41. Barry, S. M.; Mueller-Bunz, H.; Rutledge, P. *J. Org. Biomol. Chem.* **2012**, *10*, 7372.
42. Czodrowski, P.; Kriegl, J. M.; Scheuerer, S.; Fox, T. *Expert Opin. Drug Metab. Toxicol.* **2009**, *5*, 15.
43. Ekins, S.; Andreyev, S.; Ryabov, A.; Kirillov, E.; Rakhmatulin, E. A.; Sorokina, S.; Bugrim, A.; Nikolskaya, T. *Drug Metab. Dispos.* **2006**, *34*, 495.
44. Bellosta, S.; Paoletti, R.; Corsini, A.; *Circulation* **2004**, *109*, III50.
45. Calza, P.; Pazzi, M.; Medana, C.; Baiocchi, C.; Pelizzetti, E. *J. Pharm. Biomed. Anal.* **2004**, *35*, 9.
46. Medana, C., Calza, P.; Giancotti, V.; Dal, Bello F.; Pasello, E.; Montana, M.; Baiocchi, C. *Drug Test. Anal.* **2011**, *3*, 724.
47. Medana, C.; Calza, P.; Giancotti, V.; Dal, Bello F.; Aragno, M.; Baiocchi, C. *Anal. Bioanal. Chem.* **2013**, *405*, 1105.
48. Nissilä, T.; Sainiemi, L.; Karikko, M.; Kemell, M.; Ritala, M.; Franssila, S.; Kostiainen, R.; Ketola, R. A. *Lab Chip* **2011**, *11*, 1470.
49. Earle, M. D. *Phys. Rev.* **1942**, *61*, 56.
50. Agrios, A.; Pichat, P. *J. Appl. Electrochem.* **2005**, *35*, 655.
51. Fujishima, A.; Zhang, X.; Tryk, D. A. *Surf. Sci. Rep.* **2008**, *63*, 515.
52. Tang, J.; Durrant, J. R.; Klug, D. R. *J. Am. Chem. Soc.* **2008**, *130*, 13885.
53. Konstantinou, I.; Albanis, T. *Appl. Catal. B* **2004**, *49*, 1.
54. McLintock, I. S.; Ritchie, M. *Trans. Faraday Soc.* **1965**, *61*, 1007.
55. Hurum, D. C.; Agrios, A. G.; Gray, K. A. *J. Phys. Chem. B* **2003**, *107*, 4545.
56. Ahmed, S.; Rasul, M. G.; Brown, R.; Hashib, M. A. *J. Environ. Manag.* **2011**, *92*, 311.
57. Tomic, N.; Grujic-Brojcin, M.; Fincur, N.; Abramovic, B.; Simovic, B.; Krstic, J.; Matovic, B.; Scepanovic, M. *Mater. Chem. Phys.* **2015**, *163*, 518.
58. Bhatkhande, D. S.; Pangarkar, V. G.; Beenackers, A. A. *J. Chem. Technol. Biotechnol.* **2002**, *77*, 102.
59. Prieto-Rodriguez, L.; Miralles-Cuevas, S.; Oller, I.; Aguera, A.; Li, Puma G.; Malato, S. *J. Hazard. Mater.* **2012**, *211–212*, 131.
60. Fujishima, A.; Rao, T. N.; Tryk, D. A. *J. Photochem. Photobiol. C, Photochem. Rev.* **2000**, *1*, 1.
61. Calza, P.; Pelizzetti, E.; Brussino, M.; Baiocchi, C. *J. Am. Soc. Mass. Spectrom.* **2001**, *12*, 1286.
62. Ruokolainen, M.; Valkonen, M.; Sikanen, T.; Kotiaho, T.; Kostiainen, R. *Eur. J. Pharm. Sci.* **2014**, *65*, 45.
63. Ruokolainen, M.; Gul, T.; Permentier, H.; Sikanen, T.; Kostiainen, R.; Kotiaho, T. *Eur. J. Pharm. Sci.* **2016**, *83*, 36.
64. Raoof, H.; Mielczarek, P.; Michalow, K. A.; Rekas, M.; Silberring, J. *J. Photochem. Photobiol. B, Biol.* **2013**, *118*, 49.
65. Trawinski, J.; Skibinski, R.; Komsta, Ł. *Acta Chromatogr.* DOI: 10.1556/1326.2017.29.2.2.
66. Monfort, N.; Martinez, L.; Berges, R.; Segura, J.; Ventura, R. *Drug Test. Anal.* **2015**, *7*, 819.
67. Spaggiari, D.; Geiser, L.; Rudaz, S. *Trends Anal. Chem.* **2014**, *63*, 129.