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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, $[\text{DCC}]_{\text{plasma}}$

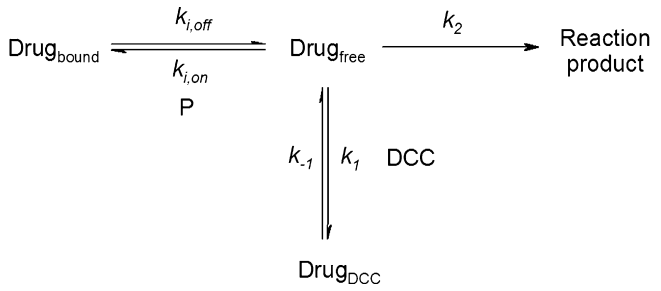

Fig. 1. Kinetic scheme for the adsorption of a plasma unstable compound to DCC in plasma. k_1 is the rate constant for drug adsorption to DCC and k_{-1} is the rate constant for drug desorption from DCC. k_2 is the rate constant for the degradation of free drug in plasma.

is the concentration of DCC within the plasma, $[\text{Drug}]_{\text{DCC}}$ is the concentration of the drug–DCC complex, k_1 is the rate constant for drug adsorption to DCC, and k_{-1} is the rate constant for drug desorption from DCC. Similarly, the rate of loss of plasma bound drug is given by Eq. (4)

$$\frac{-\text{d}[\text{Drug}]_{\text{bound}}}{\text{d}t} = -\sum_i \frac{\text{d}[\text{Drug}]_{i,\text{bound}}}{\text{d}t}$$

$$= \sum_i k_{i,\text{off}}[\text{Drug}]_{i,\text{bound}} - [\text{Drug}]_{\text{free}} \sum_i k_{i,\text{on}}[\text{P}]_i \quad (4)$$

The rate of change of total drug in plasma, $[\text{Drug}]_{\text{plasma}}$, is then given by the sum of Eqs. (3) and (4)

$$\frac{-\text{d}[\text{Drug}]_{\text{plasma}}}{\text{d}t} = -\left(\frac{\text{d}[\text{Drug}]_{\text{bound}}}{\text{d}t} + \frac{\text{d}[\text{Drug}]_{\text{free}}}{\text{d}t}\right)$$

$$= k_1\ fu[\text{Drug}]_{\text{plasma}}[\text{DCC}]_{\text{plasma}} - k_{-1}[\text{Drug}]_{\text{DCC}} \quad (5)$$

where fu is the free fraction of drug in plasma which is equal to $[\text{Drug}]_{\text{free}}/[\text{Drug}]_{\text{plasma}}$. The $[\text{Drug}]_{\text{plasma}}$ used in all plasma kinetic experiments is 20 μM (see experimental description in Sections 2.4 and 2.6), and the main contribution to the PPB is most likely due to the binding to albumin (present at approximately 600 μM), consequently fu is considered to be constant [22]. The substitution of $fu[\text{Drug}]_{\text{plasma}}$ for $[\text{Drug}]_{\text{free}}$ in Eq. (5) assumes rapid PPB equilibration which is reasonable considering typical binding kinetics to albumin [23]. If we now consider the situation where pseudo first order kinetic degradation takes place of free drug from within the plasma then Eq. (5) needs an additional term. Eq. (6) contains the additional term $k'[\text{Drug}]_{\text{plasma}}$ (see Eq. (2)) which can be determined experimentally from plasma degradation experiments

$$\frac{-\text{d}[\text{Drug}]_{\text{plasma}}}{\text{d}t} = k_1\ fu[\text{Drug}]_{\text{plasma}}[\text{DCC}]_{\text{plasma}} - k_{-1}[\text{Drug}]_{\text{DCC}}$$

$$+k'[\text{Drug}]_{\text{plasma}} \quad (6)$$

With respect to the kinetic system displayed in Fig. 1, $k' = k_2 fu$ where k_2 is the rate constant for the degradation of free drug from plasma. If we now consider only the initial rate of the reaction (where $[\text{Drug}]_{\text{DCC}} = 0$), we can derive Eq. (7)

$$\nu_{\text{plasma}} = -\left[\frac{\text{d}[\text{Drug}]_{\text{plasma}}}{\text{d}t}\right]^{t=0}$$

$$= k_1\ fu[\text{Drug}]^{t=0}_{\text{plasma}}[\text{DCC}]^{t=0}_{\text{plasma}} + k'[\text{Drug}]^{t=0}_{\text{plasma}} \quad (7)$$

If we now consider the drug binding to DCC in buffer, rather than in plasma, then the kinetics will be described by Eq. (8)

$$-\frac{\text{d}[\text{Drug}]_{\text{buffer}}}{\text{d}t} = k_1[\text{Drug}]_{\text{buffer}}[\text{DCC}]_{\text{buffer}} \quad (8)$$

Hence the initial rate will be given by Eq. (9)

$$\nu_{\text{buffer}} = -\left[\frac{\text{d}[\text{Drug}]_{\text{buffer}}}{\text{d}t}\right]^{t=0} = k_1[\text{Drug}]^{t=0}_{\text{buffer}}[\text{DCC}]^{t=0}_{\text{buffer}} \quad (9)$$

Combination of Eqs. (7) and (9) then gives to Eq. (10)

$$\frac{\nu_{\text{plasma}}}{\nu_{\text{buffer}}} = \frac{k_1\ fu[\text{Drug}]^{t=0}_{\text{plasma}}[\text{DCC}]^{t=0}_{\text{plasma}} + k'[\text{Drug}]^{t=0}_{\text{plasma}}}{k_1[\text{Drug}]^{t=0}_{\text{buffer}}[\text{DCC}]^{t=0}_{\text{buffer}}} \quad (10)$$

If the same initial concentration of drug is used (see experimental description in Section 2.6), in all the kinetic studies (i.e. $[\text{Drug}]_{\text{plasma}} = [\text{Drug}]_{\text{buffer}}$), then Eq. (10) can be simplified to Eq. (11)

$$\frac{\nu_{\text{plasma}}}{\nu_{\text{buffer}}} = \frac{fu[\text{DCC}]^{t=0}_{\text{plasma}}}{[\text{DCC}]^{t=0}_{\text{buffer}}} + \frac{k'}{k_1[\text{DCC}]^{t=0}_{\text{buffer}}} \quad (11)$$

A rearranged version of Eq. (9) (for k_1), can be substituted into Eq. (11) and subsequent rearrangement will finally give an expression for fu, Eq. (12)

$$fu = \frac{\nu_{\text{plasma}} - k'[\text{Drug}]^{t=0}_{\text{buffer}}}{\nu_{\text{buffer}}} \frac{[\text{DCC}]^{t=0}_{\text{buffer}}}{[\text{DCC}]^{t=0}_{\text{plasma}}} \quad (12)$$

Hence fu can be determined from the rates of binding of the compound to DCC in the presence and absence of plasma, along with a determination of the rate of degradation in plasma. For compounds that are stable in plasma (i.e. $k' = 0$), Eq. (12) reduces to the more simple form given by Eq. (13)

$$fu = \frac{\nu_{\text{plasma}}}{\nu_{\text{buffer}}} \frac{[\text{DCC}]^{t=0}_{\text{buffer}}}{[\text{DCC}]^{t=0}_{\text{plasma}}} \quad (13)$$

2.6. DCC adsorption methodology

For each compound two initial rate experiments were carried out. One experiment contains a solution of the compound in buffer and DCC, and the other experiment contains the compound in plasma and DCC. Into 10 centrifuge tubes was placed buffer or plasma (1980 μl) along with DCC. The DCC concentrations that were employed ranged from 0.05 to 5 mg/ml and each tube was incubated at 37 °C and stirred magnetically. A further incubation, with the DCC excluded, was also prepared in order to generate the MS response of compound at zero time. The DCC concentration was dependent on the compound and if the experiment involves buffer or plasma.

The adsorption process was initiated by the sequential addition of a 2 mM solution of the compound of interest in DMSO (20 μl) at timed intervals. The time intervals were further apart at the beginning of the experiment than at the end, since the tubes were all centrifuged together at the end of the incubation time at 11,000 × *g* for 15 s to sediment the DCC. Hence the last few reactions to be initiated became the samples with the shortest incubation times. For a buffer experiment the supernatants were then quantified by HPLC/MS without further preparation. For a plasma experiment, supernatant (250 μl) was added to acetonitrile (500 μl) and vortexed in order to quench the reactions and precipitate the proteins, followed by further centrifugation at 11,000 × g for 2 min. The final supernatant was then quantified by HPLC/MS. The incubations were followed for a maximum of 10 min. The data were then analyzed by plotting concentration versus time and carrying out a least squares fit of the data to a quadratic equation using Microsoft Excel.

Table 1
Kinetics of adsorption to DCC and equilibrium dialysis data on compounds stable in rat plasma.

Compound	$10^{-8}\nu_{buffer}$ (mol dm^{-3} s^{-1})	$10^{-8}\nu_{plasma}$ (mol dm^{-3} s^{-1})	% Bound by charcoal binding kinetic method	% Bound by equilibrium dialysis method
Sildenafil	2.64[a] ± 0.48	3.48[b] ± 0.72	86.8 ± 3.6	86.1 ± 3.4
Verapamil	1.49[a] ± x0.13	3.14[b] ± 0.34	78.9 ± 2.9	81.7 ± 4.5
Warfarin	18.2[b] ± 2.9	1.17[c] ± 0.34	99.4 ± 0.21	99.4 ± 0.20

Errors show standard deviation from 3 repeat measurements.
Initial drug concentration = 20 μM in all experiments.
[a] [DCC] = 0.05 mg/ml.
[b] [DCC] = 0.5 mg/ml.
[c] [DCC] = 5.0 mg/ml.

The initial rate of loss of compound was then derived by differentiation of the derived quadratic equation in order to find the slope of the curve at $t = 0$.

3. Results

Initial studies were carried out using 3 compounds that are stable in plasma. The rates of adsorption of sildenafil, verapamil and warfarin to DCC were determined in both buffer and rat plasma according to the method described in the experimental section. The observed rates, from triplicate experiments, are given in Table 1 along with the percent bound to plasma calculated using Eq. (13). The binding of these 3 compounds to the same batch of rat plasma was also determined using a standard equilibrium dialysis approach. These data are also shown in Table 1, and values determined from the two different approaches are consistent. The rate of degradation of enalapril was determined in human and rat plasma and the pseudo first order rate constants, k', are given in Table 2. k' for enalapril degradation in rat plasma was found to be 4.9×10^{-4} s^{-1} which corresponds to a half life of 24 min. As expected significant degradation was observed in rat plasma but not in human plasma [16,17]. The rate of adsorption of enalapril to DCC was then determined in buffer, rat plasma and human plasma, and the observed rates are given in Table 2. Since enalapril is stable in human plasma, the binding to human plasma using DCC kinetics can be calculated using Eq. (13), and the result is given in Table 2 along with the value determined using conventional equilibrium dialysis. The short rat plasma half life of enalapril complicates the kinetics of adsorption to DCC, since the observed loss of compound is due to both adsorption to DCC and plasma induced degradation. The modified kinetic model is therefore required for deriving the extent of plasma binding from the kinetic data with use of Eq. (12). This method leads to a percent bound value of 50.3 ± 12.2 in rat plasma (Table 2). Clearly, the binding of enalapril to rat plasma was not determined using equilibrium dialysis since the rate of degradation is too fast compared to the timescale of a dialysis experiment, and hence the experiment would not reach equilibrium. However, in addition to its stability in human plasma, enalapril was also found to be stable in dog and guinea pig plasma, and equilibrium dialysis was therefore used to determine the percent bound to dog plasma as 51.6 ± 9.4 and the percent bound to guinea pig plasma as 56.7 ± 7.3.

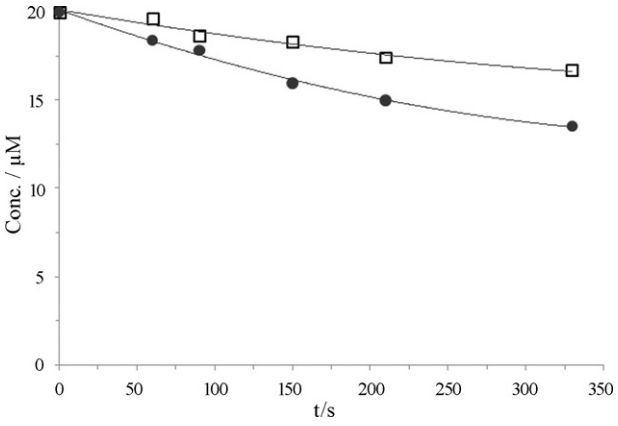

Fig. 2. Adsorption of verapamil to 0.05 mg/ml DCC in buffer (□), and to 0.5 mg/ml DCC in rat plasma (●).

4. Discussion

4.1. DCC concentration

In order to validate the use of initial rates kinetics of adsorption to DCC as a method of plasma binding determination, 3 plasma stable drugs were chosen for study. The 3 compounds (sildenafil, verapamil and warfarin) were selected since they exhibit a range of extent of plasma binding and charge type (predominantly neutral, positively charged, and negatively charged at pH 7.4, respectively). The rate of adsorption of these compounds to DCC in both buffer and rat plasma was determined, and some example data is shown for verapamil in Fig. 2. An important aspect of the DCC kinetic plasma binding method is the selection of suitable DCC concentrations in the buffer and plasma experiments. For more highly plasma bound compounds it is necessary to use a higher DCC concentration in plasma than in buffer. This is because the low free concentration of compound in plasma will lead to a very low rate of DCC adsorption, which will in turn lead to an imprecise rate measurement when using a short timescale experiment, or will necessitate a very long timescale experiment for a precise rate to be determined. A suitable rate for measurement on a convenient timescale can easily be achieved by using a higher DCC concentration in plasma than in buffer. Under the experimental conditions chosen, the rate of adsorption of verapamil to DCC is actually faster in plasma than in

Table 2
Kinetics of adsorption to DCC and equilibrium dialysis data for enalapril.

Species	$10^{-4}k'$ (s^{-1})	$10^{-8}\nu_{buffer}$ (mol dm^{-3} s^{-1})	$10^{-8}\nu_{plasma}$ (mol dm^{-3} s^{-1})	% Bound by charcoal binding kinetic method	% Bound by equilibrium dialysis method
Human	No reaction	6.8[a] ± 1.1	2.8[a] ± 0.4	59.6 ± 8.9	64.4 ± 7.6
Rat	4.9 ± 0.8	6.8[a] ± 1.1	4.4[a] ± 0.6	50.3 ± 12.2	Not determined

Errors show standard deviation from 3 repeat measurements.
Initial drug concentration = 20 μM in all experiments.
[a] [DCC] = 0.25 mg/ml.

buffer (Fig. 2). This is because the concentration of DCC was 10-fold higher in plasma than in buffer while the free concentration of verapamil in plasma is 5-fold lower than that in buffer (since it is ~80% bound in rat plasma). This results in the approximately 2-fold higher rate of adsorption to DCC in rat plasma than in buffer (Table 1).

4.2. Validation of the initial rates DCC adsorption methodology for plasma stable compounds

There is good correspondence between the plasma binding data determined using DCC adsorption and the data determined using equilibrium dialysis for the 3 plasma stable compounds. Unpublished data on a range of proprietary compounds also shows good agreement between the initial rates DCC adsorption method and equilibrium dialysis. Due to their plasma stability over the time course of the experiment these 3 compounds provide validation for Eq. (13). This should be expected since published methods have already been validated which differ only in that they either utilize the whole time course of compound adsorption or they simply use the final equilibrium concentrations [16–18].

4.3. Application of the modified method to determine the rat PPB of enalapril

The difference between Eq. (12) and Eq. (13) is that the former accounts for chemical degradation in plasma of a compound. This modified equation only needs to be applied when the plasma half life of the compound is rapid compared to the time course of the experiment. Extensive validation of this modified method is difficult since a traditional slow method such as equilibrium dialysis cannot be used to measure the plasma binding of a compound which exhibits fairly rapid degradation in plasma. However, enalapril is a compound that is useful for this problem since it is stable in human plasma but undergoes rapid ester hydrolysis in rat plasma. Therefore the binding to human plasma can be determined using both DCC adsorption kinetics and equilibrium dialysis, and the binding to rat plasma can be determined using DCC adsorption kinetics. Assuming that there is little interspecies difference in the plasma binding of enalapril, it is reasonable to expect similar binding to rat plasma, to that observed in human plasma where the compound is stable.

Using the initial rates DCC adsorption method the percent bound in human plasma for enalapril was 59.6 ± 8.9. This result compares favorably with the equilibrium dialysis value of 64.4 ± 7.6, and with the previously published value of 50% [24], again confirming the applicability of the initial rates DCC adsorption method to compounds that are stable in plasma.

The kinetics of DCC adsorption was determined in rat plasma and in buffer, and some of these data are shown in Fig. 3 along with the kinetics of degradation in rat plasma. Since low plasma binding was expected, the same DCC concentration was used in rat plasma and in buffer. Consequently it can be seen in Fig. 3 that the rate of DCC adsorption is slower in plasma than in buffer, and that these rates are slower than the rate of degradation in plasma. These three rates are then used to calculate the percent bound to rat plasma as 50.3 ± 12.2 (Table 2). It follows from the observation that there are only very small interspecies variations in the PPB value of enalapril in plasmas where it is chemically stable (i.e. human, dog and guinea pig), that this rat PPB value shows good correspondence. Furthermore, the observation that the percent bound of enalapril to rat plasma is slightly less than that to human plasma is consistent with what would typically be expected for interspecies differences in PPB [4].

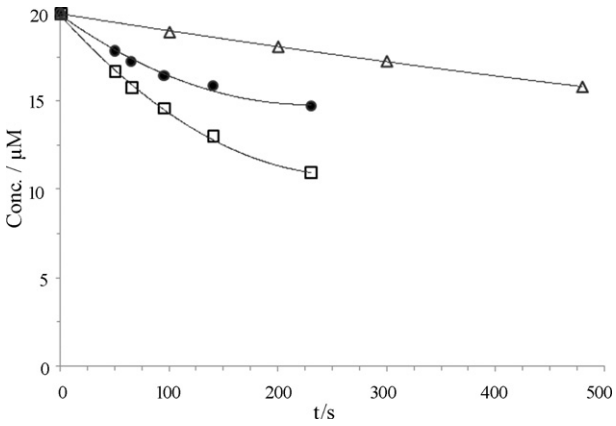

Fig. 3. Degradation of enalapril in rat plasma (△). Adsorption of enalapril to 0.25 mg/ml DCC in buffer (□), and to 0.25 mg/ml DCC in rat plasma (●).

4.4. Further work and considerations

Even though enalapril has a rat plasma half life of 24 min the initial rates DCC adsorption method along with Eq. (12) enables the rat PPB to be determined. Furthermore, unpublished work has been conducted to determine the PPB of a wide range of proprietary compounds with plasma half lives as short as 2 min, where convenient rates of DCC adsorption were achieved through the suitable manipulation of the concentration of DCC used. An assumption that is made regarding the degradation of compound in plasma is that the observed plasma degradation follows pseudo first order kinetics but if this is not the case then Eq. (12) is not applicable.

This method could in principle be applied to compounds exhibiting even more rapid degradation through the use of dilute plasma along with the method of Wan et al. [10] for the extrapolation of the data back to that in undiluted plasma. However, for compounds that predominantly bind to α_1-acid glycoprotein (AAG), which is present at approximately 10–30 μM in plasma, it is important that the AAG concentration in the diluted plasma is at least 10 times greater than the compound concentration [4]. The determination of binding to individual plasma proteins, i.e. serum albumin, may be an alternative strategy for estimating the PPB for a plasma unstable compound. However, preparations of serum albumin may still contain significant amounts of the enzymes, i.e. esterases, which could still cause a compound to significantly degrade over the experiment's time course. With respect to compounds that are substrates from esterases, the use of inhibitors could also be considered to minimize any degradation [25]. Practically, it may be difficult to obtain enough data points to fit a quadratic curve to extract the rate constants from the DCC absorption kinetics using the described methodology. In such cases, the rate constant may be extracted by fitting a linear line but it must be acknowledged that the associated experimental error to the PPB measurement will probably increase. Interestingly, when using just the first two time points to extract the DCC absorption initial rates in buffer and plasma, the rat PPB measurement for enalapril is 50.4% which is almost identical to the value determined using the more formal methodology.

5. Conclusions

Enalapril displays rapid rat plasma instability that therefore prevents its PPB being determined using conventional methods. A simple initial rates DCC adsorption method that accounts for enalapril's plasma instability has been developed and can be carried out without the use of very specialized equipment. Experiences with proprietary compounds with very rapid plasma instability have shown this method to have wider applicability. Although more

compounds are needed to properly validate this methodology it nonetheless should be of interest to groups looking for a method of determining the PPB of plasma labile prodrugs and antedrugs.

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