Journal of Pharmaceutical and Biomedical Analysis 49 (2009) 498–501

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

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

Short communication

Novel therapeutic biosensor for indinavir—A protease inhibitor antiretroviral drug

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ARTICLE INFO

Article history:
Received 3 April 2008
Received in revised form 19 October 2008
Accepted 20 October 2008
Available online 28 October 2008

Keywords:
Drug metabolism biosensor
CYP3A4
Indinavir
Amperometric biosensor
Bioelectrode

ABSTRACT

An amperometric drug metabolism biosensor consisting of cytochrome P450-3A4 (CYP3A4) encapsulated in a didodecyldimethylammonium bromide (DDAB) vesicular system on a Pt disk electrode was developed for the determination of indinavir, a protease inhibitor antiretroviral drug. Cyclic, square wave and pulse voltammetric responses of the bioelectrode showed quasi-reversible electrochemistry of the Fe^{3+}/Fe^{2+} redox species of the heme thiolate CYP3A4 enzyme under aerobic and anaerobic conditions. The biosensor exhibited excellent response to indinavir with a detection limit and response time of 6.158×10^{-2} mg L^{-1}, and 40 s, respectively. The detection limit is well below the plasma concentration of indinavir (8 h after intake) which range from 0.13 to 8.6 mg L^{-1}.

1. Introduction

Cytochrome P450s comprise a super-family of enzymes that play important roles in drug metabolism. Cytochrome P450-3A4 (CYP3A4) is known to be a major form of P450 expressed in adult human livers [1], and a majority of the drugs currently available on the market are metabolized by this isoform [2,3]. The P450s may inactivate drugs or toxic chemicals or activate them to effective, mutagenic and/or carcinogenic forms [4,5]. Thus, the characterisation of these enzymes is a key issue in pharmacokinetic and toxicokinetic metabolic pathways. Activity of P450s is currently determined from the rates of formation of metabolites. The methods that are now available for the *in vitro* monitoring of drug metabolism are time consuming and expensive. Thus there is an urgent need to develop new analytical tools that would be easy to use, capable of identifying substrates and inhibitors of an enzyme, and allow quantification of substrate turnover. The use of appropriate biosensor would be a welcome alternative, because they are generally of small size, capable of continuous measurements, and can measure analytes faster and at lower cost than traditional methods [6,7]. This study deals with an amperometric biosensor system, based on the redox properties of P450-3A4, which allows one to follow the catalytic cycle of the enzyme in the presence of its substrate.

A major advance in antiretroviral therapy for acquired immune deficiency syndrome (AIDS) has been the introduction of the human immunodeficiency virus (HIV) protease inhibitor drugs. Among protease inhibitors approved in US and Canada are saquinavir (Invirase), indinavir (Crixivan) and ritonavir (Norvir) [8–11]. These protease inhibitors, when administered in high dose and in combination with other antiretroviral (ARV) drugs, e.g. azidothymidine (AZT), considerably reduce HIV levels in the blood [12,13]. However, therapy is complicated by the fact that the protease inhibitors compete for the same CYP3A4 metabolic enzyme as many other drugs including some of those administered for AIDS-related infections [14,15]. Metabolism of indinavir by CYP3A4 has previously been characterised [10,16]. The primary oxidative metabolites (i.e. metabolites associated with CYP3A4-mediated monooxygenation of the drug) are shown in Fig. 1, in which M6 (the product of *N*-depirydomethylation) is the predominant metabolite. Other metabolites result from the hydroxylation of the phenylmethyl, pyridine *N*-oxidation and the hydroxylation of M6 [10].

The majority of CYP enzymes are located in a hydrophobic environment in the endoplasmic reticulum of cells. In order to mimic the physiological environment of CYP enzymes, a number of groups [6,17–20] have used phospholipids, such as didodecyldimethylammonium bromide (DDAB), dimeristoyl-L-α-phosphatidylcholine (DMPC), dilauroylphosphatidylethanolamine (DSPE), for the construction of biosensors. Phospholipids layer form stable vesicular

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doi:10.1016/j.jpba.2008.10.025

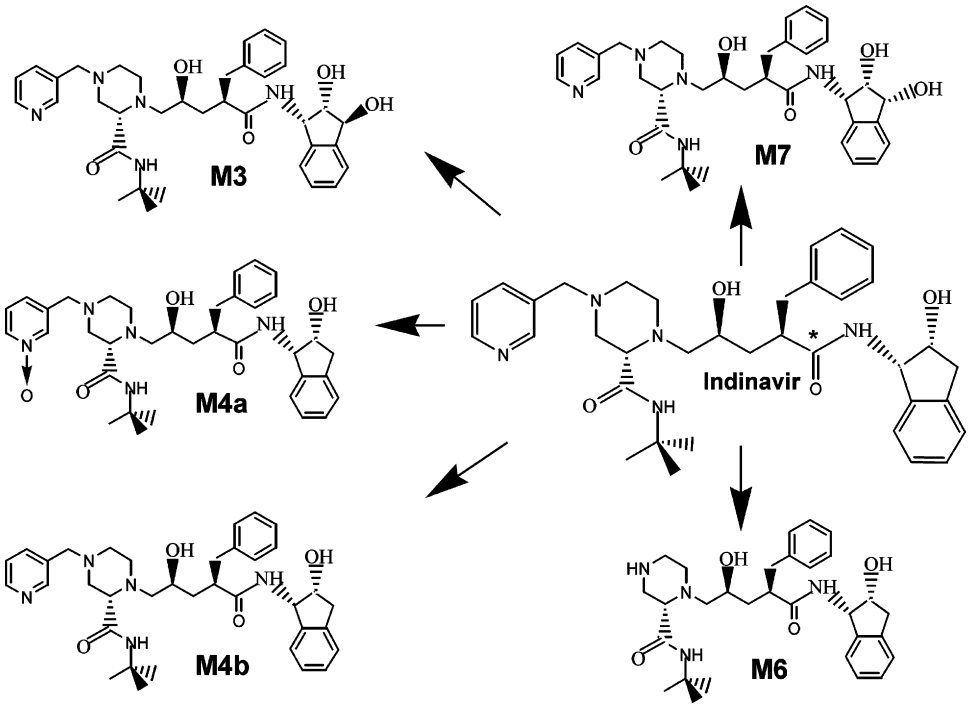

Fig. 1. Primary metabolites of indinavir [10].

dispersions that bear a structural relationship with the phospholipids components of biologically important membranes. By this way a membranous environment is created that facilitates electron transfer between the enzymes redox centre and the electrode [17]. This approach was applied in the construction of cytochrome P450-101 (CYP101)-based electrochemical biosensor for the determination of camphor [6]. The biosensor comprised a glassy carbon electrode (GCE) modified with CYP101 contained in DDAB vesicle dispersion. Glutaraldehyde in the presence of bovine serum albumin was used as the cross-linking reagent. In an earlier study, DDAB was used to investigate the direct electron transfer between pyrolytic graphite electrode and hemeproteins such as hemoglobin, myoglobin, CYP101 [18]. DDAB produces a lamellar liquid crystal surfactant film which creates a biomembrane-like microenvironment within which redox proteins reside and undergo direct electron exchange with the underlying electrode [19,20]. This study will involve the development of a DDAB-bound CYP3A4 biosensor system for determining the metabolism of indinavir, a PI drug administered for AIDS-related infections.

2. Experimental

2.1. Reagents

Cytochrome P450-3A4 was obtained from Sigma–Aldrich, Germany, and stored in the freezer at −80 °C when not in use. Didodecyldimethylammonium bromide (DDAB) was obtained from Fluka, Germany, and stored at <5 °C. A 10 mM vesicle dispersion of DDAB was prepared by sonicating 9.26 mg of the powdery compound in 2 mL of water. 25% glutaraldehyde was obtained from Aldrich. Saline phosphate buffer (PBS) of pH 7.5 was prepared with 50 mM Na_2HPO_4, 50 mM NaH_2PO_4 (Aldrich) and 100 mM KCl (Aldrich). All chemicals were of analytical grade. Ultra pure water with a resistivity of 18.2 MΩ prepared with Millipore Synergy water purification system was used to prepare all reagents. Indinavir solution was prepared by dissolving one capsule of Crixivan® 400 mg (M.W. 711.88) from Merck & Co., Inc., NJ, USA, in PBS. The solution was sonicated for 30 min and stirred at 37 °C for 30 min. Clear solution of the drug was obtained after filtration using PTFE syringe filter of 0.45 μm pore size (Whatman).

2.2. Instrumentation

A three-electrode 10 mL cell system, consisting of 0.071 cm^2 Pt disk electrode (BAS), 0.5 mm Pt wire and Ag/AgCl (3 M KCl type) working, auxiliary and reference electrodes, respectively, connected to a BASi 100B workstation (LG Fayette, USA), was used for all electrochemical measurements. The Pt electrode was polished with aqueous slurries of 1.0, 0.3 and 0.05 μm alumina before use. Cyclic voltammetry (CV) was performed over a potential range of +100 to −900 mV at a sensitivity of 10 $\mu A\,V^{-1}$ and scan rate of 300 $mV\,s^{-1}$. The experimental parameters used for 0 to −800 mV square wave voltammetry (SWV) were: 30 mV square wave amplitude, 5 Hz frequency and 10 $\mu A\,V^{-1}$ sensitivity. Differential pulse voltammetry (DPV) data were obtained for potentials of +200 to −900 mV at sensitivity, scan rate and pulse amplitude of 10 $\mu A\,V^{-1}$, 30 $mV\,s^{-1}$ and 25 mV, respectively. Steady-state amperometry was carried out at −680 mV and sensitivity of 1 $\mu A\,V^{-1}$ while stirring the cell solution continuously at 150 rpm.

2.3. Preparation of biosensor

The indinavir biosensor was prepared by the immobilisation of CYP3A4 contained in DDAB vesicular dispersion on the 0.071 cm^2 Pt disk working electrode [6]. 25 μL of 10 mM aqueous vesicle dispersion of DDAB was mixed with 1 mg of BSA to form solution A. 12.5 μL of 1 $\mu M\,L^{-1}$ CYP3A4 was mixed with 12.5 μL of solution A to form a vesicle–enzyme complex (solution B). 1.5 μL of solution B was dropped on a 0.071 cm^2 Pt disk electrode surface and dried in air for 2 h. 1.5 μL of 2.5% glutaraldehyde (Glu) was then dropped on top of the biolayer and dried for 5 h to form Pt|CYP3A4–DDAB–BSA|Glu

bioelectrode which was stored in the refrigerator at 4 °C when not in use.

3. Results

3.1. Electrocatalytic responses of biosensor

In Fig. 2 square wave (A), cyclic (B) and pulse (C) voltammetry results of Pt|CYP3A4–DDAB–BSA|Glu biosensor are shown for anaerobic (argon-degassed) and aerobic conditions. The cyclic voltammograms (Fig. 2 B) in substrate-free aerobic solution at 300 mV s^{-1} consists of oxidation and reduction waves, with cathodic peak, $E_{p,c}$, and anodic peak, $E_{p,a}$, potential values of −735 and −644 mV, respectively. The peak separation, ΔE_p ($= E_{p,a} - E_{p,c}$) value was 91 mV, which indicates a quasi-reversible system. A mid point potential, E_m {$= 1/2(E_{p,a} + E_{p,c})$} value of −689 mV vs Ag/AgCl was estimated from the cyclic voltammogram.

The midpoint potential value for the immobilized CYP3A4 estimated from voltammetry is not the same as what was reported by other workers [6,7]. This discrepancy may be caused by the possible differences in the heme environment of the P450 in solution and immobilized state. The electrostatic interaction of enzyme-polyion in the film may significantly control the redox potential of the heme iron [21]. Voltammetric techniques (SWV: Fig. 2A; CV: Fig. 2B; and DPV: Fig. 2C) were used to investigate the electrochemical conversion of CYP3A4(Fe^{III}) to CYP3A4(Fe^{II}) in enzyme-bound film in the presence and absence of oxygen. The increase in the reduction current and the cathodic shift in the peak current (Fig. 2A–C) observed in the presence of oxygen suggests the accompanying

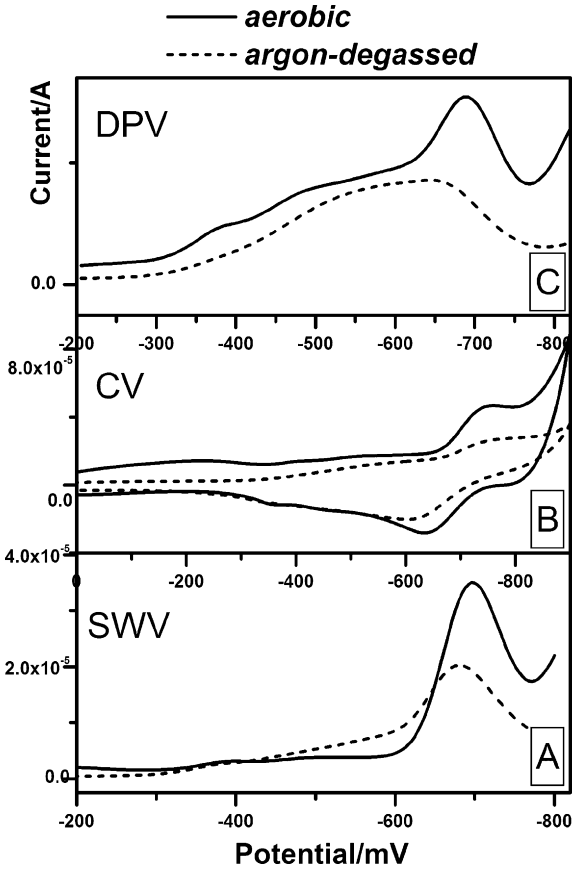

Fig. 2. Square wave (A), cyclic (B), differential pulse (C) voltammograms of Pt|CYP3A4–DDAB–BSA|Glu biosensor in aerobic (straight line) and anaerobic (dash) conditions in PBS (pH 7.5).

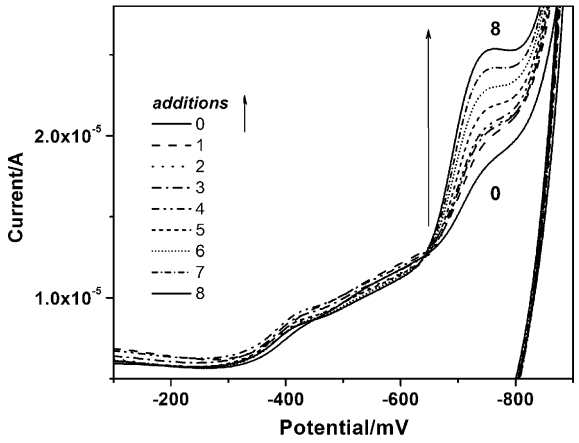

Fig. 3. Cyclic voltammograms of Pt|CYP3A4–DDAB–BSA|Glu biosensor at 300 mV s^{-1} in PBS (pH 7.5) upon the addition of indinavir in aerobic conditions.

of the reduction of CYP3A4(Fe^{III}) by an oxygenation step (i.e. the binding of dioxygen) to form CYP3A4(Fe^{II})O_2. The one-electron electrochemical reduction of Fe^{3+} to Fe^{2+} generated CYP3A4(Fe^{II}) which reacted with dioxygen in the film, followed most likely by the electrochemical reduction of the CYP3A4(Fe^{II})O_2 produced. This later reduction process (i.e. the reduction of CYP3A4(Fe^{II})O_2) involving the cleavage of the O—O bond and the formation of CYP3A4(Fe^{IV})O, is a key reaction in the mono-oxygenation catalytic cycle of heme enzymes [22]. There is the possibility of a peroxide shunt in which CYP3A4(Fe^{III}) is converted to CYP3A4(Fe^{IV})O by H_2O_2 which donates both the electron and oxygen required to short-circuit the oxygenation process. However, the auto-oxidation of CYP3A4(Fe^{II})O_2, known to be a source of H_2O_2 production, is too slow to be important in this monooxygenation process [23].

The response pattern of indinavir on the Pt|CYP3A4–DDAB–BSA|Glu biosensor was investigated by CV and steady-state amperometry techniques. Fig. 3 shows the voltammetric responses of the biosensor to aliquots of indinavir. An increase in the reduction current and a cathodic shift in the peak response potential were observed for each addition of indinavir. This CV response pattern is characteristic of electrocatalytic reaction of CYP biosensors in the presence of substrates and oxygen-saturated solutions [6]. Thus the CV responses shown in Fig. 3 are due to the electrochemical monooxygenation reaction of indinavir on Pt|CYP3A4–DDAB–BSA|Glu biosensor in aerobic conditions.

3.2. Steady-state amperometry

Amperograms 1–3 of Fig. 4 present the steady-state responses of the Pt|CYP3A4–DDAB–BSA|Glu biosensor to incremental amounts of indinavir. The response time (time required for the signal to attend a steady-state value following the addition of indinavir) was approximately 40 s (see Amperogram 2). As shown by Amperogram 1 the addition of indinavir-free blank (PBS pH 7.5) did not produce any biosensor response. On the other hand, results in Amperogram 3 portray the comparatively low responses (compared to responses shown in Amperogram 2) of the biosensor to indinavir in an oxygen-free solution. This response (Amperogram 3) is entirely due to the binding of indinavir to CYP3A4, a process which does not require oxygen but initiates the enzyme catalysed metabolic process. What happens is that indinavir binding to CYP3A4 activates the low-to-high spin transition of the heme Fe^{3+} which is required to facilitate CYP3A4$Fe^{3+\rightarrow 2+}$ electrochemistry. The consequence is that the rate of CYP3A4$Fe^{3+\rightarrow 2+}$ electrochemical reaction increases with increase

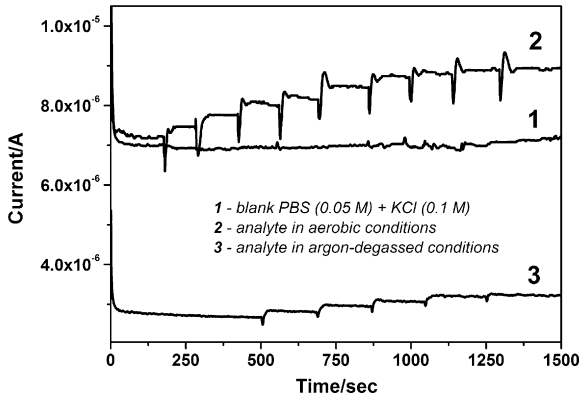

Fig. 4. Steady-state amperometry responses of Pt|CYP3A4–DDAB–BSA|Glu biosensor for indinavir in aerobic (2) and anaerobic (3) PBS (pH 7.5), at −680 mV.

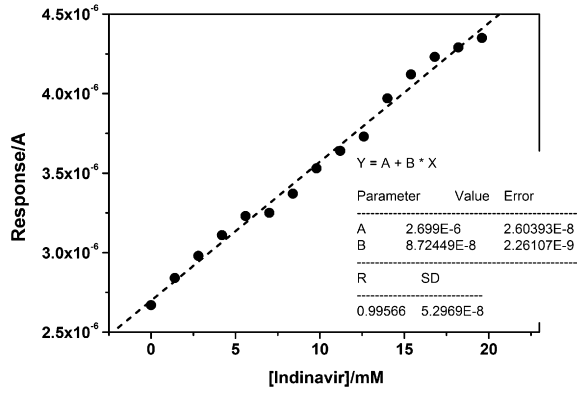

Fig. 5. The calibration curve of Pt|CYP3A4–DDAB–BSA|Glu biosensor in the presence of indinavir in PBS (pH 7.5).

in indinavir concentration as shown in the amperogram. The behaviour of the electrocatalytic process in oxygen-rich medium is depicted in Amperogram 2. The amperometric responses presented in Amperogram 2 represent the coupling of the CYP3A4$Fe^{3+\rightarrow 2+}$ electrochemistry to the monooxygenation reaction steps that only occur in the presence of oxygen. The comparatively larger response currents in the presence of oxygen, therefore, result from the combination of indinavir-dependent CYP3A4$Fe^{3+\rightarrow 2+}$ reduction current and the oxygen-dependent monooxygenation current.

Concentration-dependent response profile of the biosensor is shown in Fig. 5. The enzyme electrode gave a calibration linearity that extended to 20 mM. The detection limit value of the sensor (calculated as three times the standard deviation of the measurements divided by the sensitivity of the sensor) was 6.158×10^{-2} mg L^{-1}. From *in vivo* studies, the maximum plasma concentration (C_{max}) of indinavir (8 h after intake) range from 0.13 to 8.6 mg L^{-1} [24–26]. The C_{max} values fall within the low detection limit of the Pt|CYP3A4–DDAB–BSA|Glu biosensor system and its dynamic linear range. This means that the sensor can be successfully applied in the detection of indinavir in biological samples at very low concentrations or concentrations found in physiological samples.

4. Conclusion

Pt|CYP3A4–DDAB–BSA|Glu bioelectrode exhibited quasi-reversible electrochemistry of the heme-Fe^{3+}/Fe^{2+}. Voltammetric and amperometric results indicate that the observed response signal (current) of the biosensor can be related to the amount of substrate present in the solution. A biosensor response time of 40 s for indinavir obtained in this study, implies that the CYP3A4-based biosensing procedure can be employed as a fast alternative to the expensive and often complicated analytical methods currently used for analysing the metabolism of antiretroviral drugs in plasma. Further experiments are underway to apply the biosensor to real physiological samples.

Acknowledgements

This study was funded by grants from the National Research Foundation (NRF) of South Africa and the Medical Research Council (MRC) of South Africa.

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