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Modification of a PAMPA model to predict passive gastrointestinal absorption and plasma protein binding

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ABSTRACT

The Parallel Artificial Membrane Permeability Assay (PAMPA) is a well-known high throughput screening (HTS) technique for predicting *in vivo* passive absorption. In this technique, two compartments are separated by an artificial membrane that mimics passive permeability through biological membranes such as the dermal layer, the gastrointestinal tract (GIT), and the blood brain barrier (BBB).

In the present study, a hexadecane artificial membrane (HDM)-PAMPA was used to predict the binding of compounds towards the human plasma using a mixture of human serum albumin (HSA) and alpha-1-acid glycoprotein (AGP). The ratio of HSA and AGP was equivalent to that found in the human plasma for both proteins (~20:1). A pH gradient (5.0–7.4) was performed to increase the screening capacity and overcome the issue of passive permeability for acidic and amphoteric compounds.

With this assay, the prediction of passive GIT absorption was maintained and the compounds were discriminated according to their permeability (on a no-to-high scale). The plasma protein binding (PPB) was estimated *via* the correlation of the differences between the amount of compound crossing the artificial membrane in assays conducted with and without protein using only a two end-point measurement. The use of a mixture of HSA and AGP to modulate drug permeation was compared to the use of the same concentrations of HSA and AGP used separately. The addition of HSA alone in the acceptor compartment was sufficient for estimating PPB, while it was demonstrated that AGP alone could enable the estimation of AGP binding.

1. Introduction

It is widely known that the withdrawal of a new chemical entity (NCE) from the drug development process is mainly due to its inappropriate pharmacokinetic (PK) properties (Leucuta, 2014). Therefore, many efforts have been made to develop high throughput screening (HTS) assays to eliminate NCEs that could be critical in ADMET phases (absorption, distribution, metabolism, elimination, and toxicity) as early as possible (Balimane et al., 2006).

Because oral absorption is primarily preferred for NCEs, HTS assays predicting this property have been primarily developed (van de Waterbeemd, 2005). Absorption through the gastrointestinal tract (GIT) is governed by many factors, such as the absorption surface, blood flow at the absorption site, and the physicochemical properties and concentration of the NCE at the absorption site (Augustijns et al., 2014). The main indicators used in estimating the absorbed fraction (f_a) of a compound through the GIT are its (i) coefficient of permeability (P_e), (ii) solubility, (iii) lipophilicity, (iv) absorption time, and (v) stability (Fagerholm, 2007).

Colorectal adenocarcinoma (Caco-2) cells and the parallel artificial membrane permeability assay (PAMPA) are the major assays used to predict P_e (Kerns and Di, 2003). The use of Caco-2 cells is the standard approach commonly used by industrial companies and is recommended by the US Food and Drug Administration (FDA). Because the use of cells remains an intensive process, other methods have been developed, in association with Caco-2 cells or separately (Bhardwaj and Chandrasena, 2012; Fujikawa et al., 2005). PAMPA was first introduced in 1998 by Kansy et al. as a HTS assay (Kansy et al., 1998) and is based on a two-way flux equation where two compartments (donor and acceptor) are separated by an artificial membrane (Avdeef, 2012). Depending on the nature of the artificial membrane used, the prediction of the passive permeability value of the three major biological barriers can be made with the PAMPA technique: the dermal layer, the blood brain barrier (BBB), and the GIT (Faller, 2008). The experimental procedure takes place in a 96-well plate, allowing for the screening of 48 compounds in duplicate in one single assay. PAMPA alone only predicts passive permeability, while the use of Caco-2 values (apical to

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basolateral) can be used in association with PAMPA to identify actively transported compounds and compounds that interact with efflux proteins (Bhardwaj and Chandrasena, 2012).

After absorption, a drug reaches the blood stream and can bind to plasma proteins, such as human serum albumin (HSA) and alpha-1-acid glycoprotein (AGP) (Kratz and Elsadek, 2012). These interactions can have an impact on the free drug concentration surrounding the therapeutic target responsible for a pharmacological researched effect (Smith et al., 2010). The binding of drugs with plasma proteins mainly depends on (i) the ligand concentration, (ii) the proteins concentration, (iii) the pH, (iv) the presence of other ligands or proteins, (v) the number of interaction sites, and (vi) the abundance of different genetic variants (Israili and Dayton, 2001; Kishino et al., 2002; Kochansky et al., 2008). HSA (*ca.* 66 kDa and 585 aa (Fanali et al., 2012; Peters, 1996b)) represents approximately 60% of all plasma proteins, with a concentration ranging from 0.53 to 0.75 mM (Kratochwil et al., 2002; Zhang et al., 2012). HSA is known to bind preferentially to lipophilic, neutral, and/or acidic compounds (Liu et al., 2005). AGP (*ca.* 48 kDa and 183 aa (Fournier et al., 2000; Israili and Dayton, 2001)) is the second major plasma protein responsible for drug distribution and represents approximately 1–3% of the total plasma protein, with a concentration ranging from 10 to 30 μM in a healthy person (Zsila and Iwao, 2007). Due to its physicochemical properties, AGP preferentially binds basic compounds, but it can also bind acidic and neutral compounds (Israili and Dayton, 2001).

Several *in vitro* methods have been developed to predict drug–plasma protein interactions and are divided into two categories. The first category consists of assays that enable the use of free proteins in solution, such as equilibrium dialysis (ED), ultracentrifugation (UC), ultrafiltration (UF), isothermal titration calorimetry (ITC), and capillary electrophoresis in frontal analysis mode (CE/FA) (Vuignier et al., 2010). The second category includes assays in which the protein is bound to the experimental equipment, such as surface plasma resonance (SPR) and high performance affinity chromatography (HPAC). To the best of our knowledge, the PAMPA technique has been only referred twice in the literature for the prediction of the dissociation constant (K_d) of a drug towards HSA, and both studies required the use of complex mathematical development to estimate the K_d values of the compounds (Bujard et al., 2014; Lazaro et al., 2008).

In this study, PAMPA with a hexadecane membrane (HDM), originally developed to predict GIT passive absorption (Wohnsland and Faller, 2001), was used to not only predict passive GIT absorption but also drug–plasma protein binding (PPB). This was achieved without using complex mathematical programs, as the difference between the amount of compound crossing the artificial membrane with and without proteins was used to predict the binding of drugs with plasma proteins and AGP alone. Gradient PAMPAs were developed to circumvent the issues experienced with acidic and amphoteric compounds. The use of AGP jointly with HSA in the assay was not found to be mandatory for predicting plasma protein binding, while the use of AGP alone enabled the prediction of its binding.

2. Materials and methods

2.1. Chemicals

All tested compounds were purchased from Sigma (a division of Fluka Chemie AG, Buchs, Switzerland). Essentially fatty acid-free human serum albumin, alpha-1-acid glycoprotein, hexadecane 99%, hexane, anhydrous disodium hydrogen phosphate, potassium dihydrogen phosphate, acetic acid 99–100% and sodium hydroxide were also purchased from Sigma (a division of Fluka Chemie AG, Buchs, Switzerland). Dimethylsulfoxide (DMSO) (purity grade > 99.7%) and methanol (HPLC grade) were purchased from Fisher Chemical (Fisher Chemical UK, Leics, UK).

2.2. PAMPA experiments

All stock solutions of the tested compounds were prepared at a concentration of 20 mM in DMSO. The stock solutions were then diluted with a buffer containing 0.014 M KH_2PO_4 and 0.054 M Na_2HPO_4 (final pH 7.4) or with a buffer containing 0.048 M CH_3COOH and 0.032 M NaOH (final pH 5.0) to obtain the reference solutions at 50 μM and 1% DMSO (Bujard et al., 2014; Lazaro et al., 2008; Wohnsland and Faller, 2001). Those conditions were used to perform PAMPA both in the presence or absence of the proteins depending on the nature of the compounds as listed below.

In the absence of proteins, all compounds were tested at iso-pH 7.4 (donor and acceptor). Acidic and amphoteric compounds (Acyclovir, Chloramphenicol, Diclofenac, Doxycycline, Enalapril, Flurbiprofen, Ibuprofen, Ketoprofen, Naproxen, Piroxicam, Probenacid and Tolbutamide) were further tested at iso-pH 5.0 and gradient pH 5.0 (donor) – 7.4 (acceptor).

In the presence of proteins in the acceptor compartment, basic compounds were tested at iso-pH 7.4, while for acidic and amphoteric compounds, a gradient pH 5.0 (donor) – 7.4 (acceptor) was performed.

2.2.1. PAMPA in the absence of proteins

A 96-well microtiter polycarbonate (PC) filter plate (Millipore AG, Volketswill, Switzerland) was impregnated with 15 μL of a hexane/hexadecane (95:5, v/v) solution using a Precision 2000® 96/384 Well Automated Microplate Pipetting System (Bio-Tek Instrument Inc., Luzern, Switzerland). The PC filter specifications included 5–20% porosity with a 0.45 μm pore size and 10 μm thickness (the maximum porosity value was taken into account during this study). After membrane impregnation, the PC filter was placed under a fume hood and subject to constant shaking (75 rpm; Titramax 1000®, Huber & Co. AG, Reinach, Switzerland) for approximately 1 h to evaporate all of the hexane (Bujard et al., 2014). The filter plate constituted the donor compartment, which was filled with 280 μL of the reference solution containing the tested compounds. The donor plate was placed upon a Teflon® 96-well acceptor plate (MSSACCEPTOR, Millipore AG, Volketswill, Switzerland) that was previously filled with 280 μL of buffer at pH 7.4 or 5.0 containing 1% DMSO. The resulting sandwich was incubated at room temperature at 75 rpm for 4 h. Each compound was analyzed in quadruplicate ($n = 4$). After the incubation period, the sandwich was dissociated, and the donor and acceptor wells were transferred to black 96-well plates (MaxiSorp, Milian SA, Venier, Switzerland) prior to UHPLC (ultra-high performance liquid chromatography)-UV (ultraviolet) analysis (see Section 2.3). The membrane integrity was checked using ethidium bromide, which is known to not cross the membrane (Bujard et al., 2014) and with electrical resistance measurement (Wohnsland and Faller, 2001), where wells with lower 5 kΩ were extruded using an electrometer system especially designed for PAMPA assays (EVOMX and MULTI96, World Precision Instruments, Sarasota, USA).

2.2.2. PAMPA in the presence of proteins

The procedure was exactly the same as described in Section 2.2.1 except that the proteins were added to the acceptor compartment containing the buffer at pH 7.4 and 1% DMSO. All compounds except for the acidic and amphoteric compounds were at a pH of 7.4 in the donor compartment. The donor pH was set at 5.0 for the acidic and amphoteric compounds to obtain a gradient pH PAMPA. Performing gradient pH PAMPA did not alter the pH