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Studies of drug binding to plasma proteins using a variant of equilibrium dialysis

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Abstract

The plasma protein binding of three model compounds was investigated using a variant of equilibrium dialysis, denoted comparative equilibrium dialysis (CED), and the results were compared with those obtained with ultrafiltration (UF). In CED, the buffer that the plasma is dialysed against in traditional equilibrium dialysis is replaced by, for example, plasma from other species. The CED method has the advantage that the unbound concentration (C_u) does not need to be measured, which can be difficult for drugs with extremely small unbound fractions. Instead, the ratio of the total drug concentration (C_{tot}) on either side of the dialysis membrane at equilibrium is a direct measure of the relative binding properties of the two plasma types. For the first model compound, having an unbound fraction (f_u) of about 0.05% in human plasma, the time to reach equilibrium was too long ($\geq$40 h) to make the CED technique feasible in practice. For the second model compound, the more weakly bound drug NAD-299 (with an unbound fraction of about 2% in human plasma), the CED equilibration times were considerably shortened ($\leq$16 h), and the technique was applied to plasma from three different species. Large discrepancies between the CED and UF results were seen, CED always giving rise to much lower C_{tot} differences than expected from the UF results. It is suspected that this discrepancy was due to equilibration between the dialysis chambers of all plasma components with a molecular weight less than the cut-off of the membrane. This equilibration causes altered binding properties compared to the initial plasma. When performing ultrafiltration on plasma where drug was added to untreated plasma or added to blank plasma that was equilibrated against plasma from the same or from another species, the change of binding properties was confirmed. To ensure that the results were not specific for NAD-299, a third model compound, tolterodine, was also included. The same trends as for NAD-299 were seen.

Because of the long equilibration times for compounds with high protein binding and, in particular, the suspected partial mixture of low molecular weight compounds from the two plasma types and the subsequent change of binding properties, we cannot recommend the CED method as a tool for studying relative protein binding.

Keywords: Plasma protein binding; Unbound concentrations; Equilibrium dialysis; Ultrafiltration

1. Introduction

The two predominant techniques for studying drug protein binding in plasma are equilibrium dialysis (ED) and ultrafiltration (UF). UF today, which is probably the most common method for the determination of unbound plasma concentrations (C_u), is a rapid procedure (15–45 min being a typical range) and is very simple to use, since commercial devices are available. Recently, 96-well plates for UF were also introduced (Millipore Corp., Danvers, MA, USA), making this technique even more attractive. A major drawback is that adsorption to the UF device and the filter can be a source of significant error in this type of experiment. Non-specific adsorption must be checked for all compounds where UF is used for generating unbound fractions or concentrations. Another drawback is protein leakage across the filter, which can cause erroneously high free concentrations for highly bound compounds.

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The other common technique for determining the protein binding of a drug is equilibrium dialysis. The ED device contains two chambers, divided by a semipermeable membrane that only allows the passage of molecules with a molecular weight less than the molecular weight cut-off (MWCO) of the membrane. In a typical ED experiment, plasma (the 'retentate')—containing the drug—is on one side of the membrane and a buffer (the 'dialysate') is placed in the other dialysis chamber. While ED is not as simple to use as UF, it has the advantage that non-specific adsorption can be compensated for if the concentration at equilibrium on each side is measured, which means that the adsorption will not affect the concentration ratio at equilibrium, only the mass balance. One drawback is the relatively long times (>20 h) often needed to reach equilibrium, which could give rise to degradation and changes in the pH of the plasma in the course of the dialysis. Another drawback is that the unbound fraction at equilibrium differs from the initial unbound fraction if the binding is concentration-dependent, thus the unbound fraction should always be related to the total concentration measured on the plasma side at equilibrium. This also means that the actual free concentration of an in vivo sample cannot be determined using ED if the binding is concentration dependent. A frequently discussed problem of ED experiments is the volume shift [1], the flow of fluid from the buffer side to the plasma side. The volume shift is caused by an influx of the dialysate to the retentate, due to the osmotic pressure of the plasma proteins. The effects of volume shifts are often complicated by the fact that plasma diluted with buffer might change its binding properties in an unpredictable way, i.e. the dilution is not ideal and uniform. Factors, such as changes in ionic strength or pH, could result in significant changes in the binding properties of the proteins involved. Another commonly identified problem with the ED method is the uneven distribution of low molecular weight ions, the Gibbs–Donnan effect, due to the fact that charged proteins cannot pass through the membrane, which results in a flow of small ions across the membrane to achieve electroneutrality. The problem is reduced by using an isotone phosphate buffer to which electrolytes (i.e. NaCl) are added to diminish the difference in ionic strength on either side of the membrane [2]. ED has also the reputation as being a rather laborious and time consuming technique although some recent papers show that high throughput approaches using the 96-well format are possible [3,4].

From the above, it can be understood that both techniques have their problems and that all unbound fraction/unbound concentration results must be looked at critically. The search for a general methodology has resulted in a plethora of publications describing alternative methods, including ultracentrifugation, frontal analysis chromatography, affinity chromatography, negligible extraction methods and erythrocyte/plasma distribution [5–7].

The objective of this study was to investigate the usefulness of a recently published variant of the ED method where the drugs valproate and monoacetyl dapsone were studied, both compounds showing high plasma protein binding [8]. This method will be referred to as comparative equilibrium dialysis (CED). In CED, plasma (usually spiked) from two different sources (species, individuals, etc.) is placed on either side of the dialysis membrane instead of, as in traditional ED, plasma on one side and buffer on the other. At equilibrium, the C_{tot} measured on either side of the membrane reflects the ratio between the respective unbound fractions (see Fig. 1). This relative binding for two species would be very valuable when, for example, scaling the pharmacokinetics from animals to humans, since information about the 'equivalent total concentrations' for a given unbound drug concentration is ideally obtained in this way. Also, in the case of drugs with extremely high protein binding, the low unbound concentrations can be difficult to measure with acceptable precision. In a CED experiment, this problem is circumvented, as high total concentrations are determined instead. CED would therefore also be an excellent tool for verifying whether two determined unbound fractions are correct, e.g. whether the species with a determined protein binding of 99.4% really has a three times higher unbound fraction than the species with a protein binding of 99.8%. Another interesting possibility opens up for compounds with very large species variations in protein binding: if the absolute unbound fraction in the species with the lowest protein binding can be determined with acceptable precision, it would be possible to obtain a good estimate of the absolute unbound fraction for all species via a series of CED experiments.

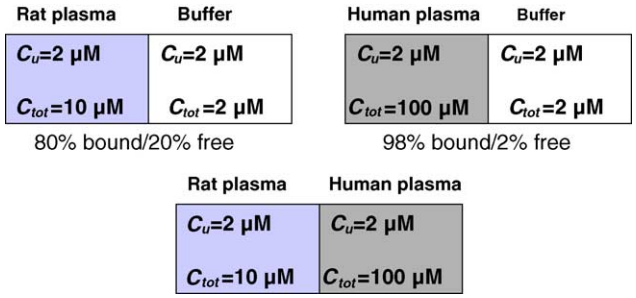

Fig. 1. Principle of comparative equilibrium dialysis [8]. The top panels represent traditional ED, where plasma is on one side of the membrane and a buffer solution is on the other side. The bottom panel describes a CED experiment, where rat and human plasma are equilibrated against each other. At equilibrium (i.e. when C_u is equal on both sides) C_{tot} for the human plasma side is 10 times higher than for the rat plasma side. This is also the inverse of the relationship for the unbound fractions (f_u) of the two species: f_u values for rat plasma and human plasma are 20 and 2%, respectively.

The relationship between the unbound and total drug concentrations in plasma in vivo is described by:

$$C_{tot} = \frac{C_u}{f_u} \quad (1)$$

where C_u is determined by dose rate and unbound clearance and f_u is a function of drug–protein affinity and the concentration of binding sites. The total plasma concentration then becomes a function of C_u and f_u.

In a CED experiment, using plasma from two different species, denoted I and II, at equilibrium the unbound concentrations (C_u) are the same on both sides:

$$C_u^I = C_u^{II} \quad (2)$$

From Eq. (1), it can be seen that at a given unbound concentration the total concentration in each chamber will be governed by the unbound fraction of the drug in the chamber. Thus,

$$C_{tot}^I f_u^I = C_{tot}^{II} f_u^{II} \quad (3)$$

or (rearranged)

$$\frac{C_{tot}^I}{C_{tot}^{II}} = \frac{f_u^{II}}{f_u^I} \quad (4)$$

Eq. (4) shows that the ratio between the determined total concentrations of two different types of plasma is the same as the inverse ratio between the unbound fractions of the same plasma types. Thus, determination of the total concentration in each chamber can be used to study the relationship between the unbound fractions of two species, for example, and if the absolute unbound fraction is known for one species, the other unbound fraction can be calculated.

Three model compounds with differing protein-binding properties were used to study this promising variant of ED, some properties being shown in Table 1. The first compound, referred to as Compound 1, shows very high plasma protein binding in humans (predominantly to albumin, unbound fraction about 0.05%). The unbound fraction in the rabbit is considerably higher, 1.5%, offering good possibilities for comparison with human plasma in CED experiments. The second compound, NAD-299, was chosen since it does not bind to plasma proteins to the same extent [9]. It is a basic drug, binding mainly to α_1-acid glycoprotein (α_1-AGP). The between-species variation of the unbound fraction is also large (Table 1), which should facilitate the interpretation and validation of the CED method. Tolterodine, which also shows large species differences in the degree of plasma protein binding [10], was chosen as a third model compound to further investigate the CED method.

Table 1
Some properties of the studied compounds

Compound	pK_a	Major plasma binding protein	Typical unbound fractions in plasma (%)
Compound 1	5.2/10.6	Serum albumin	0.05 (human) 1.5 (rabbit)
NAD-299	7.4	α_1-AGP	2 (human) 25 (dog) 36 (rat)
Tolterodine	9.9	α_1-AGP	3.7 (human) 2.1 (dog) 22 (rat)

Data for Compound 1 and NAD-299 are in-house data determined with ultrafiltration and after pH and temperature adjustment. Tolterodine data are from ref. [10]. The unbound fractions are obtained at slightly different total concentrations.

2. Experimental

2.1. Materials

All water was purified in a Milli-Q filtration system (Millipore Corp., Bedford, MA, USA). For the phosphate buffer, 34.5 g $NaH_2PO_4 \cdot H_2O$ (Merck) and 22.2 g $Na_2HPO_4 \cdot 2H_2O$ (Merck) were each dissolved in 250 mL of water, giving concentrations of 1.00 and 0.50 M, respectively. Fifteen millilitres of the solution containing Na_2HPO_4 was diluted to 0.015 M and the pH was adjusted to 7.4 with the solution containing NaH_2PO_4. For soaking of the dialysis membranes, an isotone buffer [3] was prepared: 4.00 g $Na_2HPO_4 \cdot 2H_2O$, 0.77 g $NaH_2PO_4 \cdot H_2O$ and 5.78 g NaCl (Merck) were dissolved in 1 L of water. The pH of this solution was adjusted with NaH_2PO_4 to 7.35, and the resulting isotone buffer had an ionic strength of 0.168, which is close to that of plasma. For the tolterodine ED experiments, a purchased isotone phosphate buffer solution, Dulbecco's buffer (Sigma–Aldrich, St. Louis, MO, USA) was used.

All plasma was pre-treated by thawing, mixing and centrifugation at approximately 1800 × *g* for 5 min. Prior to the UF/ED dialysis experiments, the pH of the plasma was adjusted to 7.4 by incubating the plasma for 20 min in an incubator with 9% CO_2 at 37 °C [9].

2.2. Compound 1 experiments

0.07955 g of Compound 1 (molecular weight (MW) 491.4 g, synthesized at AstraZeneca) was dissolved in 5 mL DMSO (Merck), giving a concentration of 31.57 mM (solution 1a). A 0.1 mL of solution 1a was diluted to 10 mL with DMSO, giving a concentration of 315.7 μM (solution 1b). A 0.1 mL of solution 1a was then diluted to 5 mL with DMSO, resulting in a concentration of 631.34 μM (solution 1c). The standard solutions were prepared from solutions 1a to c by appropriate dilutions with the phosphate buffer described above. The concentration range of the standard solutions was 1–100 μM. A $^{13}C_6$ isotope of Compound 1 (MW = 497.4 g, synthesized at AstraZeneca) was used as internal standard (IS). The IS was dissolved in a solution containing 75% ethanol/25% water with 2% formic acid to a concentration of 19.8 μM. The working IS was further diluted to 4.95 μM with the same solvent mixture.

For the ED experiments, pooled human and rabbit plasma was treated and pH-adjusted as above prior to the dialysis. Two sizes of teflon chambers were used, depending on the purpose of the experiment. Volume shifts can be difficult to measure if the volume is too small, and for these experiments, 1 mL chambers were used, filled with 0.8 mL plasma per chamber. If we were only interested in measuring the total concentration of Compound 1 on each side of

the membrane, smaller chambers, containing 200 μL (filled with 170 μL per chamber) were used. The membranes for the dialysis (Spectra/Por® RC, Spectrum Laboratories, Rancho Dominguez, CA, USA) had a MWCO of 12–14 kDa in all experiments. Prior to use, the membranes were soaked for 1 h in isotone buffer and thereafter rinsed twice with fresh buffer. The time to reach equilibrium was tested by collecting samples from each chamber at 1–4, 6, 16, 24 and 40 h. The ED experiments were always performed at 37 °C and at agitation.

A 50 μL sample from the dialysis experiments was mixed in a test tube with 200 μL IS solution (4.95 μM) and the mixture was incubated for 10 min. A 1 mL of a 1:1 mixture of *n*-heptane and ethylacetate was then added and the tubes were rotated for 30 min, followed by centrifugation at 1800 × *g* for 5 min. About 0.8 mL of the organic phase was withdrawn and evaporated to dryness. Prior to analysis with LC–MS/MS, the samples were dissolved in 100 μL of 0.1% acetic acid in 50% ethanol/50% water (v/v).

For the LC–MS/MS analysis, the following mobile phases were prepared: (A) 20 mL of acetonitrile and 0.3 mL of formic acid were mixed and diluted with water to 1000 mL. (B) 20 mL of water, 0.3 mL of formic acid and 100 mL of isopropanol were mixed and diluted with acetonitrile to 1000 mL. A Symmetry Shield® RP_8 2.1 mm × 50 mm, 3.5 μm LC-column (Waters Corp., Milford, MA, USA) was used, the column temperature was kept at 40 °C and the pump (LC-10AD, Shimadzu Corp., Kyoto, Japan) flow rate was 0.300 μL/min. The mobile phase gradient started with 33% mobile phase B and from 1 to 3 min the composition of the mobile phase changed to 80% mobile phase B, which resulted in a retention time of about 2.4 min for both Compound 1 and IS. Samples of 30 μL were injected into the LC system using an HTS PAL autosampler (CTC Analytics AG, Zwingen, Switzerland).

The mass spectrometer used was a Quattro Micro with QuanLynx 4.0 software (Micromass Ltd., Manchester, UK). The mass spectrometer was operated in the negative electrospray ionisation (ESI−) mode and responses were measured using multiple reaction monitoring.

2.3. NAD-299 experiments

NAD-299 [9,11] was synthesized at the Chemistry Department, AstraZeneca R&D Södertälje, Sweden. The buffer solution was prepared by adding 0.77 g of ammonium acetate and 1 mL of formic acid to 800 mL of water. A 12.632 mg of NAD-299 (MW = 486.5 g) and 12.418 g IS (a tri-deuterated NAD-299, MW = 489.5 g) were dissolved in 25.965 and 25.369 mL of buffer, respectively, giving concentrations of the stock solutions of 1.000 mM. The stock solutions of NAD-299 were diluted with the buffer to working standards in the range 1 nM–100 μM while the working IS solution was diluted to 1 μM.

The UF and ED experiments were performed as described for Compound 1, except that pooled plasma from humans, dogs and rats was used instead. After the first comparative equilibrium dialysis experiments, it was concluded that 16 h was enough to reach equilibrium. The small teflon dialysis chambers were used, whenever possible.

A 50 μL of dialysate or ultrafiltrate was mixed with 50 μL of IS (1 μM), 100 μL of 1.00 M NaOH and 2 mL of diethyl ether. The mixture was then extracted in a rotating mixer for 6 min and centrifuged at 2200 rpm for 10 min. The aqueous phase was then frozen in the extraction tubes with a mixture of ethanol/dry ice and about 1 mL of the organic phase was transferred to a 96-well plate. The organic phase was then evaporated to dryness and redissolved in 100 μL of buffer.

The mobile phase for LC–MS/MS was prepared by mixing 155 mL of the ammonium acetate–formic acid buffer (above) with 800 mL of acetonitrile. The LC pump was run in the isocratic mode using a flow rate of 0.35 mL/min. A retention time of about 2.0 min was obtained for both NAD-299 and IS. The injected volume was 20–30 μL. The mass spectrometer was run in the positive electrospray ionisation mode (ESI+) with multiple reaction monitoring. Precursor ions at *m*/*z* 319 for NAD-299 and at *m*/*z* 322 for the IS, respectively, were further fragmented into the monitored product ions at *m*/*z* 194 (NAD299) and *m*/*z* 197 (IS). The same LC-column, LC pump, autosampler and mass spectrometer as for the Compound 1 experiments were used.

2.4. Tolterodine experiments

Tolterodine tartrate (Dayang Chemicals, Shanghai, China), 0.126 mg (MW = 475.6 g), was diluted in 1.060 mL of a solution containing 25% DMSO and 75% 0.025 M formic acid in water, giving a concentration of 250 μM in the stock solution. Bupivacaine (AstraZeneca, Södertälje, Sweden), a 0.5 μM solution dissolved in 0.025 M formic acid in water, was used as internal standard. The stock solution of tolterodine was diluted with ultrafiltrate from human plasma to working standards in the range 10–1000 nM.

The ED and UF experiments were performed using pooled human and rat plasma. In view of the similar f_u values of tolterodine and NAD-299 and the results from the CED experiments of NAD-299, it was concluded that 16 h should be sufficient to reach equilibrium. The CED was run in a 96-well ED apparatus HTD96a (HTDialysis, Gales Ferry, CT, USA), with 150 μL in each dialysis chamber. Prior to dialysis, the membrane strips (MWCO = 12–14 kDa) were soaked for 1 h in Dulbecco's buffer, thereafter for 20 min in the buffer, mixed with 20% ethanol. Finally, the strips were rinsed twice with fresh buffer. After the CED, the samples on either side of the dialysis membrane were pooled five-by-five in order to produce five pooled samples of each type of CED combination. After the CED, the pooled samples were pH-adjusted as above, spiked with 1 μM tolterodine, followed by ultrafiltration using the same equipment as earlier.

The samples were prepared by taking 50 μL of ultrafiltrate and mixing it with 50 μL of IS (0.5 μM bupivacaine in 0.025 M formic acid). The analysis was performed by gradi-

ent elution LC–MS/MS using the following mobile phases: (A) 5% (v/v) acetonitrile and 95% water with 0.025 M formic acid, (B) 95% acetonitrile and 5% water with 0.025 M formic acid. An ACE 3 C18 2.1 mm × 50 mm column (ACT, Aberdeen, Scotland) was used with a flow rate of 0.4 mL/min and the injected volume was 25 μL. The mass spectrometer was run in the positive electrospray ionisation (ESI+) mode. As for the other compounds, the multiple reaction monitoring (MRM) mode was used, the precursor ions being at *m*/*z* 326.1 for tolterodine and at *m*/*z* 289.0 for the IS (bupivacaine). The precursor ions were further fragmented into the monitored product ions at *m*/*z* 147.1 (tolterodine) and at *m*/*z* 140.0 (IS). The same LC pump, autosampler and mass spectrometer as for the other experiments were used.

3. Results and discussion

3.1. Compound 1 experiments

As an initial experiment, the time required to reach equilibrium between two dialysis chambers was investigated, i.e. when the values of C_{tot} on both sides of the dialysis membrane were constant if plasma from different species was used on each side or, if the same plasma was used on both sides, when C_{tot} was the same on both sides. In Fig. 2(a) the time course of C_{tot} is shown for a CED experiment with human plasma in both chambers, where one side was spiked with 100 μM of Compound 1 and the other side was initially blank. Fig. 2(b) is similar to Fig. 2(a), but instead there was rabbit plasma in both chambers. Finally, Fig. 2(c) shows the time evolution of C_{tot}, now with spiked rabbit plasma (100 μM) in one chamber and blank human plasma in the other. The rate of equilibration was closely correlated with the extent of drug plasma protein binding, in accordance with Fick's first diffusion law, which states that the rate at which equilibrium is reached is governed by, among other parameters, the difference in concentration at both sides of the membrane. For example, for this compound, rabbit plasma is known to have the highest unbound fraction of all the species so far measured, the unbound fraction being as high as about 1.5%, compared to about 0.05% in human plasma (see Table 1). After 22 h, the system seemed to be close to equilibrium with rabbit plasma in both cham-

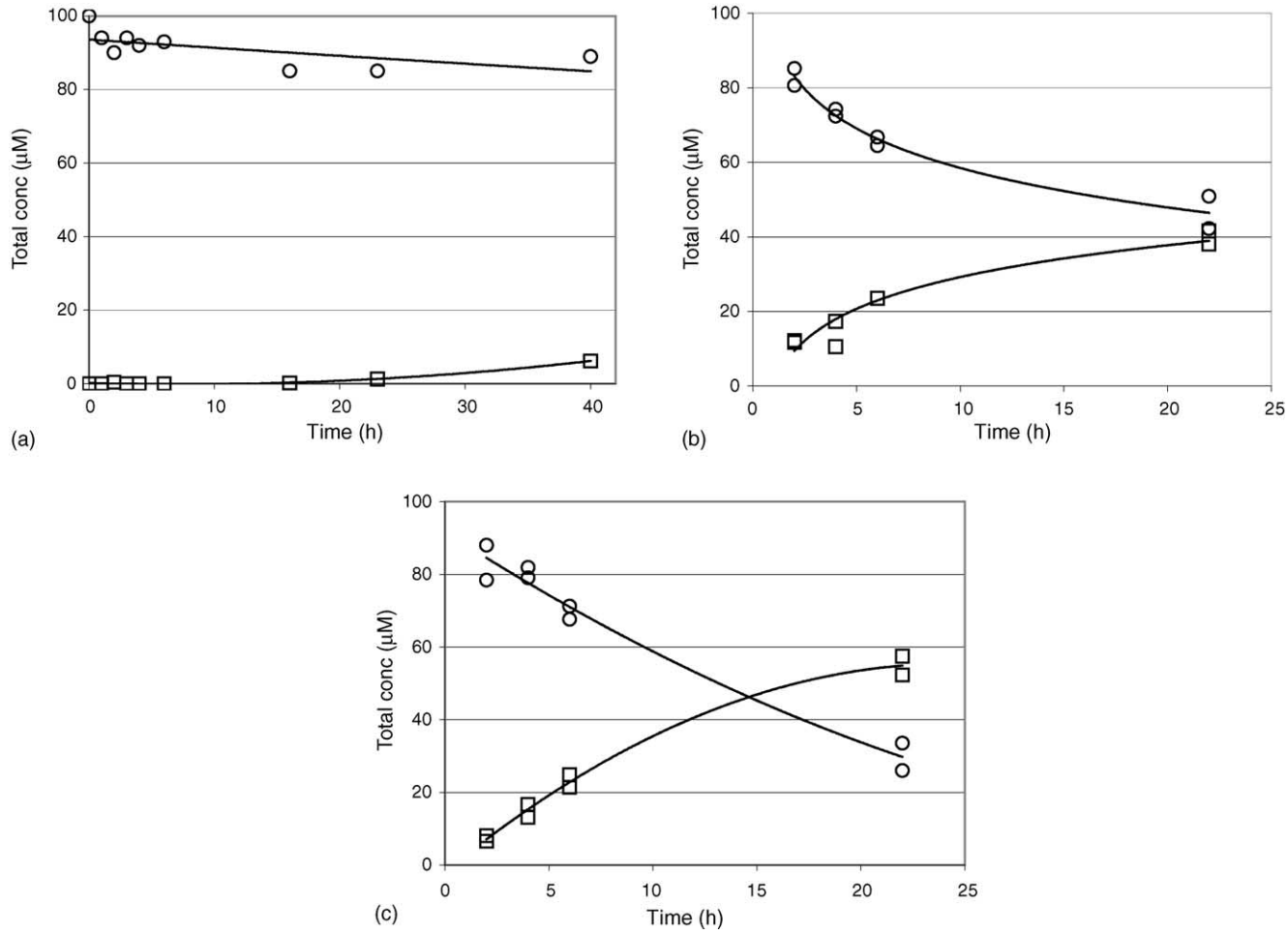

Fig. 2. CED experiments with (a) human plasma in both chambers, (b) rabbit plasma in both chambers and (c) human plasma in one chamber and rabbit plasma in the other. In all experiments one side was spiked (◯) with 100 μM Compound 1 and the other side was initially blank (□). In (c) the rabbit plasma was spiked and human plasma was blank.

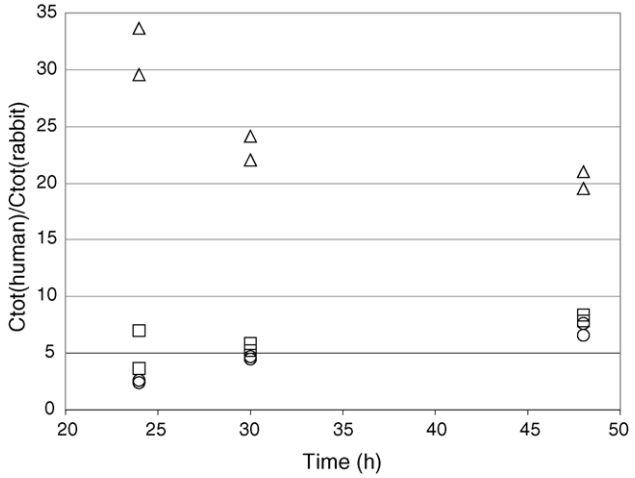

Fig. 3. Time evolution of $C_{tot}^{human}/C_{tot}^{rabbit}$ for CED experiments with human plasma in one chamber and rabbit plasma in the other, starting from different concentrations (C_{tot}^{0}) of Compound 1 in each chamber: (△) human plasma (100 μM)—blank rabbit plasma; (□) human plasma (70 μM)—rabbit plasma (30 μM); (◯) human plasma (50 μM)—rabbit plasma (50 μM).

bers (Fig. 2(b)), as compared to a system with human plasma where equilibrium had not been established even after 40 h incubation (Fig. 2(a)). For the experiment in Fig. 2(c), a ratio $C_{tot}^{human}/C_{tot}^{rabbit}$ of approximately 30 was expected, using Eq. (4) and assuming that the UF values in Table 1 are correct. Instead the ratio was about 2 at 22 h, indicating that the system is still far from equilibrium or that the ratio is influenced by other factors.

In a final attempt to shorten the equilibration time, different initial amounts of Compound 1 were added to both sides of the dialysis membrane. The time courses of the $C_{tot}^{human}/C_{tot}^{rabbit}$ ratios are shown in Fig. 3. Not surprisingly, in the experiment starting with the Compound 1 concentrations closest to those expected at equilibrium (i.e. starting with 100 μM Compound 1 in human plasma and blank rabbit plasma), the ratio after 48 h (around 20–25) was also closest to that estimated from UF data, although even in this case equilibrium was not obtained within 24 h, which we regard as the practical time limit for a CED experiment.

It can thus be concluded that even if we start with concentrations closer to equilibrium, the time to reach equilibrium will be too long for compounds with high protein binding due to the low free concentrations. Drawbacks with long CED equilibration times include compound degradation and possible changes in pH of the plasma, which might change the binding properties for the drug in question. There is also a risk of bacterial growth, which will cause changes in the composition of the plasma.

3.2. NAD-299 experiments

NAD-299 has an unbound fraction varying between 2 and 36% for different species (see Table 1). Since f_u is generally larger for NAD-299 than for Compound 1, the initial free drug concentration gradient across the membrane should be greater, which would lead to a shorter equilibration time than for Compound 1. An initial CED experiment was performed to investigate this. One chamber was filled with human plasma, spiked with 1 μM NAD-299 and the other chamber contained blank human plasma. The concentrations of NAD-299 on either side of the membrane were then determined after 16 and 24 h. From the results in Table 2, it was concluded that an equilibration time of 16 h was sufficient for this system.

Table 2
CED experiments for setting equilibration time for NAD-299, initially with 1 μM NAD-299 in human plasma on one side and blank human plasma on the other side

Time (h)	Spiked human plasma (μM)	Blank human plasma (μM)
0	1.00	0
16 ($n=6$)	0.49 ± 0.02	0.44 ± 0.02
24 ($n=4$)	0.48 ± 0.06	0.50 ± 0.06

Eq. (4) gives the relationship between the ratios of C_{tot} at equilibrium, which can be obtained from CED, and the unbound fractions which can be determined using ultrafiltration. In order to test the applicability of the CED method to a drug with high to moderate protein binding, CED experiments and ultrafiltrations were performed with plasma from three different species (human, dog and rat), spiked with three different concentrations of NAD-299. The choice of species is due to their large differences in plasma protein binding (Table 1). The CED results from this experiment are summarised in Table 3. As an example, in the human-rat experiment at 1 μM, a total concentration of 1.45 μM NAD-299 in human plasma would be equivalent to 0.31 μM NAD-299 in rat plasma as they give the same free concentration. It can also be said, without knowing the absolute unbound fractions,

Table 3
CED experiments for pooled plasma from three species: human, dog and rat, $n=6$ for all combinations

C_{tot}^{0} (μM)	C_{tot}^{human} (μM)	C_{tot}^{rat} (μM)	Ratio
Human–rat			
10.0	11.9 ± 0.4	5.15 ± 0.59	2.32
1.0	1.45 ± 0.09	0.31 ± 0.05	4.72
0.10	0.148 ± 0.008	0.033 ± 0.010	4.46
C_{tot}^{0} (μM)	C_{tot}^{human} (μM)	C_{tot}^{dog} (μM)	Ratio
Human–dog			
10.0	9.80 ± 0.91	7.98 ± 0.65	1.23
1.0	1.30 ± 0.06	0.62 ± 0.06	2.08
0.1	0.136 ± 0.007	0.060 ± 0.008	2.28
C_{tot}^{0} (μM)	C_{tot}^{dog} (μM)	C_{tot}^{rat} (μM)	Ratio
Dog–rat			
10.0	12.69 ± 0.65	6.36 ± 0.21	2.00
1.0	1.26 ± 0.06	0.522 ± 0.022	2.42
0.1	0.140 ± 0.010	0.058 ± 0.006	2.40

Initially, the same amounts (C_{tot}^{0}) of NAD-299 were added to each dialysis chamber.

Table 4
Ultrafiltrations of pooled plasma from three species: human, dog and rat

C_{tot} (μM)	C_u^{human} (μM)	f_u^{human} (%)
Human		
10.0	0.476 ± 0.004	4.76
1.0	0.017 ± 0.002	1.70
0.1	0.0022 ± 0.001	2.20
C_{tot} (μM)	C_u^{dog} (μM)	f_u^{dog} (%)
Dog		
10.0	0.97 ± 0.09	9.7
1.0	0.069 ± 0.008	6.9
0.1	0.009 ± 0.002	9.4
C_{tot} (μM)	C_u^{rat} (μM)	f_u^{rat} (%)
Rat		
10.0	3.02 ± 0.10	30.2
1.0	0.313 ± 0.011	31.3
0.1	0.03 ± 0.01	30.0

There are three samples per concentration. C_{tot} is the concentration of NAD-299 that was added to plasma prior to the ultrafiltrations.

that the unbound fraction in rats is almost five times higher than in humans.

In order to validate the results from the CED method using Eq. (4), ultrafiltrations were performed with the different plasma types, spiked with NAD-299 (Table 4). The highest C_{tot} (10 μM) in human plasma had a f_u that was more than twice the f_u at the lower concentrations. This concentration-dependent binding for NAD-299 has been observed at our laboratory previously and is not unexpected as NAD-299 binds to α_1-AGP. When the total drug concentration approaches the physiological α_1-AGP concentration, 10–15 μM, the unbound fraction will start to increase due to saturation effects. The concentration-dependent binding in human plasma can also be seen in the CED experiment (Table 3), where $C_{tot}^{human}/C_{tot}^{rat}$ and $C_{tot}^{human}/C_{tot}^{dog}$ ratios at 10 μM were only half of those at the two lower concentrations, 1 and 0.1 μM. In contrast, $C_{tot}^{dog}/C_{tot}^{rat}$ (Table 3) remained relatively constant over the studied concentration range as did their corresponding unbound fractions (Table 4). Thus, of the three species studied, only human plasma showed concentration-dependent binding.

The next step in the evaluation of the CED experiments was to compare the C_{tot} ratios in Table 3 with the inverse ratios of the unbound fractions according to Eq. (4). Generally, the ratios obtained from CED were two to three times lower than the f_u ratios from the ultrafiltrations (Table 5). The difference was most pronounced when comparing human plasma with rat plasma, where the concentration ratios from the ultrafiltrations were more than three times larger than those from the CED. It was suspected that this discrepancy was due to not only equilibration of the study drug between the two plasma chambers, but also the fact that all plasma components present with a molecular weight less than the cut-off of the membrane will be at equilibrium after the dialysis, which might change the binding properties of the proteins. Possible initial differences in ionic strength, composition of low molecular weight proteins (e.g. different amino acid sequences) and metabolites will thus, even out during the dialysis and at equilibrium the media surrounding the protein will then no longer be the same as initially, resulting in different unbound fractions.

In order to test this hypothesis, an equilibrium dialysis experiment was designed where plasma from two different sources was used as follows: (1) blank plasma from one species (human or rat) was equilibrated against plasma from the other species for 16 h, (2) blank plasma from one species was equilibrated against plasma from the same species for 16 h. The plasma from these initial equilibration experiments as well as untreated plasma were then spiked to the three concentrations 0.1, 1 and 10 μM, adjusted to a pH of 7.4, ultrafiltrated and analysed for the content of drug. These results are shown in Table 6. It should be noted that the 0.1 μM results

Table 5
Ratios of C_{tot} for NAD-299, obtained from CED and ratios of f_u determined with ultrafiltrations

Initial concentration (μM)	$C_{tot}^{human}/C_{tot}^{rat}$	f_u^{rat}/f_u^{human}	$C_{tot}^{human}/C_{tot}^{dog}$	f_u^{dog}/f_u^{human}	$C_{tot}^{dog}/C_{tot}^{rat}$	f_u^{rat}/f_u^{dog}
10.0	2.32	6.34	1.23	2.04	2.00	3.11
1.0	4.72	18.4	2.08	4.06	2.42	4.54
0.1	4.46	13.6	2.28	4.27	2.40	3.19

Table 6
Unbound fractions (%) of NAD-299

NAD-299	Unbound fractions (%), $n = 2$			
	Concentration (μM)	Direct UF	UF after ED with same species	UF after ED with opposite species
Human plasma	10	2.8, 3.4	4.3, 3.9	5.6, 5.4
Human plasma	1	0.8, 0.9	1.1, 1.1	2.0, 2.0
Human plasma	0.1	0.6, 0.7	1.1, 1.9	1.8, 1.5
Rat plasma	10	38.0, 37.9	32.2, 32.1	26.1, 24.7
Rat plasma	1	34.5, 34.0	31.3, 30.0	24.8, 25.0
Rat plasma	0.1	32.7, 33.9	32.4, 31.5	26.1, 23.3

Untreated plasma or plasma after equilibrium dialysis (16 h) were spiked at three different concentrations, adjusted for pH and temperature and ultrafiltrated.

Table 7
Unbound fractions (%) of tolterodine

Tolterodine	Unbound fraction (%), $n = 5$			
	Concentration (μM)	Direct UF	UF after ED with same species	UF after ED with opposite species
Human plasma	1	3.3 ± 0.1[a]	3.5 ± 0.2[b]	3.9 ± 0.1
Rat plasma	1	32.5 ± 0.8	28.4 ± 2.5	20.8 ± 1.0

Untreated plasma or plasma obtained after CED (16 h) were spiked with tolterodine, adjusted for pH and temperature and ultrafiltrated.
[a] One outlier.
[b] Plasma leakage in two samples.

were close to the LOQ and somewhat uncertain and that the different ratios between 1 and 10 μM when human plasma was involved were a result of the concentration-dependent binding.

The trend is clear: the binding properties were changed after CED, depending on whether plasma from the same species or from different species was in the dialysis chambers. The unbound fraction for NAD-299 decreased from 35 to 25% (average for all concentrations) when rat plasma was dialysed against human plasma, while the unbound fractions in human plasma increased from 0.8 to 2% (at 1 μM) when dialysed against rat plasma. Since the only experimental difference between these results is the plasma pre-treatment, this strongly suggests that the composition of the plasmas on each side of the dialysis membrane is changed, resulting in altered binding properties as the initial differences in composition even out after the equilibration. The average volume shift measured for the three plasma combinations was only around 4% and should not make a substantial contribution to the observed changes in binding. Compared to untreated plasma, the differences were even more obvious. These differences between untreated plasma and plasma dialysed against the same plasma were not expected but they were significant for most of the combinations. A possible explanation might be that some proteins were mechanically damaged after 16 h of agitation.

3.3. Tolterodine experiments

In order to investigate whether this partial mixture of plasma components during CED and the subsequent change of unbound fractions was not just NAD-299 specific, the last experiment was repeated for tolterodine as well, but only at the 1 μM concentration. With tolterodine, the same trend as for NAD-299 was observed (Table 7): a substantial decrease in the unbound fraction of tolterodine in rat plasma if equilibrated against human plasma (21%) compared to plasma equilibrated against rat plasma (28%) and compared to untreated plasma (33%). As for NAD-299, but to a less marked degree, the unbound fractions of tolterodine increased when comparing untreated human plasma (3.3%) with human plasma taken after equilibration with human (3.5%) or rat plasma (3.9%).

4. Conclusions

When exploring the CED method for the determination of relative unbound concentrations, several difficulties were encountered. The time to reach equilibrium was strongly dependent on the free faction. For Compound 1, unusually strongly bound in human plasma, equilibrium could no be reached within a reasonable time (<24 h). For the other two model compounds a more rapid equilibration (<16 h) was seen. CED experiments were done with plasma from three different species (human, dog and rat) and corresponding ultrafiltrations were performed to validate the CED results. The general trend was that the difference in binding seemed to be underestimated using CED. It was suspected that this phenomenon is due to a partial mixture of the plasma types during the CED, since all molecules with a molecular weight less than MWCO can cross the membrane and will be equally distributed in both dialysis chambers. This partial mixture of plasma types changes the binding properties.

Additional CED experiments were performed where the chambers were filled with blank plasma from different species. Samples were withdrawn after 16 h and thereafter spiked with NAD-299, adjusted for pH and temperature, ultrafiltrated and analysed. A substantial difference in the f_u was observed for samples that had been equilibrated against another type of plasma, compared to plasma equilibrated against the same type of plasma and compared to untreated plasma. This confirmed the changes in composition of the plasma during the CED. A final experiment with tolterodine gave similar results, confirming that the phenomenon is more general and does not apply only to NAD-299. These results are not surprising, considering that factors like the ionic strength of a protein solution affects the binding properties of the proteins by changing the surrounding electrostatics. Both NAD-299 and tolterodine are mainly bound to α_1-AGP and it would be of interest to see whether compounds binding to albumin also show the same behaviour.

Highly efficient dialysis equipment that allows a maximum equilibration rate might be a way to shorten the equilibration times; however, even though a shorter equilibration time might cause a less pronounced mixture of the plasma types, the phenomenon will still be present and will have a significant influence on the results. Thus, the CED method, which at a first glance seems to be a convenient method for

determining relative plasma protein-binding properties, has major inherent problems and from our perspective there is no easy way around this.

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