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A novel method using nuclear magnetic resonance for plasma protein binding assessment in drug discovery programs

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ABSTRACT

A new methodology based on Nuclear Magnetic Resonance (NMR) was developed to determine plasma protein binding (PPB) of drug candidates in drug discovery programs. A strong correlation was found between the attenuation of NMR signals of diverse drugs in the presence of different plasma concentrations and their fraction bound (f_b) reported in the literature. Based on these results, a protocol for a rapid calculation of f_b of small molecules was established. The advantage of using plasma instead of purified recombinant proteins and the possibility of pool analysis to increase throughput were also evaluated. This novel methodology proved to be very versatile, cost-effective, fast and suitable for automation. As a plus, it contemporarily provides a quality check and solubility of the compound.

1. Introduction

Plasma protein binding (PPB) role in modulating the effective drug concentration at the pharmacological target has been extensively discussed in the literature [1–3]. Knowledge of the unbound drug concentration across species (preclinical and human) in a drug discovery program supports interpretation of PK/PD relationships and extrapolation of efficacy and safety margins from preclinical species to the clinical setting.

Plasma is a complex matrix, containing diverse proteins, glucose, metabolites, clotting factors, electrolytes, hormones, etc. The major plasma proteins that drugs bind to are human serum albumin (HSA), acid alpha-1-glycoprotein (hAGP) and lipoproteins. HSA is the most abundant, with an average concentration in healthy subjects of 600 μM and quite stable levels except for extreme disease states. HSA has different binding sites capable of binding endogenous compounds, in particular fatty acids, as well as xenobiotics, with a preference for acidic and neutral compounds. hAGP, whose concentration varies from 15 to 30 μM, shows a preference to bind basic and neutral compounds, and its concentration and even binding capacity can significantly change in disease/inflammation states [4].

Even though it is widely accepted that reducing the binding to these plasma proteins should lead to a higher fraction of active drug (free drug theory, FDT) [5], it is yet crucial to understand if the fraction bound (f_b) is really predictive of drug efficacy or toxicity before investing resources in PPB optimization.

The accurate determination of f_b of drug candidates is not always straightforward with the currently available methods, like ultrafiltration, ultracentrifugation and rapid equilibrium dialysis (RED) [6,7]. Equilibrium dialysis is the most commonly used method. However, this technique is not suitable for compounds that are unstable in plasma (<3–5 h). Another critical aspect of RED as well as of ultrafiltration is the adsorption at membranes and on the surfaces of devices of some compounds that significantly impacts the measurement. Furthermore, using ultrafiltration, in some cases the free compound may be under-represented due to molecular sieving, especially when increasing the molecular size of the compound. On the other hand, ultracentrifugation requires long centrifugation times, presents a low throughput and is an expensive technique [6].

Recognizing the utility of NMR as a very sensitive method for detecting binding, we have focused on developing an approach particularly fast and with low cost per sample, without considering the cost of the spectrometer, alternative to those currently used for determining PPB. Among the different NMR methods for determining ligand protein binding, only ligand-observed 1D tech-

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niques with relatively high throughput and low resource usage are suitable for screening the protein binding of several compounds in a drug discovery program. Several fast, ligand-focused 1D NMR experiments exist that exploit differences in relaxation rates, diffusion rates, saturation transfers, or NOE transfers to identify protein-ligand complexes [8]. However, the fastest and simplest NMR method for evaluating protein binding is the traditional 1D ^{1}H line-broadening experiment, which additionally allows by a single-point measurement easily rank binding affinities [9].

Signals of small molecules (MW < 500 Da), as normally potential drug candidates are, in 1D ^{1}H NMR experiments are usually very sharp due to fast tumbling in solution and a consequent slow T_2 relaxation. Binding to high MW molecules, such as plasma proteins, induces peak broadening and the corresponding decrease of the ligand's NMR signal intensity because the bound ligand experiences the shorter relaxation time of the protein. This happens if the exchange regime is fast enough in NMR time scale. The observed increase in ligand line width in such an experiment will depend on several factors that include the dissociation equilibrium constant for the protein-ligand interaction, the fraction of bound ligand, the free ligand NMR line width, and the line width for the bound state of the ligand [9]. Taking into account that plasma is a complex matrix system, we decided for an empirical approach. For twenty-five drugs (see tables I and II) we observed a strong correlation between the attenuation of NMR signals in the presence of different plasma dilutions and their f_b reported in the literature. Based on these results, a protocol for a rapid and low cost calculation of f_b of small molecules was established. In this manuscript, we describe this novel validated method to determine plasma protein binding (PPB) of drug candidates in drug discovery programs.

Furthermore, the advantage of using plasma instead of purified recombinant proteins, as some other methods do, and the possibility of incorporating pool analysis to increase throughput are discussed. As a plus, this method contemporarily provides a quality check and solubility of the compound.

2. Materials and methods

2.1. Chemicals and reagents

Acetaminophen, 1-(1-Adamantyl)ethylamine, Atenolol, Bupropion, Carbamazepine, Cefaclor, Cefsulodin, Cephalexin, Chlorpromazine, Clofibrate, Diclofenac, Diflunisal, Digitoxin, Ethinyl Estradiol, Furosemide, Haloperidol, Ibuprofen, Imipramine, Ketoconazole, Labetalol, Naproxen, Nortriptyline, Omeprazole, Phenytoin, Phenylbutazone, Procainamide, Sulfamethoxazole, Salicylic Acid, Ranitidine, Tamoxifen, Trimethoprim, Urapidil, Verapamil, Warfarin and Zidovudine were purchased from Sigma Aldrich. Dimethyl sulfoxide-d_6 (99.9% D) was obtained from Euriso-top. 3-(trimethylsilyl) propionic-2,2,3,3-d_4 acid sodium salt (TSP), sodium formate, and human alpha1-acid glycoprotein were purchased from Sigma Aldrich. Plasma matrixes were pools from four healthy subjects and was obtained from CliniSciences. Phosphate Buffer Saline with 10% D_2O (PBS; pH 7.4) used was of analytical grade and proven pH.

2.2. Sample preparation

Stock solutions of the drugs in dimethyl sulfoxide-d_6 (2 mM) were prepared and diluted in PBS buffer pH 7.40 containing 200 μM sodium formate and 10% D_2O, to a final compound concentration of 20 μM in 600 μL. The pooled human plasma was thawed and centrifuged 15 min at 4000 × *g* using a refrigerated centrifuge at 4 °C for removing fibrin. Five plasma stock solutions were prepared in PBS containing 200 μM sodium formate and 10% D_2O by making serial dilutions from plasma. If considering 600 μM the HSA concentrations plasma, the final concentrations of HSA in these stock solutions ranged from 600 μM (no dilution) to 12 μM (1:50 plasma dilution). They were prepared so that 20 μL of each plasma stock solution were added to 580 μL of a free compound solution. Alternatively, 20 μL of 600 μM TSP in PBS containing 200 μM sodium formate and 10% D_2O were added to 580 μL of the working compound sample. The final concentrations were 20 μM compound and 0.4 μM, 1.5 μM, 4 μM, 16 μM and 20 μM HSA, respectively, or 20 μM TSP in PBS containing 200 μM sodium formate and 10% D_2O. These six solutions of each compound were transferred to NMR tubes.

For the NMR PPB assay, 1800 μL solution of 20.6 μM of the compound in PBS pH 7.4 containing 200 μM formate and 10% D_2O were prepared. The solution was split in three by adding 580 μL in each tube. In the first tube 20 μL of 600 μM TSP were added, in the second 20 μL of plasma stock solution, and in the latter 20 μL of diluted plasma. In this way, we obtained samples with a final compound concentration equal to 20 μM and 20 μM TSP, 1:30 or 1:400 diluted plasma, respectively. The three solutions were transferred in NMR tubes for the analysis.

In the experiments with recombinant proteins, parent 600 μM HSA and 30 μM hAGP solutions were used. The samples were prepared by adding 20 μL of 600 μM HSA and 20 μL of 30 μM hAGP, 20 μL of 600 μM HSA and 20 μL of buffer, 20 μL of 30 μM hAGP and 20 μL of buffer, or 20 μL of plasma stock solution and 20 μL of buffer to 560 μL of the compound solutions. In that way, the final concentrations were 20 μM HSA and 1 μM hAGP, 20 μM HSA, 1 μM hAGP, or 1:30 diluted plasma, respectively. The compound solution was prepared so that the final compound concentration in PBS pH 7.4 containing 200 μM sodium formate and 10% D_2O was 20 μM in 600 μL.

Pool solutions were prepared by adding 20 μL of plasma stock solution in 580 μL of a solution containing the three compounds so that the final concentration of each compound was 20 μM in PBS pH 7.4 containing 200 μM sodium formate and 10% D_2O in 600 μL.

2.3. NMR spectroscopy

All NMR spectra were collected on a Bruker 600 MHz Avance spectrometer using a 5 mm BBI probe equipped with z-axis gradient and using a SampleJet sample changer and TopSpin2.1pl8 software for automated data collection. Spectra were collected at 37 °C, using a cpmg sequence with a T_2 filter of 40 ms, a sweep-width of 7246 Hz, 32 K data points, 584 scans and a relaxation delay of 2.0 s. The residual H_2O resonance signal was suppressed using pre-saturation. The total experiment time, including sample changing for each spectrum, was approximately 20 min. Spectra were processed with Topspin. FIDs were Fourier transformed using an exponential weighting function with a line-broadening factor of 0.5 Hz and zero filled using 65 K points. NMR spectra were phase adjusted and baseline-corrected. When possible, we focused on the aromatic resonance peaks. For the quality check, the number, multiplicity and position of the peaks in the spectrum were compared with those expected considering the chemical structure of each compound. In addition, an estimation of the solubility was obtained from integration of the aromatic signals relative to the TSP signal. Overall, peak intensity was normalized relative to the formate peak at 8.52 ppm, which was used as a shimming control. Individual peak intensities in the aromatic region for each compound were summed to obtain the free (I_F) and bound (I_B) intensities at each plasma dilution point. I_B/I_F values were directly obtained using Bruker Topspin 2.1 software, after base line correction by overlapping the NMR spectra obtained in the absence and in the presence of plasma. The reported value takes into consideration all the aromatic signals of each compound, and the result represents the average of three

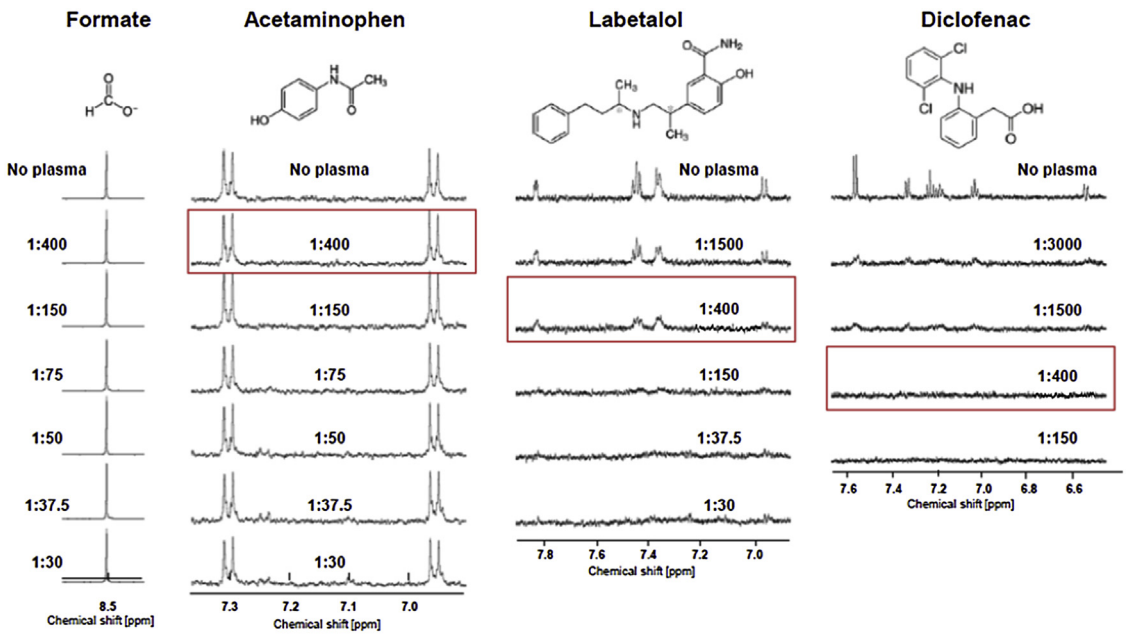

Fig. 1. 1D ^{1}H NMR line-broadening of drugs is observed in the presence of plasma dilutions. Aromatic region of the 1D ^{1}H NMR spectra for titration of 200 μM of sodium formate (**A**) and 20 μM of the drugs Acetaminophen (**B**), Labetalol (**C**), and Diclofenac (**D**) at different dilutions of human plasma. We used formate as a negative control, that does not interact with the plasmatic proteins. As the plasma concentration increases, the intensity of the NMR signals decreases, proportional to the degree of binding. Spectra obtained with the 1:400 plasma dilution is indicated with a red box for the three drugs. NMR signals of Acetaminophen, that weakly binds the plasmatic proteins, are not significantly broadened; Labetalol, an intermediate plasma protein binder, exhibits a considerable broadening at the same plasma dilution; while for Diclofenac that is a very strong binder MNR signals are broadened beyond detection in the same experimental conditions (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

replicates ± SD. I_B/I_F errors thus estimated were included and propagated in the f_b calculation, using the equations obtained from the calibration curves of Fig. 3 at the corresponding plasma dilutions.

3. Results

3.1. 1D ^{1}H NMR line-broadening of small molecules is observed in the presence of plasma

We have investigated the effect of the presence of plasma on the NMR line width of different drugs (Fig. 1). In case a fast exchange between free and bound states exists, interaction with plasmatic proteins yields broader NMR signals. This effect is due to a reduced average transversal relaxation time (T_2) of the interacting molecule, in equilibrium between the free state (long T_2) and the bound state (short T_2). Signal broadening brings with the consequent reduction of the intensity. As comparing signal intensities is more easy and straightforward than comparing line widths, we decided to evaluate the plasma protein interaction by examining the ratio of NMR peak intensities in the presence and in the absence of plasma (I_B/I_F). This relationship represents an easily measurable response of ligand binding that inversely depends on the fraction of bound ligand [9]. Spectra were obtained using an NMR experiment that includes a 40 ms T_2 filter, which enhances the effect on signal intensity caused by the interaction. A higher plasma concentration causes a decrease in the intensity of the NMR signals, consistently with a larger fraction of the drugs bound to plasmatic proteins. As Fig. 1 shows, the intensity decrease depends on the degree of binding: reported tighter binding ligands exhibit a larger broadening than weaker binding ligands for the same plasma dilution. This can be easily observed in the spectra outlined with a red box in Fig. 1. Signal intensities decrease in the series Acetaminophen ($f_b \approx$ 0-0.25) - Labetalol ($f_b \approx 0.50$) - Diclofenac ($f_b > 0.99$), reflecting their different PPB capacities.

3.2. NMR signal intensity attenuation in the presence of plasma dilutions allows discriminating compounds accordingly to their PPB capacity

To explore if it is possible to discriminate quantitatively different PPB affinities using the decrease of signal intensity, we selected a set of compounds based on the previously reported f_b in plasma. A total of twenty-five compounds, classified into four different categories related to their PPB capacities, were used to examine the ratio (I_B/I_F) between the intensity of the NMR signals of compounds in the presence (I_B) and in the absence (I_F) of plasma in ^{1}H NMR experiments using different plasma concentrations (Fig. 2).

Conditions were chosen in order to ensure that curves span a sufficiently wide range from 0.02 equivalents of HSA (dilution 1:1500) to one equivalent (dilution 1:30) with respect to the compound (20 μM). As it is evident from the figure, the four groups show a differential behaviour, allowing a discrimination of the compounds based on their f_b through the I_B/I_F ratio at any plasma dilution. For example, at the highest plasma concentration, low binders ($f_b < 0.5$) present an $I_B/I_F > 0.7$, medium binders between 0.7 and 0.2, and high and very high binders an $I_B/I_F < 0.2$. On the other hand, at the lowest plasma concentration, high and very high binders are better discriminated.

3.3. Linear correlations are found between f_b literature values and the attenuation of the NMR signal

Once ascertained the qualitative relationship between f_b and I_B/I_F, we have investigated the possibility of using the intensity ratio to obtain a quantitative value for the degree of PPB. For this purpose, we selected two dilutions that allowed the best discrimination of the different f_b values. Inspection of curves in Fig. 2 shows that measurements at a 1:400 dilution give a good separation of the I_B/I_F values within the range observed for very high and high

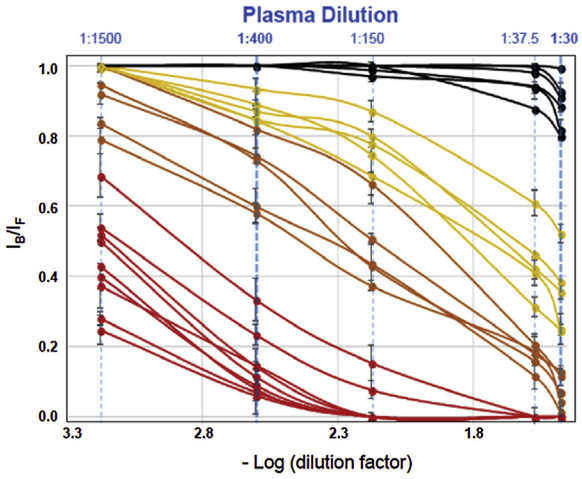

Fig. 2. NMR signal intensity attenuation in the presence of plasma dilutions allows the classification of drugs accordingly to their fraction unbound in plasma. Plot of the ratio between signal intensity in the presence and in the absence of plasma (I_B/I_F) as a function of the minus logarithm of the plasma dilution factor. Compounds were classified into four groups, based on their literature f_b values: very high binders (red, $f_b \geq 0.98$) (Tamoxifen, Warfarin, Diflunisal, Naproxen, Furosemide, Ketoconazole, Ethinyl Estradiol, and Ibuprofen), high binders (orange, $0.90 \leq f_b < 0.98$) (Urapidil, Salicylic Acid, Phenytoin, Imipramine, and Haloperidol), medium binders (yellow, $0.40 \leq f_b < 0.90$) (Verapamil, Carbamazepine, Trimethoprim, Bupropion, and Labetalol), and weak binders (black, $f_b < 0.40$) (Cephalexin, Ranitidine, Procainamide, Atenolol, Cefaclor, and Acetaminophen) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

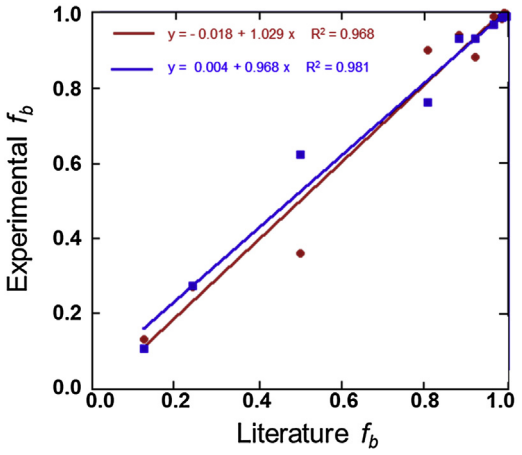

Fig. 4. Agreement between experimental human plasma protein binding using NMR (blue) or RED (red) and literature values for a set of standard compounds. The best-fit lines obtained with the corresponding equations and the R^2 are shown. Table 3 shows the compounds used for the fitting (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

binders, whereas at a 1:30 dilution the same condition is achieved for medium and weak binders.

Compounds in Tables 1 and 2 were selected to determine an empirical relationship between f_b and I_B/I_F in the case of medium-low and very high-high binders, respectively. To this end, we have measured the intensity ratio at the corresponding plasma dilution and plot the literature f_b as a function of the I_B/I_F average of three replicates using three different plasma pools. This is important to cope also with the possible variation of plasma protein affinities in different plasma samples. Graphs in Fig. 3 show that there is a significantly strong correlation between I_B/I_F and f_b for the two cases, with Pearson correlation coefficients of -0.9894 ($P<10^{-5}$) and -0.9592 ($P<10^{-5}$), for medium-low and very high-high binders, respectively. Data were fitted using linear equations, as the use of quadratics polynomials did not show a significant decrease in the variance between literature f_b and calculated values (F-test, results not shown). Fig. 3 shows the corresponding empirical equations that allow the calculation of the f_b value. Last columns of Tables 1 and 2 show the corresponding f_b values determined using these equations. From these results, we can estimate an average uncertainty of 0.04 and 0.004 on the f_b value of medium-low and very high-high binders, respectively.

3.4. Comparison of plasma protein binding determined by NMR with values obtained using RED and values reported in literature for a set of standard compounds

We have then measured the f_b values of ten compounds, that were not previously used for the derivation of the linear equations shown above, using the NMR method and a well-established methodology, rapid equilibrium dialysis (RED) [10]. These f_b values were then compared with literature values. As observed in Fig. 4 and Table 3 there is a very good agreement with the literature with only a few deviations. In particular, we have measured a lower f_b value than that reported for Labetalol by RED, whereas measured

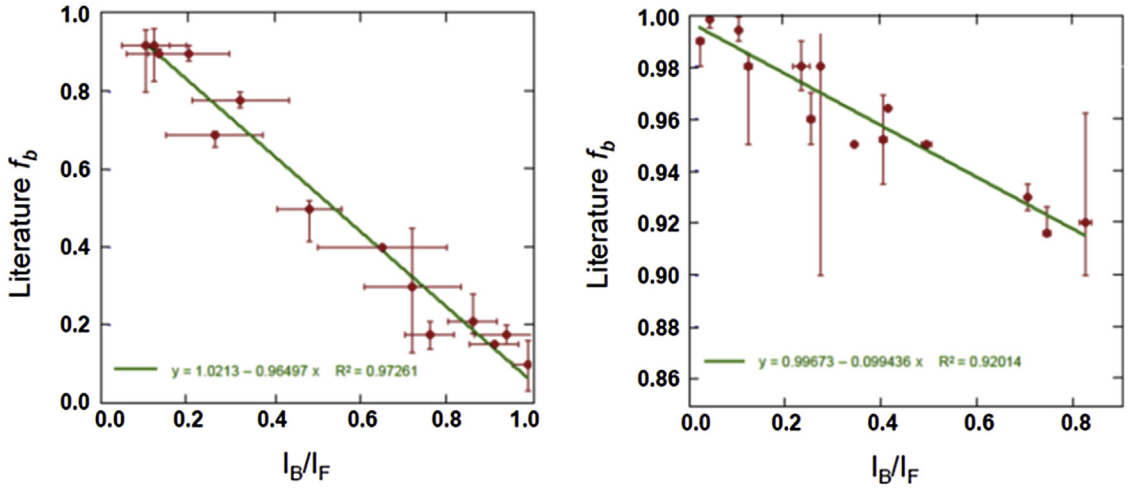

Fig. 3. Calculation of PPB for medium and weak ligands by NMR. Linear correlation of I_B/I_F and literature f_b values for selected medium and weak binders (left panel, 1:30 plasma dilution, listed in Table 1) and strong binders (right panel, 1:400 plasma dilution, listed in Table 2). These equations are used for obtaining f_b values from the experimental I_B/I_F value at the corresponding plasma dilution.

Table 1
Data for obtaining the human plasma protein binding of medium and weak binders.

Compound	I_B/I_F (1:30)[a]	Literature [ref] $\%f$[b]	NMR[b] $\%f$[b]
Urapidil	0.10 ± 0.03	80 [15], 90 [16], 94 [17]	92 ± 3
Imipramine	0.12 ± 0.04	92.6 [15], 90-95 [18], 96.2 [19], 0.87 [20]	91 ± 4
Phenytoin	0.13 ± 0.04	91 [15], 90 [19], 90 [21]	90 ± 4
Verapamil	0.20 ± 0.05	90 ± 2 [15], 90.6 [18], 90 [19], 83 [22]	83 ± 5
Sulfamethoxazole	0.26 ± 0.06	66 [15], 70 [19], 72 [23]	77 ± 6
Carbamazepine	0.32 ± 0.06	76 [15], 76 [19]	71 ± 6
Trimethoprim	0.48 ± 0.04	41.5 [15], 42-46 [19], 50 ± 2 [24]	56 ± 4
N-(1-Adamantyl) ethyleneamine	0.65 ± 0.08	40 [15]	39 ± 8
Cefsulodin	0.72 ± 0.06	45 [15], 13-30 [25]	33 ± 6
Cephalexin	0.76 ± 0.03	14 [19], 20.8 [26]	29 ± 3
Zidovudine	0.86 ± 0.03	19.0 ± 0.7 [27], 23 ± 5 [28], 30-38 [19]	19 ± 3
Ranitidine	0.91 ± 0.03	15 [19], 15 [29]	14 ± 3
Procainamide	0.95 ± 0.04	15 [19], 15-20 [30]	11 ± 4
Atenolol	0.99 ± 0.02	<5 [15], 6-16 [19], 3 [31]	7 ± 2

[a] **1:30** plasma dilution was used for I_B. Data are the average value of three replicates using different plasma samples.
[b] Experimental plasma protein binding obtained from the best-fit line (Fig. 3).

Table 2
Data for obtaining the human plasma protein binding of strong binders.

Compound	I_B/I_F (1:400)[a]	Literature [ref] $\%f$[b]	NMR[b] $\%f$[b]
Diflunisal	0.02 ± 0.04	99 [15], 98-99 [19]	99.5 ± 0.4
Diclofenac	0.04 ± 0.03	99.9 [19], 99.5 [15]	99.3 ± 0.3
Naproxen	0.10 ± 0.02	99.4 [15], >99 [19]	98.7 ± 0.2
Ethinyl Estradiol	0.12 ± 0.04	95-98 [15], 97 [19]	98.5 ± 0.4
Ketoconazole	0.23 ± 0.09	99 [15], 99 [19], 97.1 [32]	97.4 ± 0.9
Clofibrate	0.25 ± 0.04	96.5 [15], 95-97 [19]	97.2 ± 0.4
Ibuprofen	0.27 ± 0.03	99 [15], 90-99 [19], 99.5 [33]	97.0 ± 0.3
Omeprazole	0.34 ± 0.02	95 [15], 95 [19]	96.3 ± 0.2
Digitoxin	0.40 ± 0.04	93.5 [15], 97 [34]	95.7 ± 0.4
Phenylbutazone	0.41 ± 0.02	97.8 [15], 95 [35]	95.6 ± 0.2
Salicylic Acid	0.49 ± 0.05	95 [15]	94.8 ± 0.5
Nortriptyline	0.70 ± 0.03	93.5 [15], 93 [19]	92.7 ± 0.3
Phenytoin	0.74 ± 0.04	91 [15], 90 [19], 90 [21]	92.3 ± 0.4
Imipramine	0.82 ± 0.06	92.6 [15], 90-95 [18], 60-96 [19], 87 [20]	91.5 ± 0.6

[a] **1:400** plasma dilution was used for I_B. Data are the average value of three replicates using different plasma samples.
[b] Experimental plasma protein binding obtained from the best-fit line (Fig. 4).

Table 3
Comparison of human plasma protein binding values for a set of standard compounds determined by NMR, RED, and reported in the literature.

Compound	Literature [ref] $\%f_b$	NMR[a] $\%f_b$	RED $\%f_b$
Warfarin	99 [15], 99 [19]	99.5 ± 0.4	99.6 ± 0.2
Tamoxifen	>98 [15], >99 [36]	98.7 ± 0.5	99.5 ± 0.2
Furosemide	98.4 [15], 95 [19]	98.6 ± 0.3	98.1 ± 0.4
Chlorpromazine	97.8 [15], >90 [19]	96.5 ± 0.3	98.7 ± 0.2
Haloperidol	92 [15], 90.3 [18], 90-94 [19]	93 ± 1	88 ± 1
Urapidil	80 [15], 94 [37]	93 ± 1	94 ± 1
Bupropion	84 [15], 84 [19], 75-80 [16]	78 ± 8	90 ± 3
Labetalol	50 [15], 50 [19]	63 ± 3	36 ± 2
Cefaclor	24.7 [15], 23.5 [19]	28 ± 6	27 ± 9
Acetaminophen	0 [15], 15-21 [18], 25 [19]	10 ± 5	13 ± 2

[a] Data are the average value of three replicates using different plasma samples and measured in three different days.

f_b values resulted higher for Labetalol by NMR and Bupropion by RED. Overall, the R^2 obtained for RED is very close to that originally presented by Singh et al. [10], and not statistically different from that obtained by the NMR method (0.968 vs 0.981 for RED and NMR, respectively).

3.5. Medium through-put protocol for measuring plasma protein binding by NMR

The protocol of the NMR based method for measuring PPB is depicted in Fig. 5. A first spectrum is obtained using a 20 μM solution of the compound in PBS buffer, deuterated TSP for the measurement of the concentration, and formate as a control of field homogeneity. The control spectrum can be used to determine compound solubility and as quality check of the purity and structure of the molecule under study. The PPB for compounds showing a solubility significantly below 10 μM cannot be measured by this method.

A second spectrum is obtained using a 1:30 plasma dilution, in the same conditions. Formate linewidth comparison between the first and second spectrum is crucial, because f_b measurements rely on peak broadening occurring solely because of protein interaction, hence the need to work at constant field homogeneity. In this sense, TSP cannot be used because it interacts with plasmatic proteins, whereas formate does not [11]. If I_B/I_F is greater than 0.15, f_b is directly obtained using the corresponding equation, and the compound is classified as a medium-weak binder. For I_B/I_F lower than 0.15, a third spectrum is acquired using a 1:400 dilution of plasma, from which f_b is determined, and the compound is classified as a strong or very strong binder. Acquisition time for each

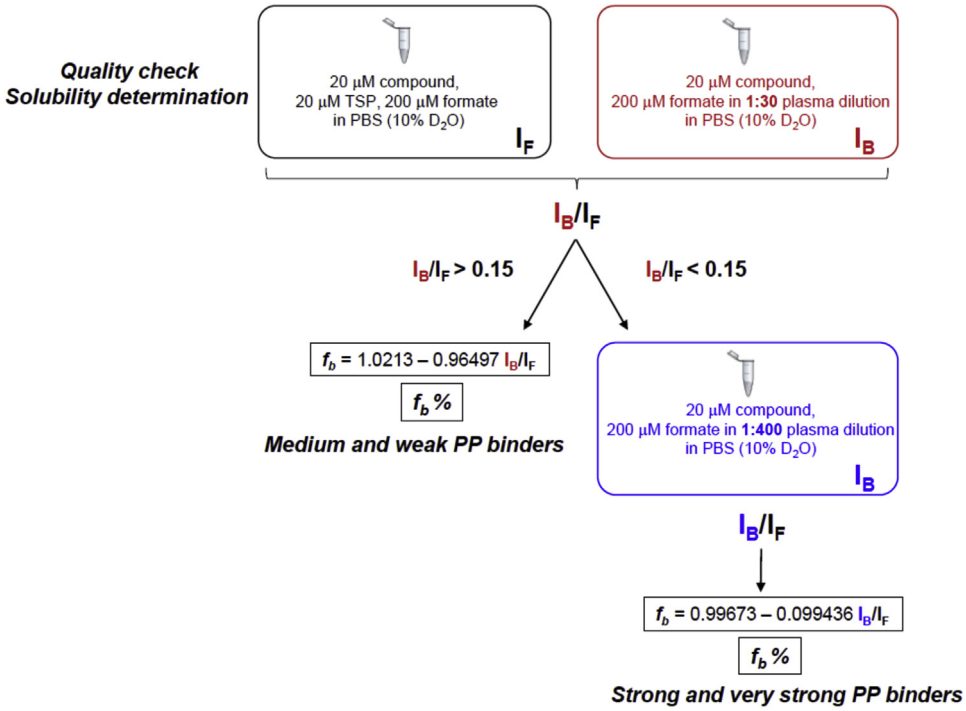

Fig. 5. Diagram showing the protocol for the NMR PPB assay. Two or three NMR spectra of the drug are recorded. The first spectrum, in the absence of plasma and containing TSP (black box sample in the figure), is used as a quality check and provides an estimation of the solubility of the compound in the experimental conditions. It also constitutes the reference for calculating the free ligand intensities. The second spectrum of the compound is recorded in the presence of 1:30 plasma dilution (red box). From the corresponding I_B/I_F measurement, the fraction bound of medium and weak binders (I_B/I_F>0.15) is obtained using the corresponding equation. For the strong and very strong binders a third spectrum in the presence of 1:400 plasma dilution is registered (blue box), and from the I_B/I_F measurement and using the corresponding equation, f_b is obtained (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

NMR spectrum is 20 min using a 600 MHz spectrometer; therefore 40 min or one hour are required for recording the two or three NMR spectra and testing one compound, depending if it results a medium-weak or a strong-very strong binder. This makes it possible to test around 20 compounds per day using plasma from one species using a sample changer in order to acquire spectra under automation. The total price of this analysis is very low, as it includes only very little amount of plasma, buffer solutions and one NMR tube.

3.6. Differences in using plasma vs recombinant proteins

We have then compared the f_b values obtained employing recombinant purified HSA and/or hAGP with those using diluted plasma. PPB of different compounds, including Urapidil, Verapamil, Ranitidine and two compounds that were synthesized for internal drug discovery programs (compounds Cpd-a and Cpd-b), were measured using four different conditions: a solution containing 1:30 dilution of plasma, two solutions containing the expected concentrations of HSA and hAGP at this plasma dilution (20 µM and 1 µM, respectively) and a solution containing both HSA and hAGP (Fig. 6). Our results indicate that for compounds like Urapidil and Cpd-a that present a medium to high affinity for HSA, no significant differences exists in the values obtained for the mixture of the two proteins or plasma (Fig. 6A). Interestingly, we have measured statistically different f_b values for Ranitidine, Cpd-b and Verapamil when using plasma or purified proteins. For the first two compounds, our measurements indicate that they mainly interact with hAGP, resulting the apparent f_b the same for hAGP alone or a mixture of HSA and hAGP (Fig. 6B). On the other hand, PPB measured in plasma resulted significantly lower than those measured in the solution containing the two proteins. To exclude the possibility that the concentration of hAGP in the plasma pool used was lower than that expected, we repeated the PPB measurement for Ranitidine adding an extra concentration of 1 µM recombinant hAGP to the plasma. The resulting f_b for Ranitidine resulted higher than that observed for pure plasma (0.24 vs. 0.17, respectively), but still lower than that obtain for the solution containing only hAGP 1 µM (0.37). This suggests that these compounds exhibit a reduced affinity for hAGP in plasma compared to that for the purified protein, probably due to the presence in plasma of competitors for the binding site. On the contrary, Verapamil, which binds with higher affinity to HSA than to hAGP, shows a higher f_b when using plasma than in the case of the two purified proteins together (Fig. 6C). This result can be explained considering that Verapamil interacts also with lipoproteins [12], which is not taken into account when measuring PPB using only HSA and/or hAGP.

3.7. Increasing the throughput: Measuring pools of compounds

Finally, we have investigated the possible measurement of PPB using pools of compounds, with the purpose of increasing the throughput of the method. This can be important for screening a large number of compounds and efficiently detecting high binders of plasma proteins, using an f_b value of 0.80 as a threshold. Fig. 7 shows the f_b values obtained employing 1:30 plasma dilutions of different pools of three compounds with different PPB capacities. Results were then compared with those of the single compound. We observed a lower apparent f_b only for Trimethoprim in pool 2 and Phenytoin in pool 3, without changes in the classification of the compounds. This lower apparent f_b is the consequence of the competition with the other compounds present in the pool. With this kind of approach, it is possible to increment the throughput of the method to around 60–80 compounds per day in one plasma species or to have the possibility of testing 20 compounds in up to four different plasma matrices.

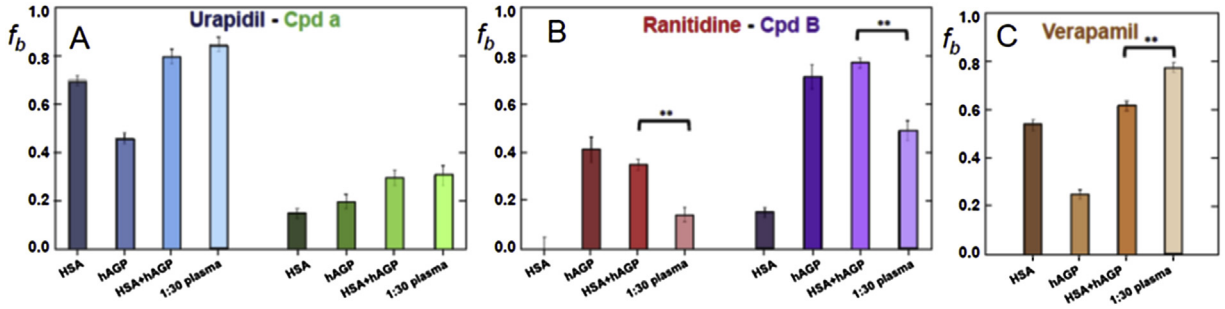

Fig. 6. Using plasma *vs.* recombinant proteins. Comparison between the fraction bound obtained using 20 μM HSA, 1 μM hAGP, both proteins at the same concentrations, or 1:30 plasma dilution for Urapidil and Cpd-a (**A**), Ranitidine and Cpd-b (**B**) or Verapamil (**C**).

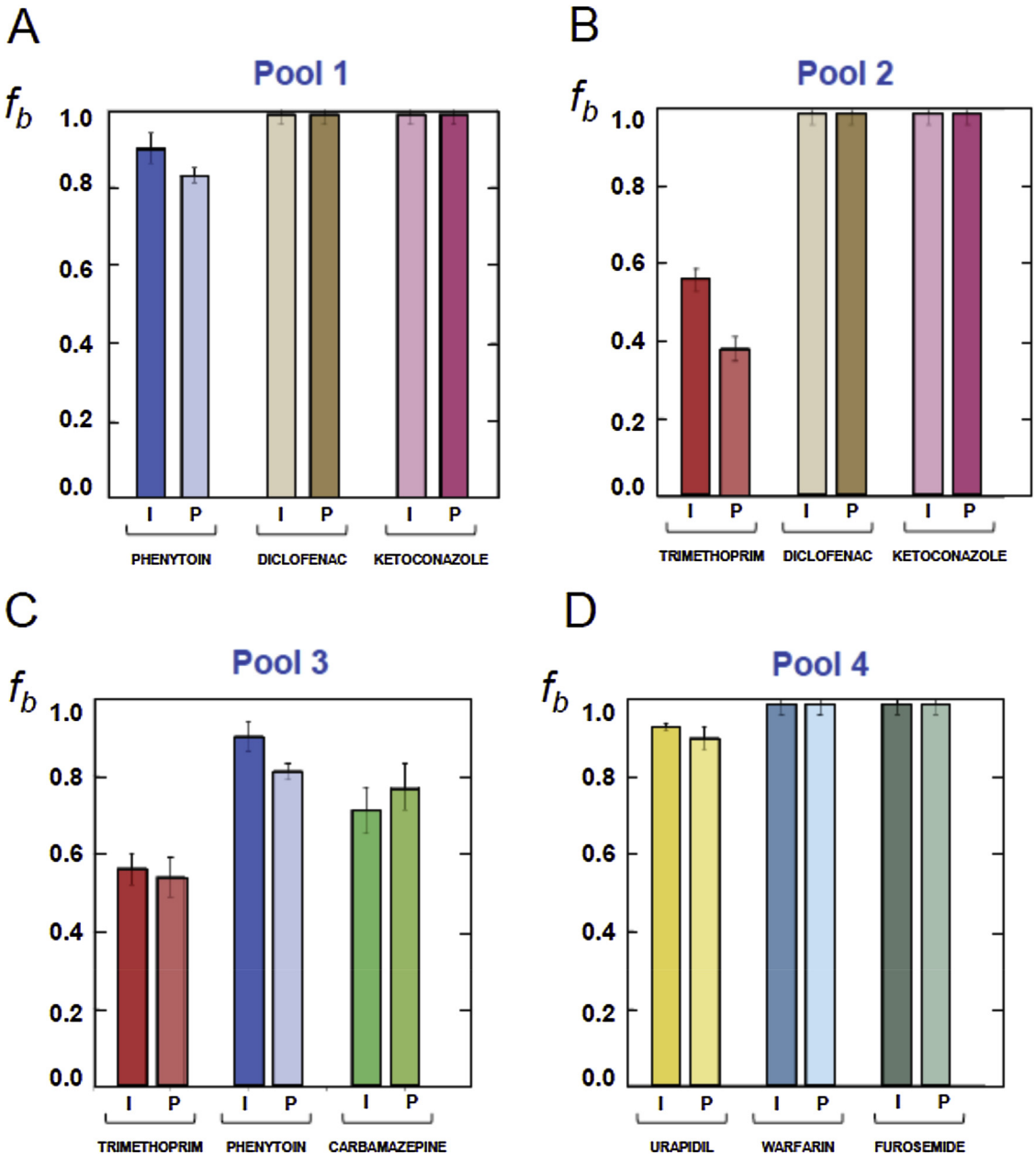

Fig. 7. Pools of compounds. Different pools of three compounds were tested using a 1:30 plasma dilution. The f_b is plotted when measured for the isolated compound and for the same compound in the pool. (**A**) Pool containing two very high and one high binder. (**B**) Pool containing one medium, one high, and one very high plasmatic protein ligand. (**C**) Pool with two medium and one high binder. (**D**) Pool contain three high binders. Compounds were classified based on their literature f_b value as in Fig. 2.

4. Discussion

In this work, we present a new method for assessing PPB of drug candidates using NMR spectroscopy based on the degree of line perturbation of the small molecule signals caused by interaction with plasmatic proteins. Differently from most of the known NMR-based methods to detect ligand-protein interaction, our method uses plasma and not purified proteins, and can provide a quantitative information about the bound fraction of the molecule using empirically derived linear relationships. The agreement with literature data is equivalent to that achievable with a well-established methodology like RED. As the main advantages of this method, it is worth mentioning that it involves a fast measurement with minimum of sample preparation and at a very low price per sample. In addition, the spectrum obtained for the isolated small molecule gives information about its solubility in buffer and serves as a quality check of the proposed structure. Finally, it is possible to assess the role of HSA and/or hAGP in the binding of the molecule. This information is particularly important in cases of binding to hAGP, because it can lead to dose-dependent behaviour of the pharmacokinetics (PK) parameters [4], mainly due to saturation at high concentrations.

A significant number of methods were proposed to measure PPB [2]. All the methods currently in use present drawbacks: equilibrium dialysis has its limitations particularly for compounds with very high non-specific binding, plasma instability, or inability to diffuse through the dialysis membrane. Ultracentrifugation may be an alternative method for these types of compounds, but presents a long experiment duration and can yield false high binding [2]. In the case of the NMR method presented here, we can foresee that problems will arise in the case of a slow exchange between free and bound compounds. This scenario was not very frequently observed for interactions with albumin, the most concentrated protein in plasma, except for tryptophan (our experiments, data not shown) and fluoro-tryptophan [13]. It can be more common for compounds interacting with alpha-glycoprotein, although no examples are known. If exchange between the free and HSA bound forms of the small molecule is slow, which normally occurs for compounds showing a high affinity for the target, values of f_b calculated at 1:400 dilution will be very low, and, contradictory, spectra obtained using the 1:30 dilution will show the almost complete disappearance of the small molecule signals. This behaviour, when detected, can be used to hypothesize a slow off-rate for dissociation of plasma proteins. This is not a minor point, since kinetics of the binding is an essential information to establish the role that PPB will play on the efficacy of a drug candidate [1].

A particularly important feature of any method used for PPB measurement is its ability to distinguish between high (f_b values between 0.90 and 0.99) and very high binders (f_b >0.99). This is because it is mostly very strong binding that might have an influence on the therapeutic effect of drugs, making recognition of these compounds essential. The empirical relationship obtained between f_b and I_B/I_F using a 1:400 plasma dilution shown in Fig. 3 makes the identification of very high binders feasible. A difference of 0.01 in f_b between two compounds will translate into a change of 0.10 in I_B/I_F, well within the accuracy of the measurement. The use of higher plasma dilutions can lead to larger differences in the signal intensity ratio, making it possible to differentiate more accurately candidates in drug discovery programs dealing with very high binders.

Another important point underlined by our results is the advantage of using plasma for assessing PPB instead of purified recombinant proteins. Although some approaches like ultrafiltration and equilibrium dialysis employ plasma, others like Biacore technology or chromatography and capillary electrophoresis are constrained to the use of recombinant proteins [2]. This limitation also holds for all NMR methods developed so far to study the interaction between proteins and small molecules [8]. Given the multiple actors and conditions that determine PPB capacity, we decided to use plasma, which showed to be particularly important for compounds interacting with hAGP or lipoproteins. Our results show that, in some cases, f_b values measured using plasma for compounds interacting almost exclusively with hAGP are lower than those obtained using the purified protein. This effect can be explained if one assumes that other compounds in plasma can compete for hAGP binding site. It is known that basic compounds can efficiently bind hAGP [14]. A large collection of small and positively charged compounds present at relatively high concentration in plasma can act lowering the apparent affinity of the tested compound. On the other hand, if the ligand interacts with lipoprotein, the use of solely HSA or hAGP will give a lower apparent f_b as compared to that measured using plasma, like in the case of Verapamil. Taken altogether, these observations strengthen the importance of developing methodologies that use plasma and not recombinant proteins.

PPB values of drug candidates are usually monitored during the drug discovery phase with the intent to prioritize compounds that show a low f_b. This common practice is based on the assumption that reducing PPB leads to a higher fraction of the active drug available to perform its pharmacological action. However, optimization of PPB needs to be strongly related to a good understanding of PK and PD parameters, which is the only way to evaluate the effect of protein binding on drug efficacy [2]. In many cases, a drug can show adequate tissue levels required for its pharmacological action in spite of showing a high PPB. For this reason, and many others described in the excellent review by Bohnert & Gan [2], it is not recommended the use of significant resources to perform detailed PPB evaluation during the early stages of drug discovery. Fast, cost-effective and highly reproducible methods like the one we present in this manuscript are perfectly suited to evaluate the PPB of compounds in the lead identification/optimization stages.

5. Conclusions

This manuscript describes a new method for assessing PPB of drug candidate using NMR that involves a fast measurement (20 min for each spectrum) with minimum of sample preparation; is cheaper than other methods for PPB calculation; includes a quality check of the chemical structure and solubility of compound and is suitable for automation. Particularly important is the use of plasma and not purified proteins, which can give PPB values different from those observed in physiological conditions and opens the possibility to measure PPB in plasma of different species.

Compliance with ethical standards

All authors are currently employed at IRBM Science Park and University of Rome “Tor Vergata” and declare no conflicts of interest.

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