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A kinetic method for the determination of plasma protein binding of compounds unstable in plasma: Specific application to enalapril

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ABSTRACT

Traditional methods for the determination of plasma protein binding (PPB), such as equilibrium dialysis and ultrafiltration, normally operate on a timescale ranging from tens of minutes to several hours and are not suitable for measuring compounds that have significant chemical degradation on this timescale. One such compound is enalapril. Although stable in human plasma enalapril is subject to rapid esterase-catalyzed hydrolysis in rat plasma. A method has been developed which allows the extent of rat PPB of enalapril to be determined from initial rates kinetics of the adsorption of the unstable compound to dextran coated charcoal (DCC). The method has been applied to stable compounds, and the results are consistent with those from traditional equilibrium dialysis experiments. The experimental method is simple to run, requires no specialized equipment, and can potentially be applied to other compounds that show instability in plasma where traditional experimental techniques are unsuitable.

1. Introduction

The extent of binding of a drug to plasma proteins is an important property which has a large influence on the efficacy, pharmacokinetics and toxicology of the compound *in vivo* [1–4]. It is a property which undergoes much measurement and optimization during the drug discovery process, but has suffered in the past because the experimental methodology was very labor intensive and lacked automation [5]. This has recently been addressed with the development of higher throughput technologies based on multi-well equilibrium dialysis [4,6–8] and ultrafiltration [9] systems, and also with the use of mixtures of compounds in each incubation [4,9,10], facilitated by modern mass spectrometry detectors with high sensitivity and fast scanning rates. These new methods are becoming more widespread and will greatly facilitate the optimization cycle in drug discovery. However, the routine application of these methods is not suitable for compounds that are chemically unstable in plasma, particularly when the chemical reaction is fast compared with the long equilibration time (typically 4 h or more) of the experiment. Chemical instability of research compounds in plasma is not an uncommon phenomenon, and is often a consequence of hydrolysis of ester groups catalyzed by esterases in the plasma [11]. Plasma instability is not necessarily a property which will render a drug unsuitable for use. If a compound is much more unstable in plasma than in other tissues but has a high volume of distribution, then it can have a much longer pharmacokinetic terminal half life than the half life in plasma *in vitro*. Hence plasma instability will not necessarily lead to poor pharmacokinetics due to short half life. Furthermore, in developing effective prodrugs and antedrugs therapies, plasma instability can be a purposefully designed feature where upon systematic exposure a drug is either activated (prodrug) or deactivated (antedrug) [12–14]. In order to gain greater understanding of the efficacy, pharmacokinetics and toxicology of plasma unstable compounds, it is valuable to generate a good estimate of the free concentration of the compound in plasma, and a determination of the extent of PPB will be a key component of the free concentration estimate.

Enalapril is a prodrug that contains an ester group that is hydrolyzed by esterases to enalaprilat an angiotensin-converting enzyme inhibitor [15]. Enalapril displays very little hydrolysis in human plasma but rapid hydrolysis in rat plasma [16,17]. Hence equilibrium dialysis could be employed to determine the extent of PPB in human plasma but not in rat plasma. Therefore to measure the rat PPB of enalapril an experimental PPB method that could operate on a short timescale was developed by the modification of existing methods based on adsorption of compounds to DCC [18–20]. The use of DCC in plasma binding determinations is based on the fact that compounds will adsorb more slowly onto DCC in the presence of plasma than in the absence of plasma due to the lowered free concentration of compound in plasma. The first reported DCC method required determination of the full time course of adsorption of the drug to DCC both in the presence and absence of plasma [18,19]. Nonlinear curve fitting of the data to a derived kinetic model then allowed the extraction of the extent of plasma

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binding from the kinetic data. The DCC absorption kinetic method was modified to an equilibrium method where only the final extent of adsorption to DCC in the presence and absence of plasma needs to be determined [20]. None of these methods account for the degradation of a compound within plasma and they are unsuitable for those compounds that are very unstable in plasma due to the time required for the DCC binding process to reach equilibrium. Of the 3 reported methodologies the shortest time course, and hence exposure of a compound to plasma, is approximately 30 min [18] and this will be unsuitable for compounds with plasma half-lives of <30 min as significant decomposition would occur on this timescale. The original kinetic method [18] has been modified to only consider the initial rate of DCC adsorption rather than analysis of the full time course, and the chemical degradation process has further been incorporated into the kinetic modeling. This methodology benefits greatly from experimental simplicity and can be applied to compounds where the plasma half life is only a few minutes. To validate this initial rates methodology the PPB measurements for compounds that were stable in plasma were compared to the measurements obtained from using a standard equilibrium dialysis methodology. This included the rat PPB of 3 compounds and the human PPB of enalapril. The rat PPB of enalapril was then estimated using the initial rates methodology.

2. Materials and methods

2.1. Materials

Potassium dihydrogenphosphate, disodium hydrogenphosphate, sodium chloride, formic acid, enalapril, verapamil, HPLC grade acetonitrile and DCC were purchased from Sigma-Aldrich Company (Dorset, UK). Warfarin was purchased from Fisher Scientific (Leicestershire, UK). Sildenafil was obtained from the AstraZeneca compound collection. Frozen human (pooled from 3 donors), rat (Sprague–Dawley), dog (Beagle) and guinea pig (Dunkin–Hartley) plasmas were sampled and processed by the Clinical Pharmacology Unit and Animal Units at AstraZeneca R&D Alderley Park (Cheshire, UK). Isotonic phosphate buffered saline (buffer) at pH 7.4 was prepared from potassium dihydrogenphosphate (1.77 g), disodium hydrogenphosphate (7.67 g), sodium chloride (4.38 g), and water (1 l).

2.2. Instrumentation

Centrifugations were carried out using a Heraeus Biofuge Fresco. Incubations were carried out in a Heraeus B15 incubator at 37 °C. A Dianorm® system with cells of 1 ml volume was used for equilibrium dialysis experiments, along with Diachema cellulose membranes with molecular weight cut off of 5 kDa (Dianorm, Munich, Germany). All HPLC analyses were carried out using a Waters 2777 auto-sampler, a Waters 2690 separations module and a Waters Quattro Ultima mass spectrometer using a selected ion recording quantitation method. Waters symmetry C8 5 μm × 3.9 mm × 20 mm columns were used along with a gradient of acetonitrile-aqueous (0.1%) formic acid (1:99, v/v) to acetonitrile-aqueous (0.1%) formic acid (99:1, v/v) at a flow rate of 2 ml/min over 5 min.

2.3. PPB using equilibrium dialysis

To one compartment of each of the dialysis cells were added 1 ml of plasma and 10 μl of a solution of the compound of interest at a concentration of 2 mM in DMSO. The other compartment of each dialysis cell was filled with 1 ml buffer. The cells were then sealed, clamped to the Dianorm unit, and rotated in a water bath at 37 °C for 18 h. The dialysis cells were then emptied and the plasma and buffer compartments solutions were treated in the following way such that the samples for HPLC/MS analysis were all present in an identical matrix of 6-fold diluted plasma. 100 μl of the plasma solution from the dialysis cell was added to 500 μl buffer. 500 μl of the buffer solution from the dialysis cell was added to 100 μl blank plasma. Four standards covering a 100-fold range in concentration were prepared for each compound using the 2 mM DMSO stock solution and 6-fold diluted plasma. The 6-fold diluted plasma samples were then directly injected into the HPLC/MS system for analysis. The plasma and buffer compartment concentrations were interpolated from the 4 point calibration line derived from the standards. These interpolated concentrations were then multiplied by the necessary factors to account for the sample dilutions prior to analysis, finally giving the concentration in plasma compartment of the dialysis cell ($[\text{Drug}]_{\text{plasma cell}}$) and concentration in the buffer compartment of the dialysis cell ($[\text{Drug}]_{\text{buffer cell}}$). The percent bound was then calculated using Eq. (1), where the factor of 1.05 accounts for the small dilution of the plasma which takes place through the osmotic volume shift during the dialysis experiment [21].

$$\%\ \text{Bound} = 100 \times \frac{1.05 \times ([\text{Drug}]_{\text{plasma cell}} - [\text{Drug}]_{\text{buffer cell}})}{1.05 \times ([\text{Drug}]_{\text{plasma cell}} - [\text{Drug}]_{\text{buffer cell}}) + [\text{Drug}]_{\text{buffer cell}}} \quad (1)$$

2.4. Kinetics of degradation in plasma

The reactions were initiated by addition of a 2 mM solution of the compound of interest in DMSO (50 μl) to plasma of the relevant species (5 ml), with incubation at 37 °C. Aliquots of the solution (250 μl) were removed at timed intervals and added to acetonitrile (500 μl) and vortex mixed to quench the reaction and precipitate the plasma proteins. These solutions were then centrifuged at 11,000 × *g* for 5 min before quantitation of the supernatants by HPLC/MS. It was assumed that the degradation of the compound in plasma followed pseudo first order kinetics, and this process is described by Eq. (2)

$$\frac{-\text{d}[\text{Drug}]_{\text{plasma}}}{\text{d}t} = k'[\text{Drug}]_{\text{plasma}} \quad (2)$$

where k' is the pseudo first order rate constant. $[\text{Drug}]_{\text{plasma}}$ is the concentration of drug in plasma. k' was then derived from the slope of a plot of ln(MS response) against time.

2.5. Kinetics for DCC adsorption

Fig. 1 shows the kinetic system in question. The drug undergoes reversible binding with plasma proteins and with DCC. The drug can also undergo irreversible chemical degradation in the plasma. If we first consider the situation where degradation does not occur then according to this scheme, the rate of loss of free drug concentration in the plasma, $[\text{Drug}]_{\text{free}}$, is given by Eq. (3)

$$\frac{-\text{d}[\text{Drug}]_{\text{free}}}{\text{d}t} = [\text{Drug}]_{\text{free}} \sum_i k_{i,\text{on}}[\text{P}]_i - \sum_i k_{i,\text{off}}[\text{Drug}]_{i,\text{bound}} + k_1[\text{Drug}]_{\text{free}}[\text{DCC}]_{\text{plasma}} - k_{-1}[\text{Drug}]_{\text{DCC}} \quad (3)$$

where the summations are over all of the binding sites on each of the proteins in the plasma, $[\text{P}]_i$ is the concentration of each of the protein binding sites, $k_{i,\text{on}}$ is the rate constant for binding of the drug to each of the binding sites, $[\text{Drug}]_{i,\text{bound}}$ is the concentration of bound drug at each of the binding sites, $k_{i,\text{off}}$ is the rate constant for dissociation of the drug from each of the binding sites, $[\text{DCC}]_{\text{plasma}}$