A method for estimation of plasma protein binding using diffusion ordered NMR spectroscopy (DOSY)

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RSC Medicinal Chemistry

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- Studies which examine the effect of the molecular structure of a compound on **pharmacokinetic behaviour and pharmacodynamics**.

UNIVERSITY OF WEST LONDON
School of Biomedical Sciences

Dear Editor,

First of all, my co-authors and I would like to sincerely thank the editorial staff and reviewers for the consideration of our manuscript entitled "A Method for Estimation of Plasma Protein Binding Using Diffusion Ordered NMR Spectroscopy (DOSY)" for publication in *RSC Medicinal Chemistry* journal. The following is a point-by-point reply to the concerns raised by the reviewers:

Response to Reviewer 1

(1) The influence of temperature on DOSY results is significant, and the author needs to clarify whether temperature control was implemented during the experiments and specify the temperature settings. Additionally, setting experimental parameters correctly is crucial. It is suggested that the author provides more details on parameter settings such as pulse sequence, gradient range, and diffusion delay (Δ).	We can confirm temperature control was maintained for all experiments and thank the reviewer for pointing out this was not mentioned in the manuscript. The instrument was set to 298.2 K, and monitored for deviations from this baseline. We have amended the manuscript to state: "The DOSY spectra were collected using a Bruker Spectrospin 400 Ultrashield NMR spectrophotometer operating at 400 MHz, with samples maintained at 298.2 K via a condensed gas feed (400 lph). ^{1}H DOSY NMR measurements were set up with 16 gradient scans, with 16 N repeat samples across each gradient. All measurements initiated using Bruker TopSpin (version 3.7) with IconNMR automation. The pulse sequence ledbpgp2s was used (see **Supporting Information** for more details)." See track changed version of the manuscript for the changes. We have also provided additional information in **Supporting Information** file.
(2) In the methodology section, the description of the experimental materials is not sufficiently detailed.	We have now identified the commercial sources of all materials.
(3) In the Abstract: "corelates" should be corrected to "correlates".	This has been amended
(4) In the Introduction "benefits" should be in verb form, so it should be changed to "does benefit".	This has been amended
(5) In the Introduction: It should use the plural form of the verb, "measures".	This has been amended
(6) Reviewer 1 had also asked for a discussion of possible experimental errors and limitations to the method.	In the discussion, we have added a paragraph to discuss the issue of convection/temperature variation and viscosity.

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Response to Reviewer 2

The authors need to justify the importance of the manuscript in light of the work reported in above references, how this work is different from that already reported.	Whilst it is true that DOSY is a well-established technique to investigate molecular binding, it has not previously used to determine PPB, which has significant implication in medicinal chemistry. We thank the reviewer for the three manuscript they brought to our attention. The paper by Tanoli *et al*. ("The exploration of interaction studies of smaller size, mostly ignored yet intrinsically inestimable molecules towards BSA; An example of STD and DOSY NMR" *Cent Eur J Chem*, **2014**, *12*, 332-340.) investigates binding of 2-aminopyridine and isovanillin to BSA and primarily uses saturation transfer difference technique. There is a brief mention in this paper (section 3.5) that presence of BSA induced a gradual shift in diffusion coefficient (D) of these two molecule. The D values though are NOT used to determine binding affinities. It purely uses the change in diffusion as evidence that the molecules have 'an interaction' with proteins. The goal/output of this work is very distinct from the purposes of our work which uses DOSY to study the bound and unbound fractions of drug molecules in a quantitative manner. Nevertheless, we have cited this work as it confirms changes in diffusion speed of small molecules in presence of a protein (see later) Paper by Liu, *et al.* ("Measurement of Biomolecular Diffusion Coefficients in Blood Plasma Using Two-Dimensional ^{1}H-^{1}H Diffusion-Edited Total-Correlation NMR Spectroscopy" *Anal. Chem.* **1997**, *69*, 1504-1509) describes a method to measure diffusion coefficients of individual small molecules (for example glucose, amino acids etc), which are in a complex biofluid mixture. There is no mention of drugs or PPB, and no binding affinity of these molecules are measured. Paper by Lee, *et al.* ("High-Resolution Diffusion Measurements of Proteins by NMR under Near-Physiological Conditions" *Anal. Chem.* **2020**, 92, 5073-5081) describes a method to recoup information lost during NMR

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	structural studies of large biomolecules due to proton exchange. Again, it has no mention of PPB and no binding affinity of small molecules are included. We feel neither of these two papers are sufficiently relevant to the scope of our study to be cited. However, in view of the reviewer's comments we considered framing our work within the published literature relevant to the use of DOSY in determining molecular interactions. We have added two paragraphs to describe this use of DOSY.
The authors need to add how the diffusion coefficients as shown in the graph quantitatively determine PPB. This needs to be explained in the discussion part.	Firstly, we would like to emphasise that the data we provide in table 1 are the values for fraction of drug bound to BSA (f_b) at given drug:BSA ratio in comparison to published PPB. As we outline in the text, albumin is the major component of plasma protein and so the f_b values are a close estimation of PPB. We believe we have provided clear explanation of the calculation for f_b in **Figure 5** and associated text. However, to remove any misapprehension of this point, we have explicitly stated values in the table are for f_b to BSA.

Additional changes

In addition to the above changes, we have made two minor amendments to two sentences in pages 6 and 19 to clarify the points we are making.

We would like to thank the reviewers again for their helpful advice and hope that the revisions that we have made will address the issues raised by them.

Sincerely,

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A Method for Estimation of Plasma Protein Binding Using Diffusion Ordered NMR Spectroscopy (DOSY)

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Abstract:

The plasma protein binding (PPB) of a drug plays a key role in both its pharmacokinetic and pharmacodynamic properties. During lead optimisation, medium and high throughput methods for the early determination of PPB can provide important information about potential PKPD profile within a chemotype or between different chemotype series. Diffusion ordered spectroscopy (DOSY) is an NMR spectroscopic technique that measures the diffusion of a molecule through the magnetic field gradient, according to its molecular size/weight. Here, we describe the use of DOSY for a rapid and straight forward method to evaluate the PPB of drug molecules, using their binding to bovine serum albumin (BSA) as a model.

Introduction

Human blood contains about 6-8% of soluble proteins which are essential for its function.[1,2] In particular, these proteins (commonly referred to as plasma proteins) bind and transport many biologically important molecules such as lipids and hormones. Administered drugs also bind plasma proteins and are transported *via* blood to various tissues and organs. Albumin accounts for just over half of all human blood proteins.[1,2,3,4] This is followed by various globulins which account for just under 40%.[1,2,5] The main component of the remainder is fibrinogen, which is involved in the coagulation of blood in the case of injury.

The plasma protein binding (PPB) of a drug plays a key role in both its pharmacokinetic (PK) and pharmacodynamic (PD) properties.[6,7,8,9] Without energy-dependent processes, the free drug concentration in plasma correlates to that found in tissues, and it is this tissue drug concentration that promotes binding to the target, which in turn elicits a pharmacological effect.[8] The level of unbound drug in the plasma therefore correlates to the concentration of available drug in the tissues and is pharmacological action. Although low plasma protein binding translates to higher fraction of unbound drug, f_u, and lower volume of distribution, it can also promote faster clearance or metabolism. So, an understanding of the PPB enables a better appreciation of the factors that can contribute to the *in vivo* efficacy of a drug or it's safety profile. Therefore, methods that enable determination of PPB are highly desirable.[9]

There are a number of existing methods for determining PPB of drug molecules, each with their own advantages and drawbacks. Many of these protocols use binding to purified albumin in place of plasma proteins, partly because it is cheaper and more easily accessible but also because its quality does not change from one batch to another, reducing variability

in experimental results and enabling a more reliable comparison between the results.[10,11,12,13,14,15,16,17]

The gold standard for measuring the extent of PPB is equilibrium dialysis (ED).[15] ED involves partitioning of a drug between two compartments, separated by a semi-permeable membrane that functions as a molecular filter based on differences in molecular size and weight.[18] Only molecules below a particular molecular weight threshold have the ability to permeate through the membrane. So, whilst drug molecules permeate through the membrane, heavier proteins or drug-protein complexes do not. Equilibration times are typically long (12-48 hours),[18] so one drawback of ED is the slowness of the method. Rapid equilibrium dialysis (RED) is a high-throughput, 96-well plate version which enhances the efficiency of the ED method. RED allows for equilibration times ranging from 1.5 to 4 hours, depending on the rate of agitation.

However, the adsorption of some compounds at surfaces and membranes of ED devices can have an impact on the measurement, particularly since this 'parasitic' binding sometimes exceeds 50% of the total concentration.[17,18] The Gibbs-Donnan effect, whereby charged particles in close proximity to a semi-permeable membrane do not evenly distribute on either side of the membrane, is also problematic in ED methods and results in an uneven distribution of charged moieties across the membrane.[8,16] This effect can however be prevented through the use of a sufficiently strong ionic buffer, or the dilution of whole plasma prior to dialysis. Other drawbacks for ED are the limitations for non-specific, high binding compounds, as well as issues of plasma instability, and the inability of some compounds to diffuse through the dialysis membrane.[18]

Other commonly used methods for the evaluation of PPB include ultrafiltration and ultracentrifugation. Ultrafiltration is one of the simplest and fastest methods for f_u determination, making it a useful tool in drug monitoring studies.[8] It is similar to ED except

in that it utilises the application of pressure to increase the speed of analysis by forcing the solution through the membrane.[18] Ultrafiltration still suffers from the same drawbacks as ED, including the Gibbs-Donnan effect, protein leakage through the membrane, and nonspecific binding of compounds to the filter membrane. Due to the high pressure enforced for rapid analysis times, the volumes of ultrafiltration cannot exceed 10% of the total sample volume in order to maintain equilibrium.[8,16] Ultracentrifugation is also related to ED, but instead of separation *via* a membrane, centrifugal force is used to separate the protein from the free drug.[18] This method's advantage is that it eliminates membrane effects, such as the Gibbs-Donnan effect and adsorption, but ultracentrifugation also presents its own issues. A lipid layer can form as high-density proteins like albumin will sink, but lower-density lipoproteins will float, leaving the protein-free drug layer in the middle. This can cause difficulties in the sampling for this method. Moreover, the method requires expensive equipment, is low-throughput, and can give falsely high results for binding due to free-drug sedimentation, viscosity, or back-diffusion.[8,18]

Each of the above methods involves physical separation of constituent molecules, but various spectroscopic techniques [17,18,19,20] particularly nuclear magnetic resonance (NMR) spectroscopy [17,21,22,23] which do not involve separation, can also be employed in the determination of PPB. NMR methods are used to determine PPB by analysing information on changes that occur in the spectrum of an unbound drug (or unbound protein) following protein-drug-binding particularly at high-affinity binding sites. For example, NMR studies measure the degree of line perturbation of small molecule signals following plasma protein interaction.

One drawback of using NMR methods is that due to the timescale of experiments, signals are often averaged of the bound and unbound molecules. For instance, one-dimensional ^{1}H Nevertheless, this can allow for the derivation of linear relationships, providing quantitative

information on the bound fraction of the molecule.[17] However, using NMR to determine PPB binding does benefit~~s~~ from a number of important features compared to its counterparts. To start with the sample preparation is minimal and fast, and the NMR measurement data acquisition speed is comparatively fast compared to other techniques.~~this is enhanced by the speed of the NMR measurements themselves~~. In addition, running of samples, and the materials required, are inexpensive, and the role of each of the protein components of plasma can be separately assessed in binding *via* NMR method.[17,21,22,23] NMR spectrometers are already ubiquitously used in most medicinal chemistry laboratories. Perhaps most importantly though, NMR based techniques can relatively easily be adapted for automation which allows rapid collection of data on a series of chemotypes. However, one-dimensional NMR methods also have numerous drawbacks. In particular, spectra of most proteins are complex and it is often difficult to distinguish signals for individual groups of protons within the spectra of a mixture of protein, unbound drug and drug-protein adduct.[18] To overcome this many groups favour high power, high field instruments to improve resolution of complex overlapping protein peaks.[24] Whilst this does not affect per sample complexity, cost or run time it does require a vast increase in capital expenditure to both access and house these instruments.

On the other hand, two dimensional NMR spectroscopy such as DOSY, enable us to discern signals from mixtures of compounds. DOSY measures diffusion constants of molecules by measuring the rate by which they are displaced within a magnetic field gradient. The diffusion constant for a molecule depends on a number of factors in particular viscosity of the medium, and its size (molecular volume or weight).

Here, we describe a quick, straightforward and cost effective DOSY-based method to rank binding affinity of different molecules, including drug molecules, to bovine serum albumin

(BSA) and show that this data is in agreement with information from the literature on PPB binding of these molecules.

Method

Caffeine (C0750), diclofenac (SML3086), propranolol (P8688), acetyl salicylic acid (S5922), theophylline (T1633), D-glucosamine hydrochloride (G1514), D-ribose (R7500), cimetidine (C4522), esomeprazole (E7906), DMSO (472301), bovine serum albumin (A7030) and deuterium oxide (151882), were all purchased from sigma. The relevant quantity of each drug was dissolved in D_2O (>99.5% isotope purity, 5 mL), to afford a 10 mM solution (solution A). BSA (332.4 mg) was dissolved in D_2O (5 mL), yielding a 1 mM solution (solution B). The required solutions of BSA, drug and BSA plus drug were prepared by combining aliquots of solutions A, B and D_2O to a total of 600 µL volume in a 5 mm diameter NMR tube. For example, five 600 µL solutions were prepared by adding 100 µL of solution B (BSA), x µL of solution A (drug) and (500-x) µL of D_2O, where x is 0, 100, 200, 300, 400 µL. In this example, the final concentration of BSA is 167 µM and the final concentrations of drug, which depends on quantity of x, are zero, 10 fold (1.67mM), 20 fold (3.34 mM), and 40 fold (6.68 mM). Concentration of solution A can be adjusted to afford a wide range of drug:BSA ratios. The DOSY spectra were collected using a Bruker Spectrospin 400 Ultrashield NMR spectrophotometer operating at 400 MHz, with samples maintained at 298.2 K via a condensed gas feed (400 lph). ^{1}H DOSY NMR measurements were set up with ~~at~~ 16 gradient scans with 16 N repeat samples across each gradient. All measurements were initiated ~~16 sampling for each scan~~ using Bruker TopSpin (version 3.7) with IconNMR automation. The pulse sequence ledbpgp2s was used (see **Supporting Information** for more details).~~software~~. Calculation of the diffusion coefficient were done using a Bruker Dynamic Centre (version 2.4.11)~~ software~~. All experiments were carried out in triplicate. All fits were carried out using a single exponential diffusion decay with an R^2 > 99%. GraphPad Prism 8 was used for the

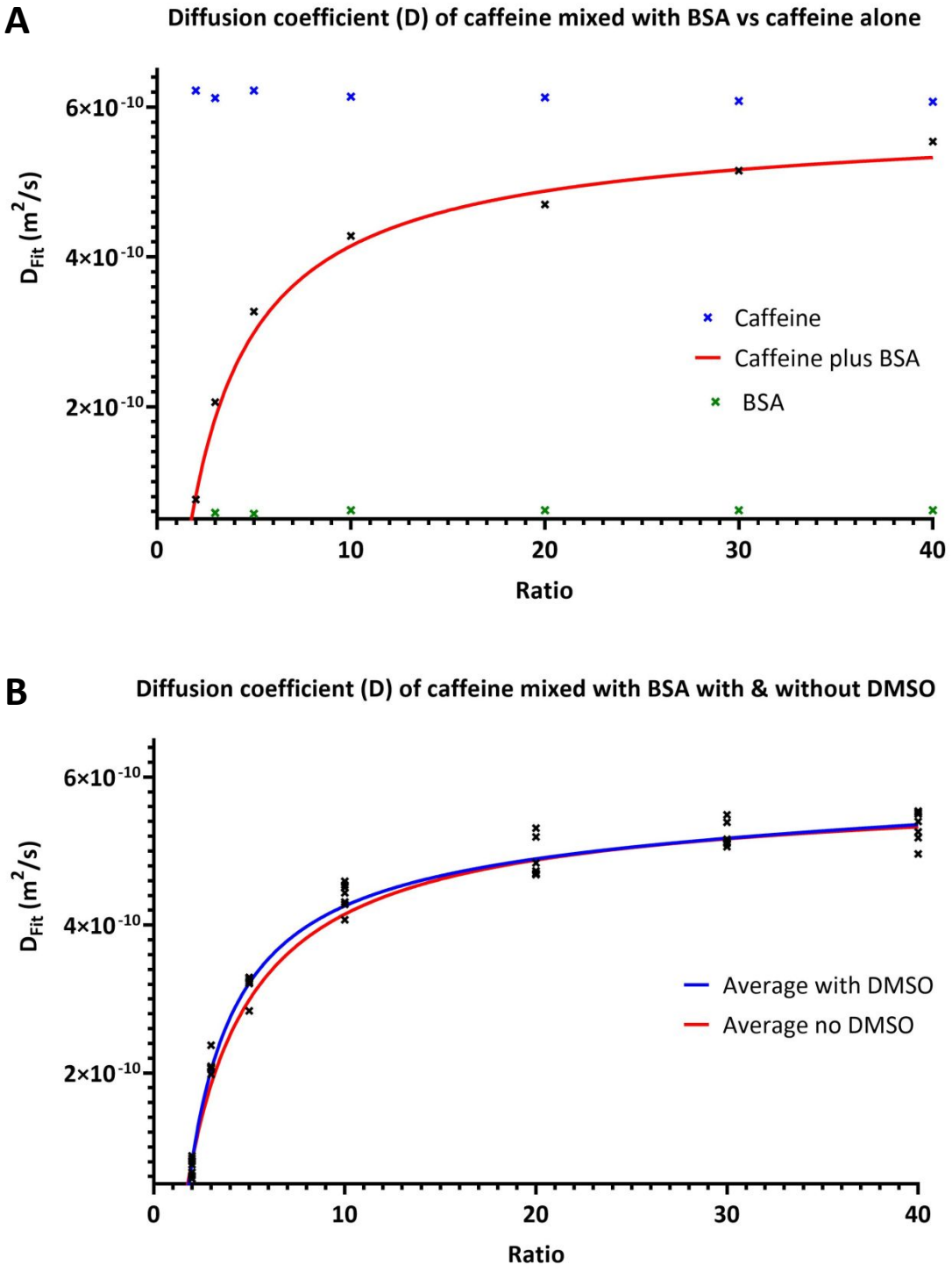
production of graphs and quantification of the results. ~~An~~ Multiple exemplar analysis ~~is~~ of raw data are provided in **Supporting Information** file.

Results

Investigation of Binding of Caffeine to BSA

We first set out to test the binding of caffeine to BSA. Caffeine has a relatively low-medium binding affinity to plasma proteins[25,26] and is often used as a standard/control in the existing methods for PPB determination. DOSY was used to determine diffusion coefficients (D) on samples containing different concentration of caffeine only, BSA only and caffeine/BSA mixtures which correspond to caffeine:BSA ratios ranging from 2:1 to 40:1 (**Figure 1A**). For both caffeine and BSA, diffusion coefficients are constant over the concentration range (**Figure 1A**, blue crosses for caffeine and green crosses for BSA). As expected however (see discussions), there is a steady rise in the observed, averaged diffusion coefficient for caffeine in the presence of BSA which eventually reaches a plateau at just below the diffusion coefficients of pure caffeine (**Figure 1A** black crosses). An observed D_{max} value can be calculated by fitting a hyperbolic curve on these datapoints. Repeating experiment (n=3) afforded very similar observed D_{max} values (standard deviation = 1.67×10^{-11} m^2/s or 2.5% of the mean) confirming the reproducibility of the experiments (**Figure 1B**). Although caffeine is quite soluble in (deuterated) water, many investigative molecules are not. In these instants, it is commonplace to dissolve test articles in dimethyl sulfoxide (DMSO), prior to addition to aqueous media to assist with their solubilisation. Therefore, we investigated the effect of adding deuterated DMSO (up to 3% v/v). Again, the experiment afforded very similar observed D_{max} value (**Figure 1B**) confirming small quantities of DMSO do not affect the values for observed D_{max}. Interestingly, although there is a steady increase in the observed D_{max} value from these experiments, the values were still just below the theoretical D_{max} (that for caffeine alone). To show that the observed D values eventually

converge to the D value for caffeine alone, we repeated the experiment at 100:1 ratio of caffeine to BSA. As expected, the observed D_{max} value raised to nearer the value for theoretical D_{max} for caffeine alone (**Figure 1C**).



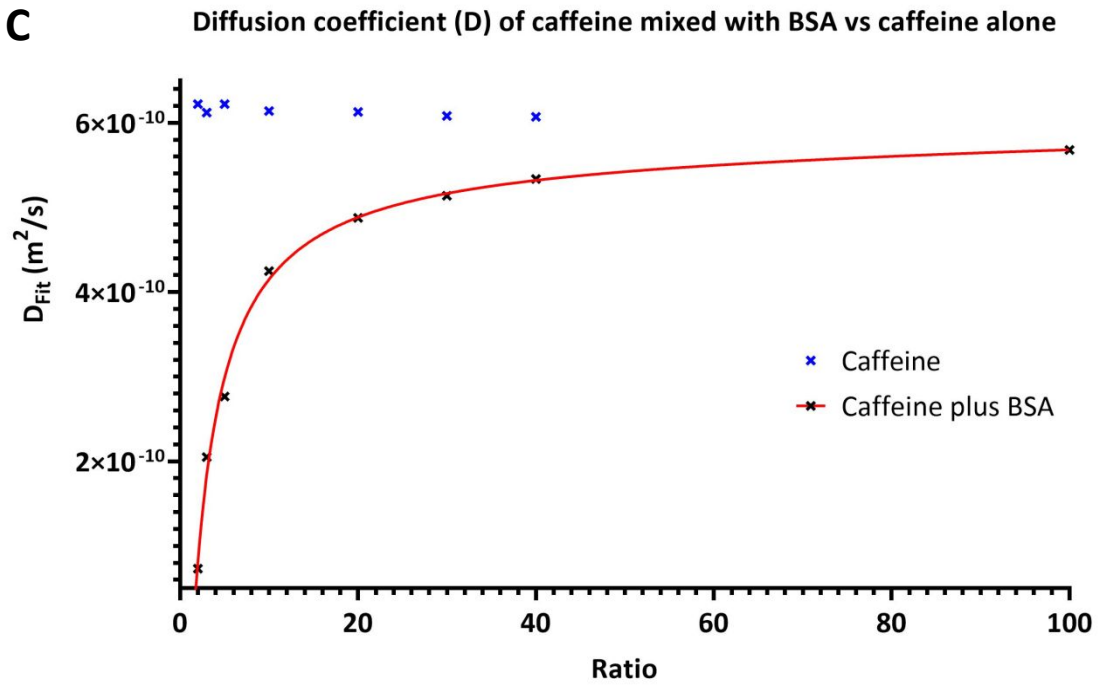


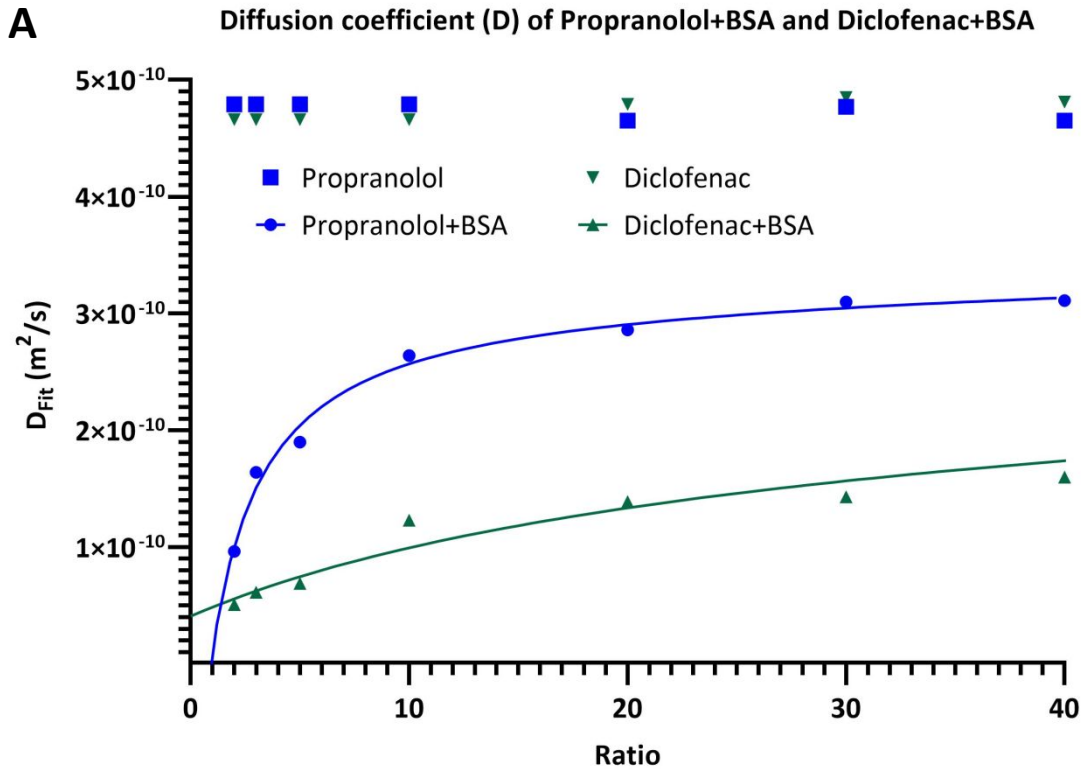
Figure 1 (**A**) Changes in diffusion coefficient, D, of caffeine over a concentration range in the absence of BSA (blue crosses) and in the presence of BSA (black crosses). (**B**) Comparison between the diffusion coefficients of caffein/BSA mixtures in the presence or absence of 3% v/v DMSO (**C**) The maximum value for diffusion coefficient, D_{max}, of a mixture of caffeine and BSA steadily rises towards the value for caffeine alone as the ratio of caffeine:BSA increases.

Determination of Binding of Diclofenac and Propranalol to BSA

In contrast to caffeine which is reported to have a low-medium PPB binding, diclofenac is reported to have a high PPB binding.[25,27] So we set out to investigate the D value for diclofenac:BSA mixtures and compare it with that of caffeine:BSA mixtures over the same range (2:1 to 40:1). Again, DOSY was used to determine diffusion coefficients of diclofenac over the concentration range to show that these values remain constant (**Figure 2A**). As expected, there is a steady rise in the observed, averaged diffusion coefficient for diclofenac with an increase in the ratio of diclofenac:BSA. However, in contrast to the observation with caffeine, the fitted curve is shallower and shows a significantly slower rise. Because of this, and to show that the D values have not yet reached a plateau at 40:1 ratio, we increased the ratio first to 100:1 and then to 600:1. As expected, the observed D value raises steadily towards the D_{max} value for diclofenac (**Figure 2B**). This observation is wholly consistent with

a higher PPB binding for diclofenac than for caffeine. Because of the higher affinity of diclofenac, the proportion of free drug to bound drug is low, meaning that contribution of the free drug to the observed D remains small unless a much larger ratio of diclofenac is used.

Propranolol (295.80 g mol^{-1}) has a similar molecular weight to diclofenac (296.15 g mol^{-1}), however it is reported to have a weaker binding to BSA.[25,28] To demonstrate that the differences in observed D values for diclofenac and caffeine where not merely a consequence of the two molecules' different molecular weights, we measured the D value for propranolol:BSA mixtures and compare it with that of diclofenac:BSA mixtures over the 2:1 to 40:1 concentration range (**Figure 2A**). Averaged diffusion coefficient for propranolol increases with the ratio of drug:BSA. However, the fitted curve is steeper and shows a significantly faster rise than observed for diclofenac. This suggests that propranolol has a weaker binding to BSA and in deed the reported value for PPB of propranolol [25,28] is lower than that for diclofenac.[25,27]



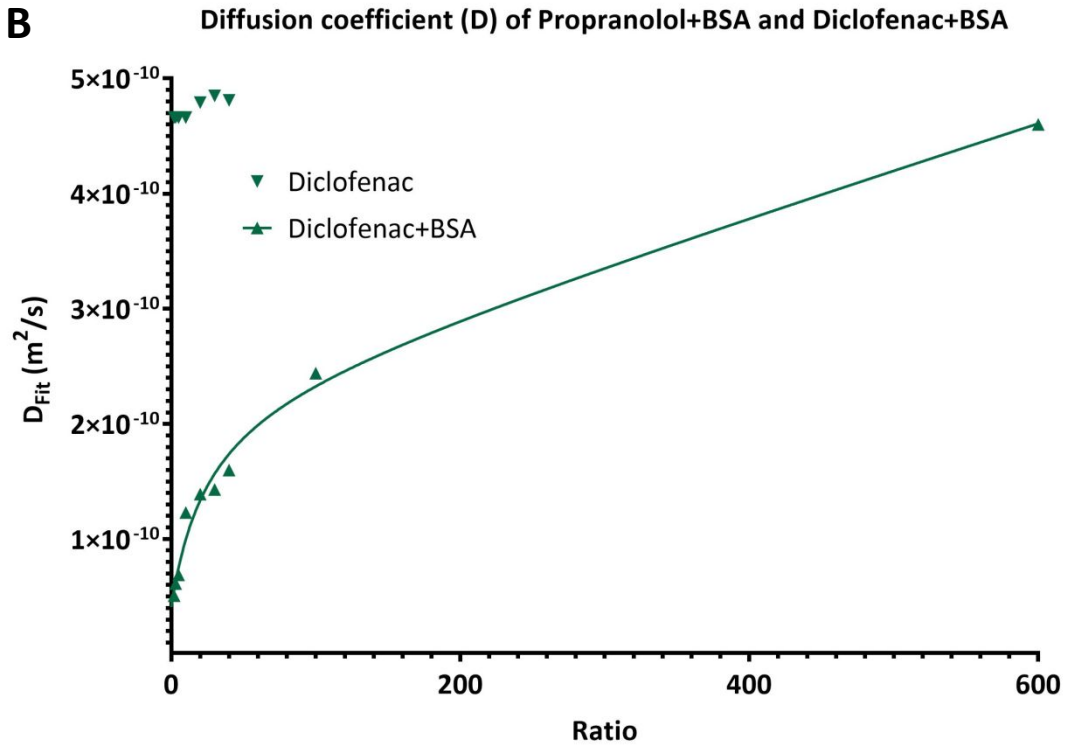


Figure 2 (**A**) Changes in diffusion coefficient, D, of propranolol (blue) and diclofenac (green) over a concentration range in the presence of BSA (blue crosses). Control values for absence of BSA are also shown. (**B**) The maximum value for diffusion coefficient, D_{max}, of a mixture of caffeine and BSA steadily rises towards the value for diclofenac alone as the ratio of diclofenac:BSA increases.

Determination of Binding of theophylline, acetylsalicylic acid and glucosamine to BSA

As was the case for propranolol and diclofenac, theophylline (180.16 g mol^{-1}), glucosamine (179.17 g mol^{-1}) acetylsalicylic acid (180.16 g mol^{-1}) have similar molecular weight but very different physical and chemical properties which means we expect each of them to bind very differently to BSA. Theophylline is however structurally very similar to caffeine which means we expect it's binding to BSA to be very similar to that of caffeine.

DOSY was used to determine diffusion coefficients of theophylline, acetylsalicylic acid and glucosamine over the concentration range (2:1 to 40:1). As can be seen below (**Figure 3A**), theophylline and caffeine not only have similar observed D_{max} values, but their respective fitted curves are also similar in shape. In contrast, acetylsalicylic acid has a very different observed D_{max} and a much shallower fitted curve suggesting it binds to BSA more strongly than theophylline. (**Figure 3B**).

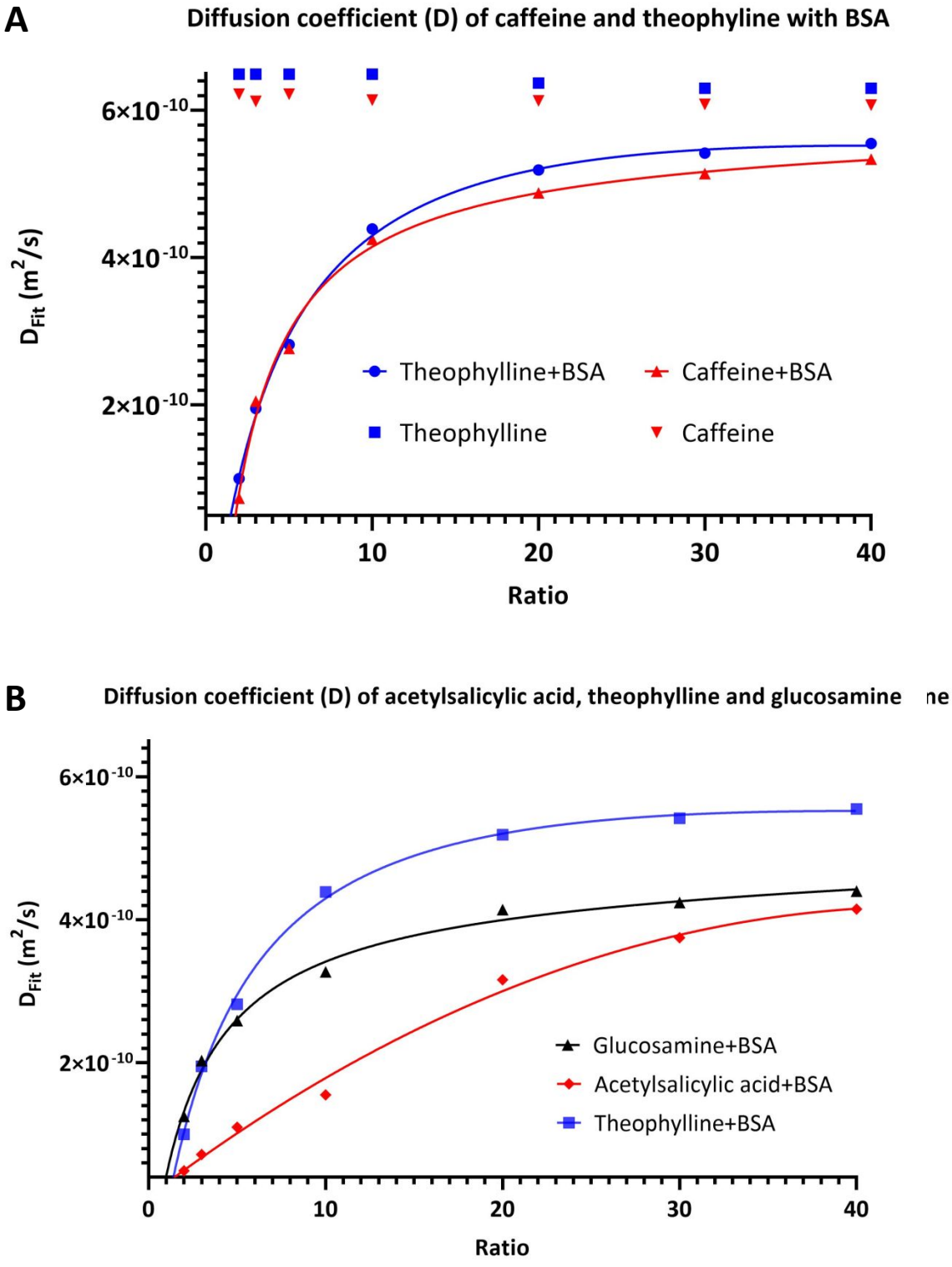
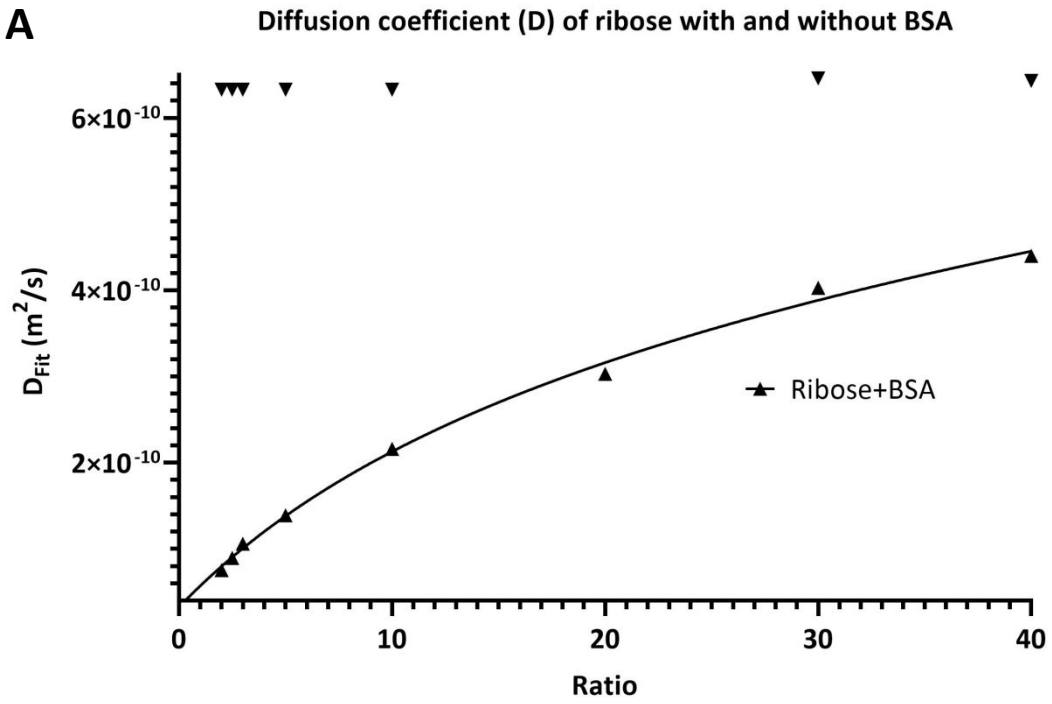


Figure 3 (**A**) Similarity in changes in diffusion coefficient, D, of theophylline (blue) and caffeine (black) over a concentration range in the presence of BSA. (**B**) Changes in diffusion coefficient, D, of theophylline (blue), acetylsalicylic acid (black) and glucosamine over a concentration range in the presence of BSA. Control values for absence of BSA are not shown for clarity.

Quantitative Comparison of Binding to BSA.

In addition to the drugs and chemical compounds mentioned above, we also investigated the binding of ribose, cimetidine and esomeprazole (as magnesium sulfate salt) to BSA (**Figure 4**). Examination of the curves confirms that esomeprazole is a high binding molecule whilst cimetidine is a low binding drug. Ribose, which appears as medium binding in comparison with the other two molecules has not been previously investigated for PPB. Interestingly, in contrast to the similarity between theophylline and caffeine, the curves for ribose and glucosamine are quite different, suggesting very different binding affinities to BSA, even though the molecules are both carbohydrate sugars.



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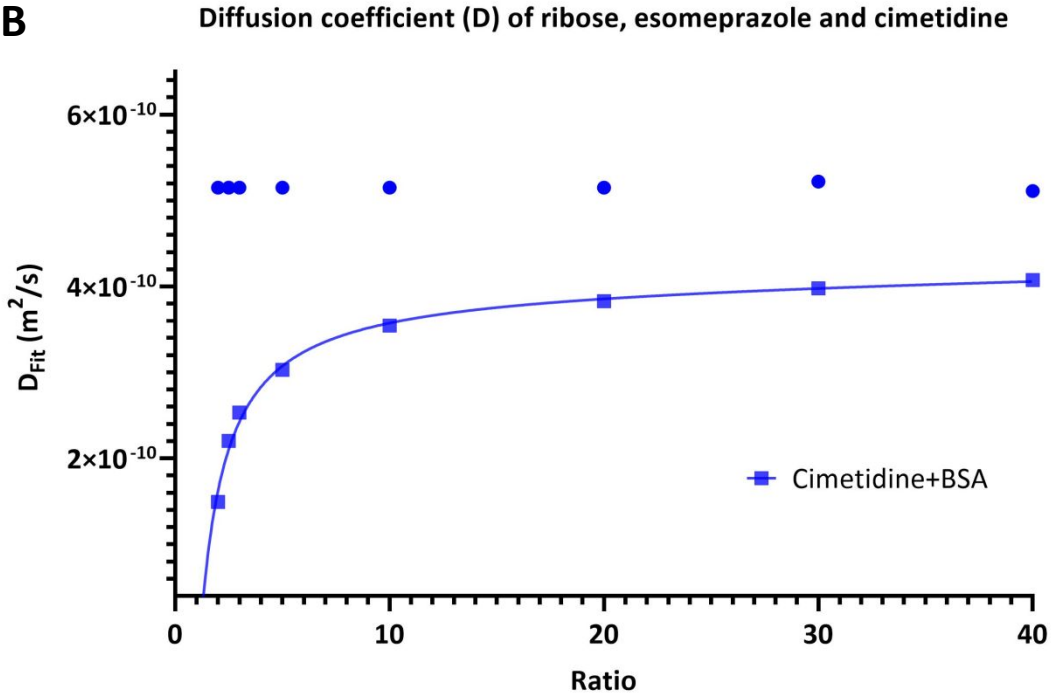
Diffusion coefficient (D) of ribose, esomeprazole and cimetidine
6×10-10
4×10-10
2×10-10
DFit (m2/s)
Cimetidine+BSA
0
10
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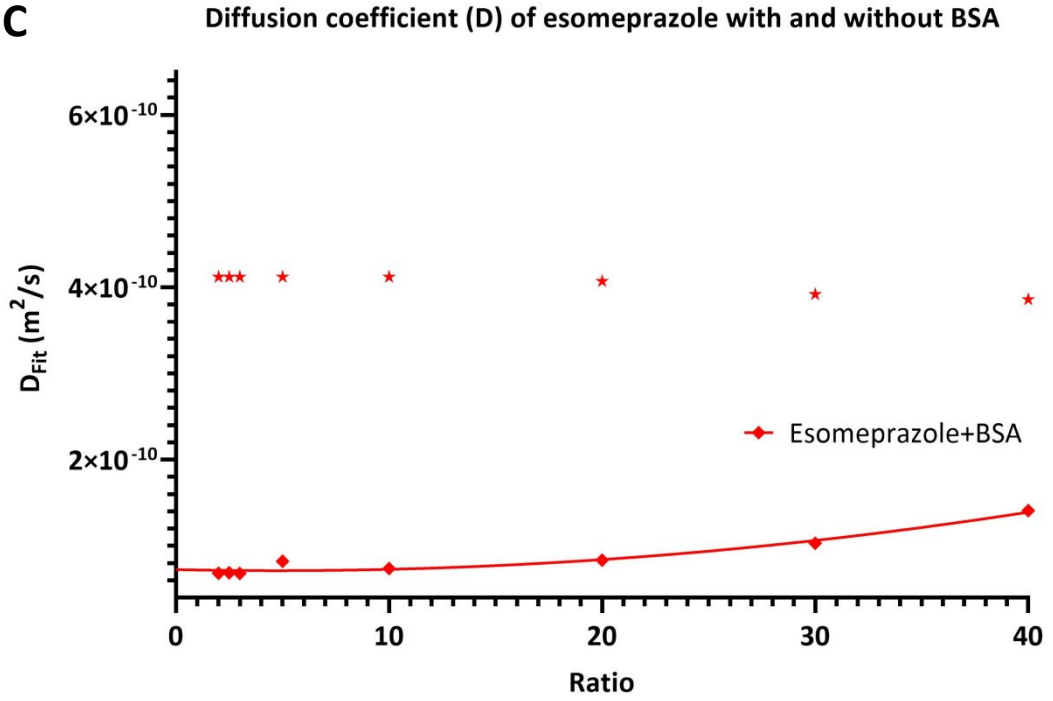
Ratio

C


Diffusion coefficient (D) of esomeprazole with and without BSA
6×10-10
4×10-10
2×10-10
DFit (m2/s)
Esomeprazole+BSA
0
10
20
30
40
Ratio

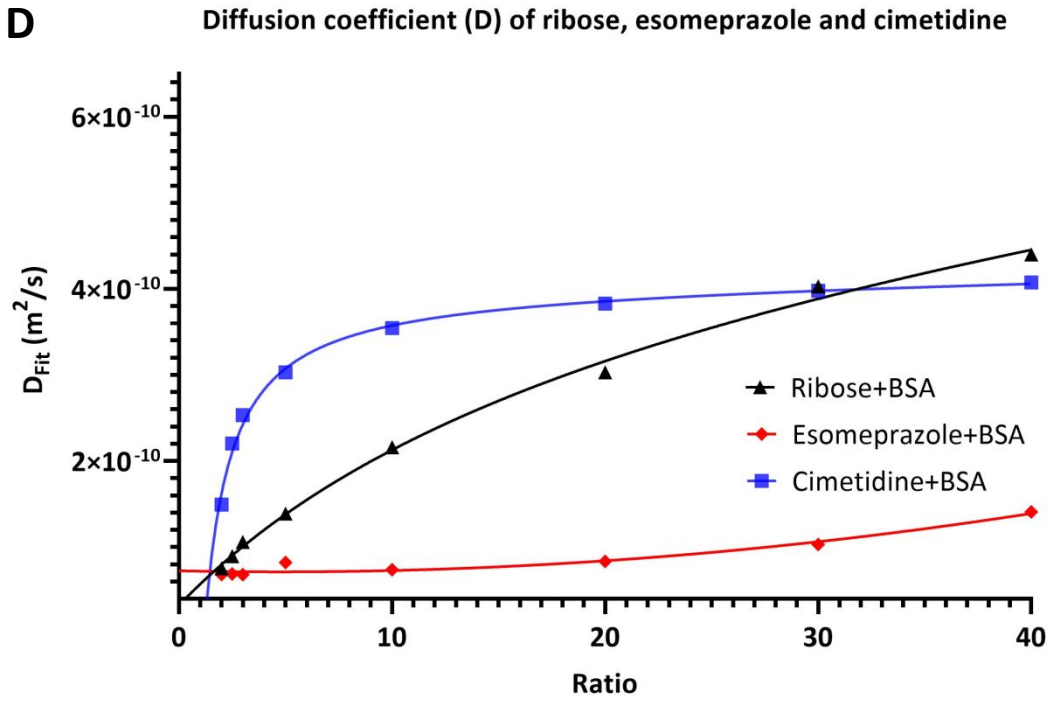


Figure 4 Differences in changes to the averaged diffusion coefficient of different molecules in the presence of BSA: (**A**) Diffusion coefficient, D, of ribose over a concentration range in the presence of BSA. (**B**) Diffusion coefficient, D, of cimetidine over a concentration range in the presence of BSA. (**C**) Diffusion coefficient, D, of esomeprazole over a concentration range in the presence of BSA. (**D**) Changes in diffusion coefficient, D, of ribose, cimetidine and esomeprazole over a concentration range in the presence of BSA. Control values for absence of BSA are not shown for clarity.

Clearly, examination of the curves provides a qualitative estimation of the binding affinities of different drug molecules to BSA. As the concentration of drug relative to a fixed concentration of BSA is increased the observed averaged diffusion coefficient for the molecule also increases approaching the value for the diffusion coefficient for the molecule in solution on its own. For molecules with low binding to BSA, in this example cimetidine, the rise to its corresponding maximum is sharper, whereas for molecules with high binding to BSA, in this example esomeprazole, the rise to its corresponding maximum is shallow.

Although the observation of fitted curves allows a qualitative measure of binding of different molecules to BSA, we wanted to quantitatively estimate the fraction of bound drug. One way to do this is to measure the relative observed diffusion coefficient (D_{obs}) to the D_{max} value at a given ratio of drug:BSA for each drug/molecule.

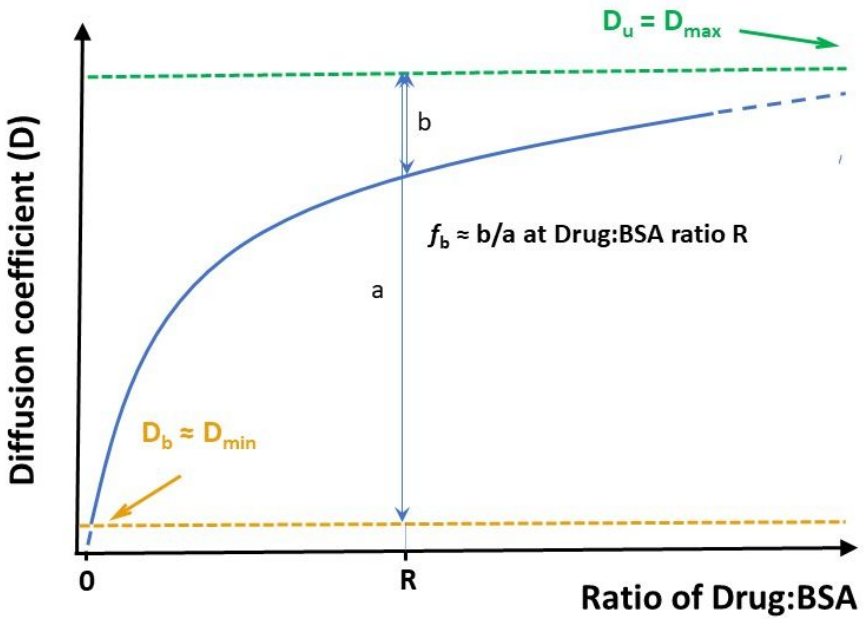


Figure 5 Estimation of the relative values of f_b at a given Drug:BSA ratio

We argued that the D_{obs} at any given drug concentration is an averaged value from all bound and unbound molecules, or $D_{obs} = (D_b \times f_u) + (D_u \times f_b)$ where D_b would be the diffusion coefficient of entirely bound drug, D_u would be the diffusion coefficient of entirely unbound drug, and f_b and f_u would be the fraction of bound and unbound drug respectively. Since $f_b + f_u = 1$, then $f_b = (D_u - D_{obs})/(D_u - D_b)$. The value of D_u is the same as the diffusion coefficient of the drug in the absence of BSA (D_{max}) which can be experimentally determined (**Figure 5**). The value of D_b however, can only be estimated. Value of D_b corresponds to when there is a large excess of BSA so that even if the drug molecule has little affinity, majority of it would be bound to BSA. However, as the ratio of Drug:BSA is reduced, it would be more difficult to discern the peaks due to the small molecule drug from protein, and hence not possible to determine diffusion coefficient. We found that for all molecules in this study, determination of diffusion coefficient was only possible at drug:BSA ratios >2.

Table 1 shows the estimated values of relative BSA binding (f_b) at 5:1, 10:1 and 20:1 ratio of drug:BSA for molecules in this study, as well as previously reported values for the whole human plasma binding of the drugs from the literature. Obviously, this qualitative

comparison carries a number of caveats. There may be differences between human and bovine serum albumin. Furthermore, albumin is only one, albeit the major component of blood proteins, and therefore, binding to other protein components of plasma can significantly change values for PPB than the ones estimated from binding to BSA. Nevertheless, as can be seen the ranking of BSA binding from our study closely mirrors the PPB binding values reported for these drugs.

Molecule	PPB reported in literature (%)	Fraction bound (f_b) to BSA (%) from this study		
		at 5:1 (%)	at 10:1 (%)	at 20:1 (%)
Diclofenac	99.5[25,27]	100	94	90
Esomeprazole	97[28]	100	98	96
Propranolol	88[25,29], 86-88[30]	79	59	51
Acetylsalicylic Acid	55[25]; <50[31]	90	84	52
Theophylline	56,[25] 40[32]	71	41	23
Caffeine	37.5[33]	73	33	29
Cimetidine	20,[34] 22.5[35]	58	34	26
D-Glucosamine	N/A	74	54	29
Ribose	N/A	89	75	64

Table 1 Comparison of the PPB values reported in the literature to the values for fraction binding to BSA (f_b) in this study.

Discussion

Poor pharmacokinetic/pharmacodynamic (PKPD) profile is one of the main reasons for the lack of *in vivo* efficacy and failure of clinical progression of drugs. [36, 37,38] PPB values have a strong association with PK/PD parameters which is the reason their evaluation is an integral part of any drug development programme. It has been recognised that during lead identification and lead optimisation steps, tools that can inform the likely levels of PPB are useful in guiding the direction of a drug discovery programme.[7,8] However, the scale and speed of lead identification and optimisation programmes dictates that methods for estimating PPB must be rapid, simple and cost-effective, in order to be able to properly inform the progression of potential drugs through preclinical stages. As the demand for

evaluation of PPB data for drug development programmes has grown, so too has an interest in the development of newer methods that meet these criteria.

Diffusion based NMR experiments were first proposed as a broad technique for measuring ligand-protein binding in 2004.[39] Since then, several studies have employed DOSY to study molecule-protein interactions. For example, changes in molecular diffusion have been used as evidence of chemical interaction between proteins and two small molecules.[40] DOSY has been shown as an excellent quantitative indicator of binding constants between small molecules,[40,41] and between small molecules and synthetic polymers.[42]

In 2015 Aroulmoji *et al*. demonstrated that DOSY studies on hyaluronate – methotrexate conjugates with bovine serum albumin produced dissociation constants of an equivalent order of magnitude to that from classical fluorescence titrations,[43] and shortly afterwards Denis-Quanguin *et al*. demonstrated that the diffusion constants between paramagnetic inorganic complexes and proteins could be easily extrapolated to monitor protein binding to tris-dipicolinate lanthanide complexes.[44] Although these are some examples where DOSY has been employed to provide binding information, NMR method traditionally used to ascertain this information is primarily via ^{1}H chemical shift changes.[44]

The work described here, builds on these earlier studies to use of DOSY to determine binding affinity of drug molecules to bovine serum albumin, as a model for human plasma proteins~~,~~. Therefore, it can quickly and easily provide information that is critical to understanding PK profile of potential drugs. ~~does to a large extend address some of the key requirement for this demand~~. The methodology is straight forward, experimentally simple to perform, and reasonably quick. As we have shown here, the method is reproducible and using binding to BSA as a model, gives quantitative estimation of plasma protein binding. The issue with solubility of test articles can be avoided since up to 3% v/v deuterated DMSO is tolerated in the experiments.

We should note here that the exact values of diffusion coefficients can be affected by temperature gradient within the sample which lead to convection[45] and sample viscosity.[46,47] There are pulse sequences that can further compensate for convection within a sample,[48] however strict control of the rate of gas flow used for temperature regulation, is required to ensure repeatability in diffusion coefficient measurements. The method provided maintains a constant concentration of protein, reducing issues with variation of viscosity between samples, which is a known concern when sampling chemical diffusion, particularly if mixed solvents are involved. In this study we have observed no significant between diffusion coefficient values were seen in 3% v/v deuterated DMSO in D_2O compared with D_2O alone. ~~The~~ Despite these issues, methodology we have described ~~also~~ has significant versatility and a wide scope. For example, whilst we have focused on binding to BSA as a model system representing blood plasma proteins, the methodology can also be applied, in principle to other individual proteins in plasma, to human serum albumin as a better measure of PPB binding in humans, or even to whole human plasma. Therefore, we plan to further expand the use of DOSY for estimating PPB binding of drugs and will report our results in due course.

Competing interests

The authors declare no competing interests.

Data Availability

The data generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Author Contribution

Experimental work was carried out by R.T. and T.S., R.T. D.W. and K.A. designed experiments. Manuscript was written by K.A. and all authors have reviewed the manuscript.

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Figure legends

Figure 1 (**A**) Changes in diffusion coefficient, D, of caffeine over a concentration range in the absence of BSA (blue crosses) and in the presence of BSA (black crosses). (**B**) Comparison between the diffusion coefficients of caffein/BSA mixtures in the presence or absence of 3% v/v DMSO (**C**) The maximum value for diffusion coefficient, Dmax, of a mixture of caffeine and BSA steadily rises towards the value for caffeine alone as the ratio of caffeine:BSA increases.

Figure 2 (**A**) Changes in diffusion coefficient, D, of propranolol (blue) and diclofenac (green) over a concentration range in the presence of BSA (blue crosses). Control values for absence of BSA are also shown. (**B**) The maximum value for diffusion coefficient, Dmax, of a mixture of caffeine and BSA steadily rises towards the value for diclofenac alone as the ratio of diclofenac:BSA increases.

Figure 3 (**A**). Similarity in changes in diffusion coefficient, D, of theophylline (blue) and caffeine (black) over a concentration range in the presence of BSA. (**B**) Changes in diffusion coefficient, D, of theophylline (blue), acetylsalicylic acid (black) and glucosamine over a concentration range in the presence of BSA. Control values for absence of BSA are not shown for clarity.

Figure 4 Differences in changes to the averaged diffusion coefficient of different molecules in the presence of BSA: (**A**) Diffusion coefficient, D, of ribose over a concentration range in the presence of BSA. (**B**) Diffusion coefficient, D, of cimetidine over a concentration range in the presence of BSA. (**C**) Diffusion coefficient, D, of esomeprazole over a concentration range

in the presence of BSA. (**D**) Changes in diffusion coefficient, D, of ribose, cimetidine and esomeprazole over a concentration range in the presence of BSA. Control values for absence of BSA are not shown for clarity.

Figure 5 Estimation of the relative values of f_b at a given Drug:BSA ratio.

Table 1 Comparison of the PPB values reported in the literature to the values for fraction binding to BSA (f_b) in this study.

A Method for Estimation of Plasma Protein Binding Using Diffusion Ordered NMR Spectroscopy (DOSY)

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Abstract:

The plasma protein binding (PPB) of a drug plays a key role in both its pharmacokinetic and pharmacodynamic properties. During lead optimisation, medium and high throughput methods for the early determination of PPB can provide important information about potential PKPD profile within a chemotype or between different chemotype series. Diffusion ordered spectroscopy (DOSY) is an NMR spectroscopic technique that measures the diffusion of a molecule through the magnetic field gradient, according to its molecular size/weight. Here, we describe the use of DOSY for a rapid and straight forward method to evaluate the PPB of drug molecules, using their binding to bovine serum albumin (BSA) as a model.

Introduction

Human blood contains about 6-8% of soluble proteins which are essential for its function.[1,2] In particular, these proteins (commonly referred to as plasma proteins) bind and transport many biologically important molecules such as lipids and hormones. Administered drugs also bind plasma proteins and are transported *via* blood to various tissues and organs. Albumin accounts for just over half of all human blood proteins.[1,2,3,4] This is followed by various globulins which account for just under 40%.[1,2,5] The main component of the remainder is fibrinogen, which is involved in the coagulation of blood in the case of injury.

The plasma protein binding (PPB) of a drug plays a key role in both its pharmacokinetic (PK) and pharmacodynamic (PD) properties.[6,7,8,9] Without energy-dependent processes, the free drug concentration in plasma correlates to that found in tissues, and it is this tissue drug concentration that promotes binding to the target, which in turn elicits a pharmacological effect.[8] The level of unbound drug in the plasma therefore correlates to the concentration of available drug in the tissues and is pharmacological action. Although low plasma protein binding translates to higher fraction of unbound drug, f_u, and lower volume of distribution, it can also promote faster clearance or metabolism. So, an understanding of the PPB enables a better appreciation of the factors that can contribute to the *in vivo* efficacy of a drug or it's safety profile. Therefore, methods that enable determination of PPB are highly desirable.[9]

There are a number of existing methods for determining PPB of drug molecules, each with their own advantages and drawbacks. Many of these protocols use binding to purified albumin in place of plasma proteins, partly because it is cheaper and more easily accessible but also because its quality does not change from one batch to another, reducing variability

in experimental results and enabling a more reliable comparison between the results.[10,11,12,13,14,15,16,17]

The gold standard for measuring the extent of PPB is equilibrium dialysis (ED).[15] ED involves partitioning of a drug between two compartments, separated by a semi-permeable membrane that functions as a molecular filter based on differences in molecular size and weight.[18] Only molecules below a particular molecular weight threshold have the ability to permeate through the membrane. So, whilst drug molecules permeate through the membrane, heavier proteins or drug-protein complexes do not. Equilibration times are typically long (12-48 hours),[18] so one drawback of ED is the slowness of the method. Rapid equilibrium dialysis (RED) is a high-throughput, 96-well plate version which enhances the efficiency of the ED method. RED allows for equilibration times ranging from 1.5 to 4 hours, depending on the rate of agitation.

However, the adsorption of some compounds at surfaces and membranes of ED devices can have an impact on the measurement, particularly since this 'parasitic' binding sometimes exceeds 50% of the total concentration.[17,18] The Gibbs-Donnan effect, whereby charged particles in close proximity to a semi-permeable membrane do not evenly distribute on either side of the membrane, is also problematic in ED methods and results in an uneven distribution of charged moieties across the membrane.[8,16] This effect can however be prevented through the use of a sufficiently strong ionic buffer, or the dilution of whole plasma prior to dialysis. Other drawbacks for ED are the limitations for non-specific, high binding compounds, as well as issues of plasma instability, and the inability of some compounds to diffuse through the dialysis membrane.[18]

Other commonly used methods for the evaluation of PPB include ultrafiltration and ultracentrifugation. Ultrafiltration is one of the simplest and fastest methods for f_u determination, making it a useful tool in drug monitoring studies.[8] It is similar to ED except

in that it utilises the application of pressure to increase the speed of analysis by forcing the solution through the membrane.[18] Ultrafiltration still suffers from the same drawbacks as ED, including the Gibbs-Donnan effect, protein leakage through the membrane, and nonspecific binding of compounds to the filter membrane. Due to the high pressure enforced for rapid analysis times, the volumes of ultrafiltration cannot exceed 10% of the total sample volume in order to maintain equilibrium.[8,16] Ultracentrifugation is also related to ED, but instead of separation *via* a membrane, centrifugal force is used to separate the protein from the free drug.[18] This method's advantage is that it eliminates membrane effects, such as the Gibbs-Donnan effect and adsorption, but ultracentrifugation also presents its own issues. A lipid layer can form as high-density proteins like albumin will sink, but lower-density lipoproteins will float, leaving the protein-free drug layer in the middle. This can cause difficulties in the sampling for this method. Moreover, the method requires expensive equipment, is low-throughput, and can give falsely high results for binding due to free-drug sedimentation, viscosity, or back-diffusion.[8,18]

Each of the above methods involves physical separation of constituent molecules, but various spectroscopic techniques [17,18,19,20] particularly nuclear magnetic resonance (NMR) spectroscopy [17,21,22,23] which do not involve separation, can also be employed in the determination of PPB. NMR methods are used to determine PPB by analysing information on changes that occur in the spectrum of an unbound drug (or unbound protein) following protein-drug-binding particularly at high-affinity binding sites. For example, NMR studies measure the degree of line perturbation of small molecule signals following plasma protein interaction.

One drawback of using NMR methods is that due to the timescale of experiments, signals are often averaged of the bound and unbound molecules. For instance, one-dimensional ^{1}H Nevertheless, this can allow for the derivation of linear relationships, providing quantitative

information on the bound fraction of the molecule.[17] However, using NMR to determine PPB binding does benefit from a number of important features compared to its counterparts. To start with the sample preparation is minimal and fast, and the NMR measurement data acquisition speed is comparatively fast compared to other techniques.. In addition, running of samples, and the materials required, are inexpensive, and the role of each of the protein components of plasma can be separately assessed in binding *via* NMR method.[17,21,22,23] NMR spectrometers are already ubiquitously used in most medicinal chemistry laboratories. Perhaps most importantly though, NMR based techniques can relatively easily be adapted for automation which allows rapid collection of data on a series of chemotypes. However, one-dimensional NMR methods also have numerous drawbacks. In particular, spectra of most proteins are complex and it is often difficult to distinguish signals for individual groups of protons within the spectra of a mixture of protein, unbound drug and drug-protein adduct.[18] To overcome this many groups favour high power, high field instruments to improve resolution of complex overlapping protein peaks.[24] Whilst this does not affect per sample complexity, cost or run time it does require a vast increase in capital expenditure to both access and house these instruments.

On the other hand, two dimensional NMR spectroscopy such as DOSY, enable us to discern signals from mixtures of compounds. DOSY measures diffusion constants of molecules by measuring the rate by which they are displaced within a magnetic field gradient. The diffusion constant for a molecule depends on a number of factors in particular viscosity of the medium, and its size (molecular volume or weight).

Here, we describe a quick, straightforward and cost effective DOSY-based method to rank binding affinity of different molecules, including drug molecules, to bovine serum albumin (BSA) and show that this data is in agreement with information from the literature on PPB binding of these molecules.

Method

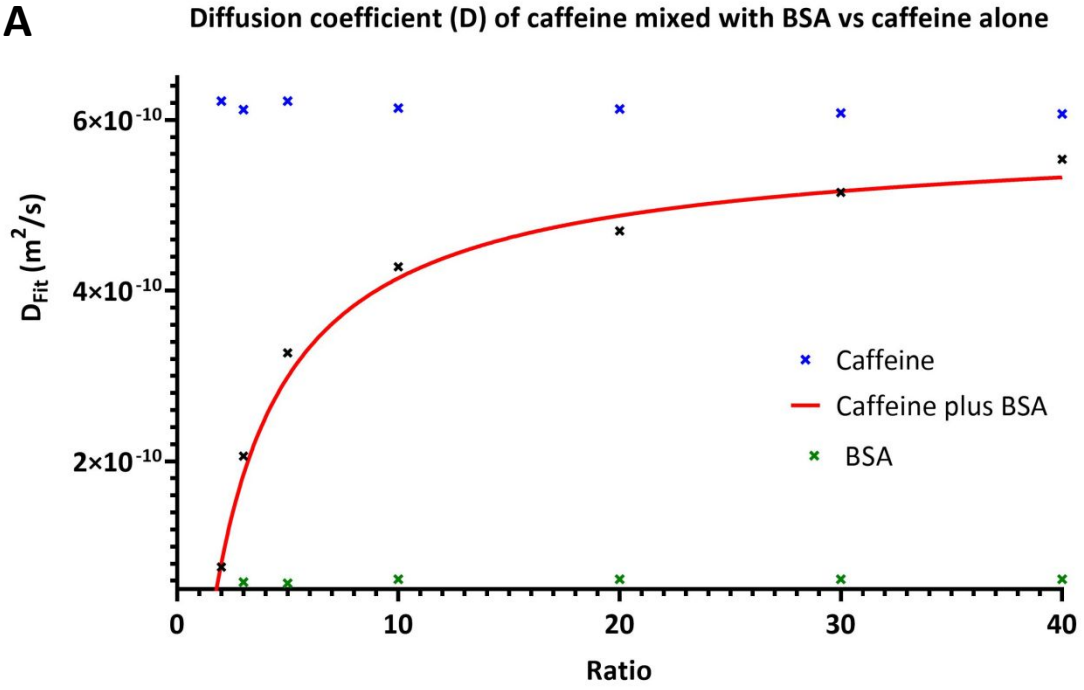
Caffeine (C0750), diclofenac (SML3086), propranolol (P8688), acetyl salicylic acid (S5922), theophylline (T1633), D-glucosamine hydrochloride (G1514), D-ribose (R7500), cimetidine (C4522), esomeprazole (E7906), DMSO (472301), bovine serum albumin (A7030) and deuterium oxide (151882), were all purchased from sigma. The relevant quantity of each drug was dissolved in D_2O (>99.5% isotope purity, 5 mL), to afford a 10 mM solution (solution A). BSA (332.4 mg) was dissolved in D_2O (5 mL), yielding a 1 mM solution (solution B). The required solutions of BSA, drug and BSA plus drug were prepared by combining aliquots of solutions A, B and D_2O to a total of 600 µL volume in a 5 mm diameter NMR tube. For example, five 600 µL solutions were prepared by adding 100 µL of solution B (BSA), x µL of solution A (drug) and (500-x) µL of D_2O, where x is 0, 100, 200, 300, 400 µL. In this example, the final concentration of BSA is 167 µM and the final concentrations of drug, which depends on quantity of x, are zero, 10 fold (1.67mM), 20 fold (3.34 mM), and 40 fold (6.68 mM). Concentration of solution A can be adjusted to afford a wide range of drug:BSA ratios. The DOSY spectra were collected using a Bruker Spectrospin 400 Ultrashield NMR spectrophotometer operating at 400 MHz, with samples maintained at 298.2 K via a condensed gas feed (400 lph). ^{1}H DOSY NMR measurements were set up with 16 gradient scans with 16 N repeat samples across each gradient. All measurements were initiated using Bruker TopSpin (version 3.7) with IconNMR automation. The pulse sequence ledbpgp2s was used (see **Supporting Information** for more details).. Calculation of the diffusion coefficient were done using a Bruker Dynamic Centre (version 2.4.11). All experiments were carried out in triplicate. All fits were carried out using a single exponential diffusion decay with an R^2 > 99%. GraphPad Prism 8 was used for the production of graphs and quantification of the results. Multiple exemplar analysis of raw data are provided in **Supporting Information** file.

Results

Investigation of Binding of Caffeine to BSA

We first set out to test the binding of caffeine to BSA. Caffeine has a relatively low-medium binding affinity to plasma proteins[25,26] and is often used as a standard/control in the existing methods for PPB determination. DOSY was used to determine diffusion coefficients (D) on samples containing different concentration of caffeine only, BSA only and caffeine/BSA mixtures which correspond to caffeine:BSA ratios ranging from 2:1 to 40:1 (**Figure 1A**). For both caffeine and BSA, diffusion coefficients are constant over the concentration range (**Figure 1A**, blue crosses for caffeine and green crosses for BSA). As expected however (see discussions), there is a steady rise in the observed, averaged diffusion coefficient for caffeine in the presence of BSA which eventually reaches a plateau at just below the diffusion coefficients of pure caffeine (**Figure 1A** black crosses). An observed D_{max} value can be calculated by fitting a hyperbolic curve on these datapoints. Repeating experiment (n=3) afforded very similar observed D_{max} values (standard deviation = 1.67×10^{-11} m^2/s or 2.5% of the mean) confirming the reproducibility of the experiments (**Figure 1B**). Although caffeine is quite soluble in (deuterated) water, many investigative molecules are not. In these instants, it is commonplace to dissolve test articles in dimethyl sulfoxide (DMSO), prior to addition to aqueous media to assist with their solubilisation. Therefore, we investigated the effect of adding deuterated DMSO (up to 3% v/v). Again, the experiment afforded very similar observed D_{max} value (**Figure 1B**) confirming small quantities of DMSO do not affect the values for observed D_{max}. Interestingly, although there is a steady increase in the observed D_{max} value from these experiments, the values were still just below the theoretical D_{max} (that for caffeine alone). To show that the observed D values eventually converge to the D value for caffeine alone, we repeated the experiment at 100:1 ratio of caffeine to BSA. As expected, the observed D_{max} value raised to nearer the value for theoretical D_{max} for caffeine alone (**Figure 1C**).

A


Diffusion coefficient (D) of caffeine mixed with BSA vs caffeine alone
D_{Fit} (m^2/s)
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2×10^{-10}
0
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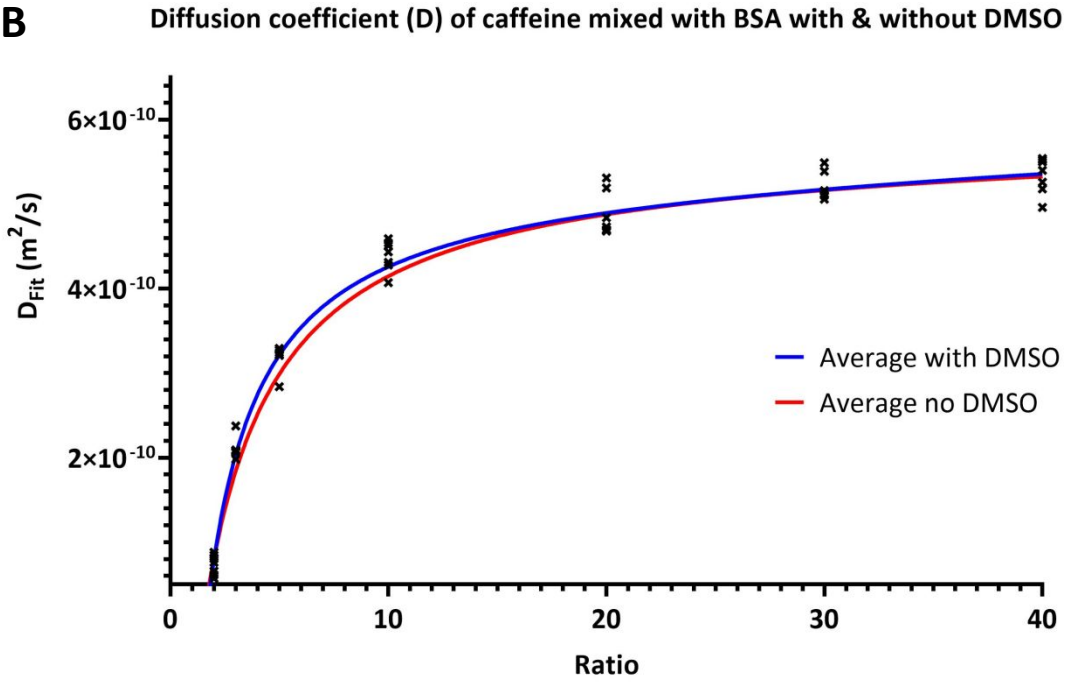
20
30
40
Ratio
Caffeine
Caffeine plus BSA
BSA

B


Diffusion coefficient (D) of caffeine mixed with BSA with & without DMSO
D_{Fit} (m^2/s)
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4×10^{-10}
2×10^{-10}
0
10
20
30
40
Ratio
Average with DMSO
Average no DMSO

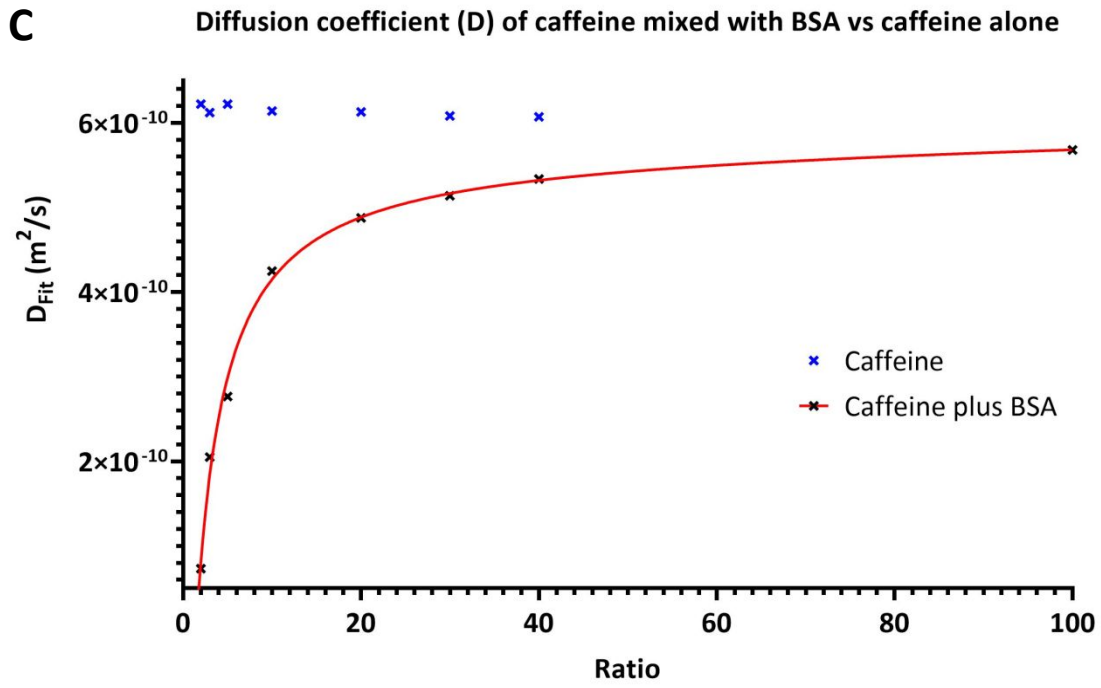


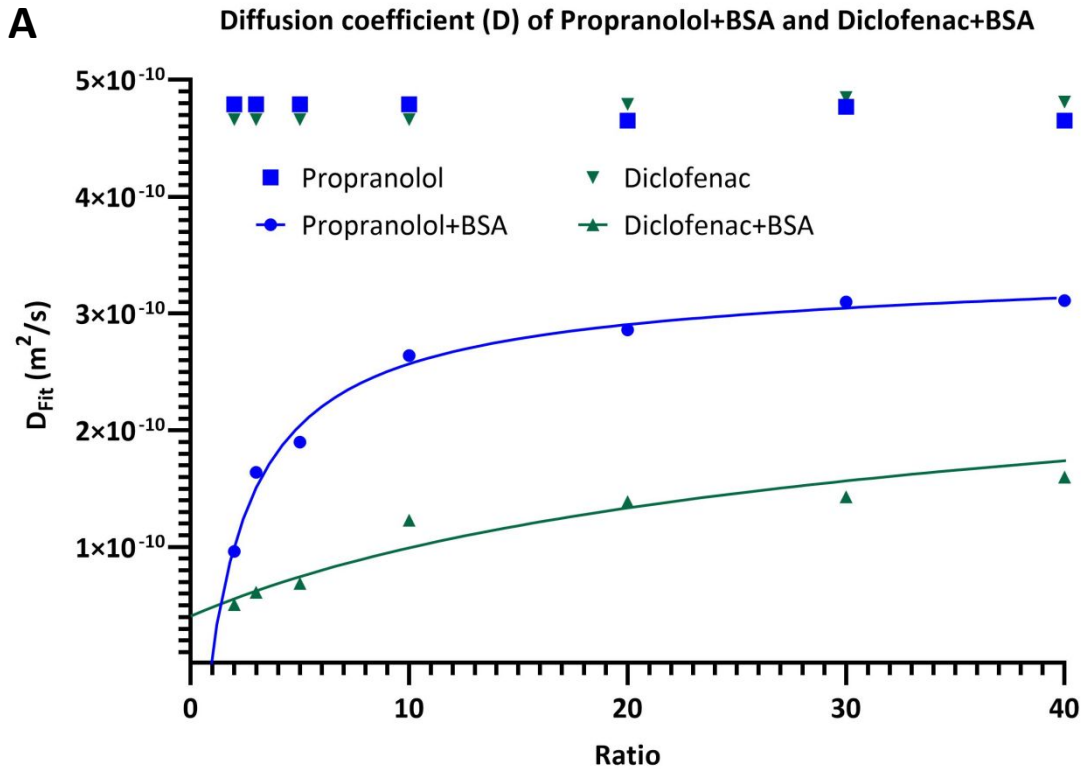
Figure 1 (**A**) Changes in diffusion coefficient, D, of caffeine over a concentration range in the absence of BSA (blue crosses) and in the presence of BSA (black crosses). (**B**) Comparison between the diffusion coefficients of caffein/BSA mixtures in the presence or absence of 3% v/v DMSO (**C**) The maximum value for diffusion coefficient, D_{max}, of a mixture of caffeine and BSA steadily rises towards the value for caffeine alone as the ratio of caffeine:BSA increases.

Determination of Binding of Diclofenac and Propranalol to BSA

In contrast to caffeine which is reported to have a low-medium PPB binding, diclofenac is reported to have a high PPB binding.[25,27] So we set out to investigate the D value for diclofenac:BSA mixtures and compare it with that of caffeine:BSA mixtures over the same range (2:1 to 40:1). Again, DOSY was used to determine diffusion coefficients of diclofenac over the concentration range to show that these values remain constant (**Figure 2A**). As expected, there is a steady rise in the observed, averaged diffusion coefficient for diclofenac with an increase in the ratio of diclofenac:BSA. However, in contrast to the observation with caffeine, the fitted curve is shallower and shows a significantly slower rise. Because of this, and to show that the D values have not yet reached a plateau at 40:1 ratio, we increased the ratio first to 100:1 and then to 600:1. As expected, the observed D value raises steadily towards the D_{max} value for diclofenac (**Figure 2B**). This observation is wholly consistent with

a higher PPB binding for diclofenac than for caffeine. Because of the higher affinity of diclofenac, the proportion of free drug to bound drug is low, meaning that contribution of the free drug to the observed D remains small unless a much larger ratio of diclofenac is used.

Propranolol (295.80 g mol^{-1}) has a similar molecular weight to diclofenac (296.15 g mol^{-1}), however it is reported to have a weaker binding to BSA.[25,28] To demonstrate that the differences in observed D values for diclofenac and caffeine where not merely a consequence of the two molecules' different molecular weights, we measured the D value for propranolol:BSA mixtures and compare it with that of diclofenac:BSA mixtures over the 2:1 to 40:1 concentration range (**Figure 2A**). Averaged diffusion coefficient for propranolol increases with the ratio of drug:BSA. However, the fitted curve is steeper and shows a significantly faster rise than observed for diclofenac. This suggests that propranolol has a weaker binding to BSA and in deed the reported value for PPB of propranolol [25,28] is lower than that for diclofenac.[25,27]



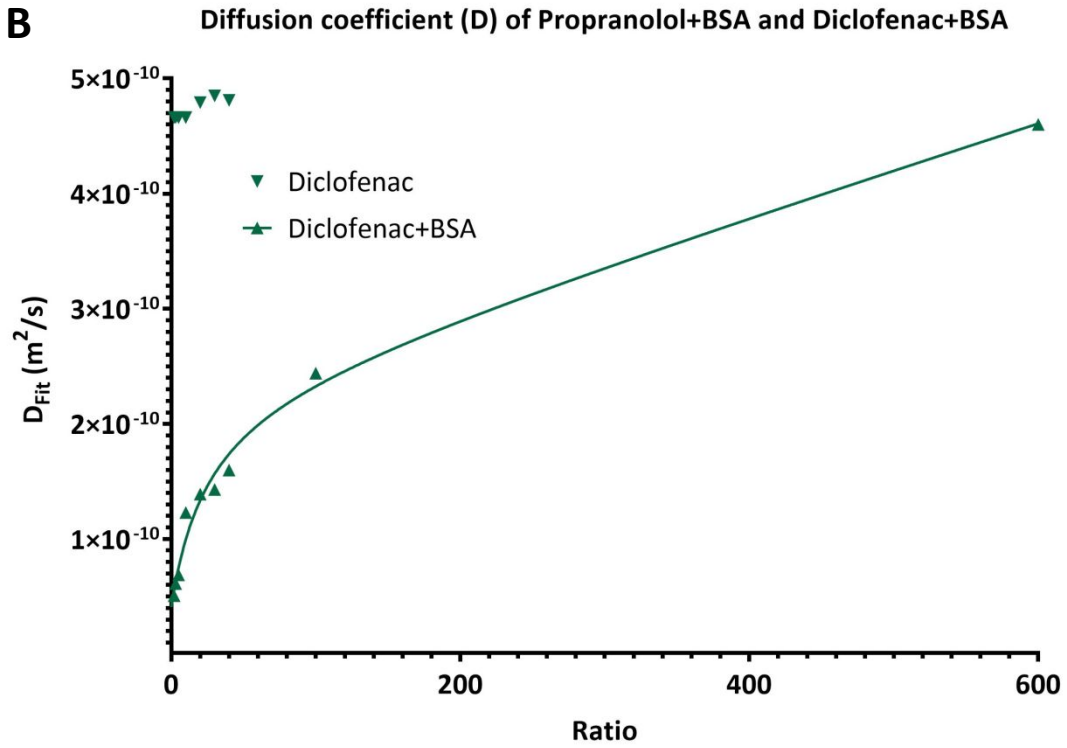


Figure 2 (**A**) Changes in diffusion coefficient, D, of propranolol (blue) and diclofenac (green) over a concentration range in the presence of BSA (blue crosses). Control values for absence of BSA are also shown. (**B**) The maximum value for diffusion coefficient, D_{max}, of a mixture of caffeine and BSA steadily rises towards the value for diclofenac alone as the ratio of diclofenac:BSA increases.

Determination of Binding of theophylline, acetylsalicylic acid and glucosamine to BSA

As was the case for propranolol and diclofenac, theophylline (180.16 g mol^{-1}), glucosamine (179.17 g mol^{-1}) acetylsalicylic acid (180.16 g mol^{-1}) have similar molecular weight but very different physical and chemical properties which means we expect each of them to bind very differently to BSA. Theophylline is however structurally very similar to caffeine which means we expect it's binding to BSA to be very similar to that of caffeine.

DOSY was used to determine diffusion coefficients of theophylline, acetylsalicylic acid and glucosamine over the concentration range (2:1 to 40:1). As can be seen below (**Figure 3A**), theophylline and caffeine not only have similar observed D_{max} values, but their respective fitted curves are also similar in shape. In contrast, acetylsalicylic acid has a very different observed D_{max} and a much shallower fitted curve suggesting it binds to BSA more strongly than theophylline. (**Figure 3B**).

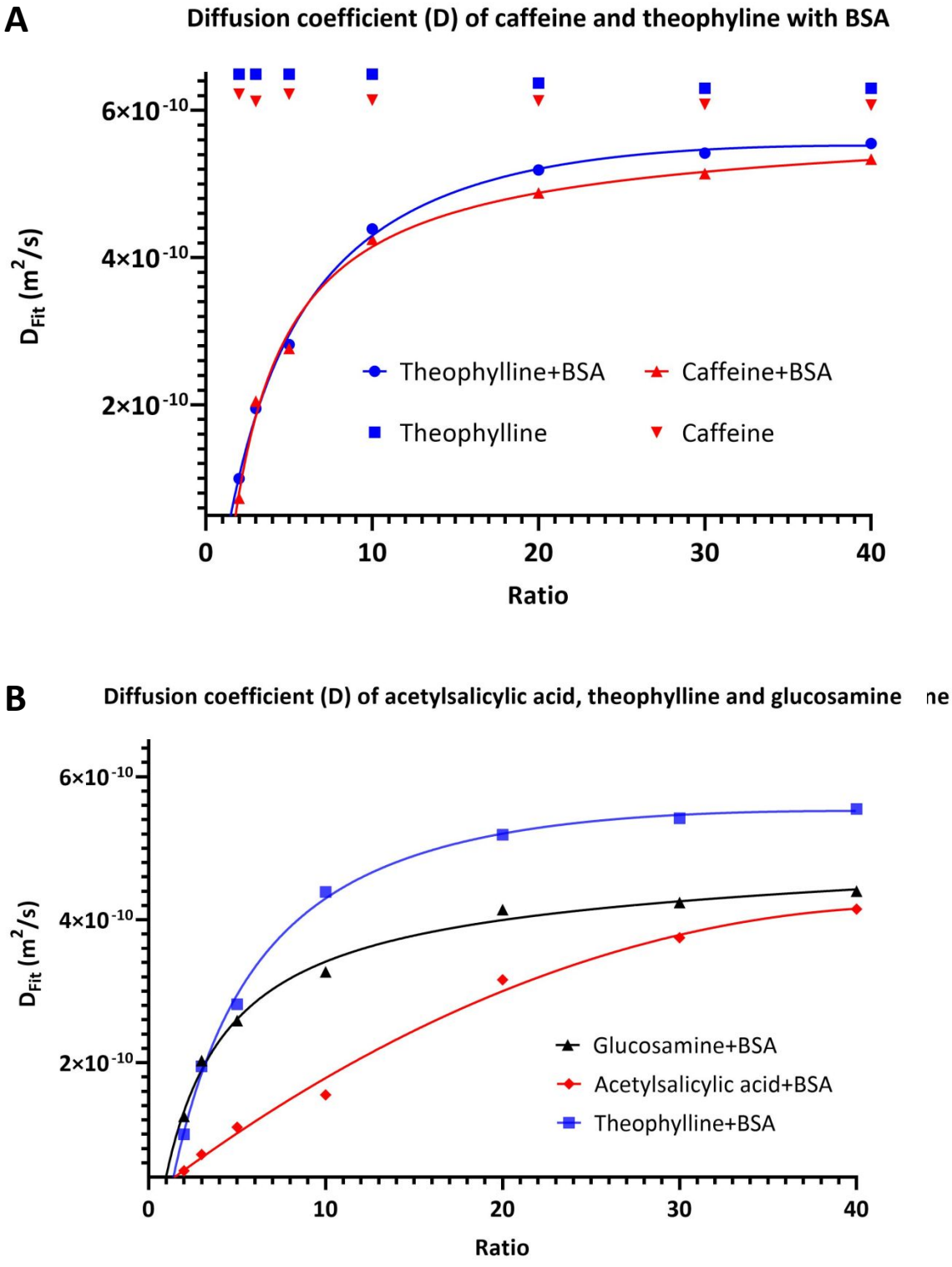
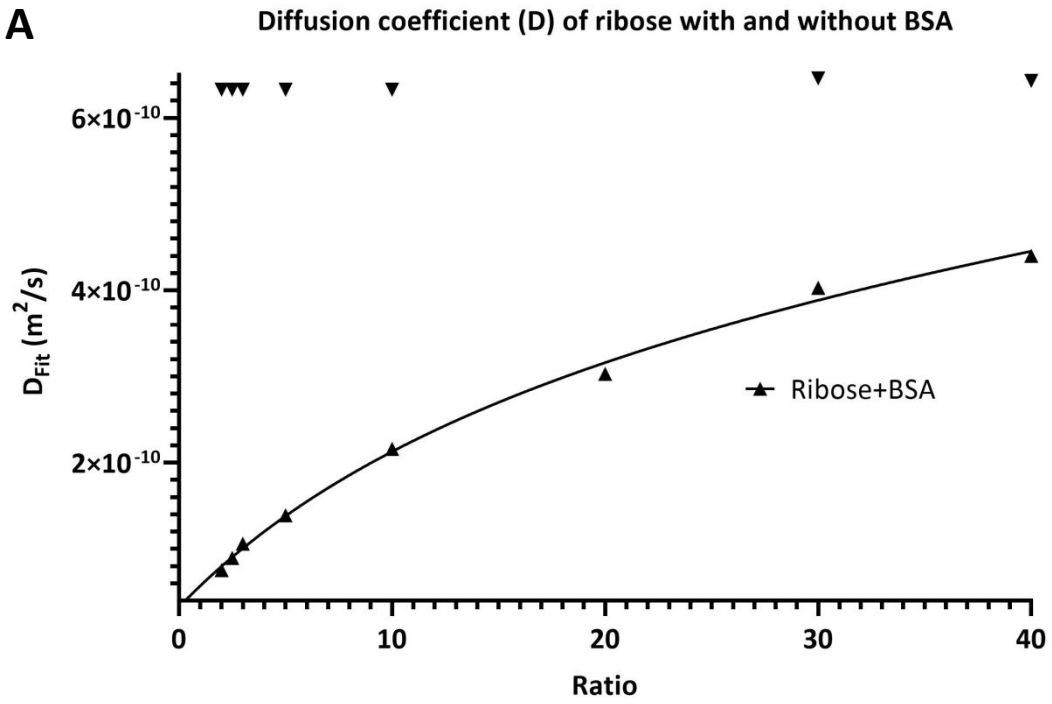


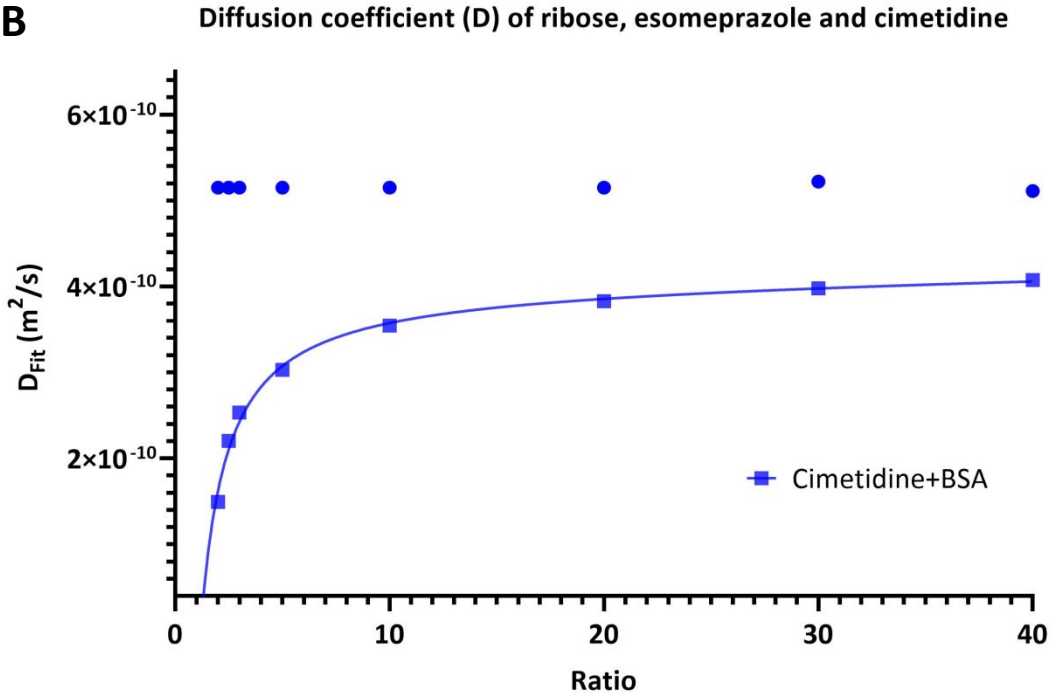
Figure 3 (**A**) Similarity in changes in diffusion coefficient, D, of theophylline (blue) and caffeine (black) over a concentration range in the presence of BSA. (**B**) Changes in diffusion coefficient, D, of theophylline (blue), acetylsalicylic acid (black) and glucosamine over a concentration range in the presence of BSA. Control values for absence of BSA are not shown for clarity.

Quantitative Comparison of Binding to BSA.

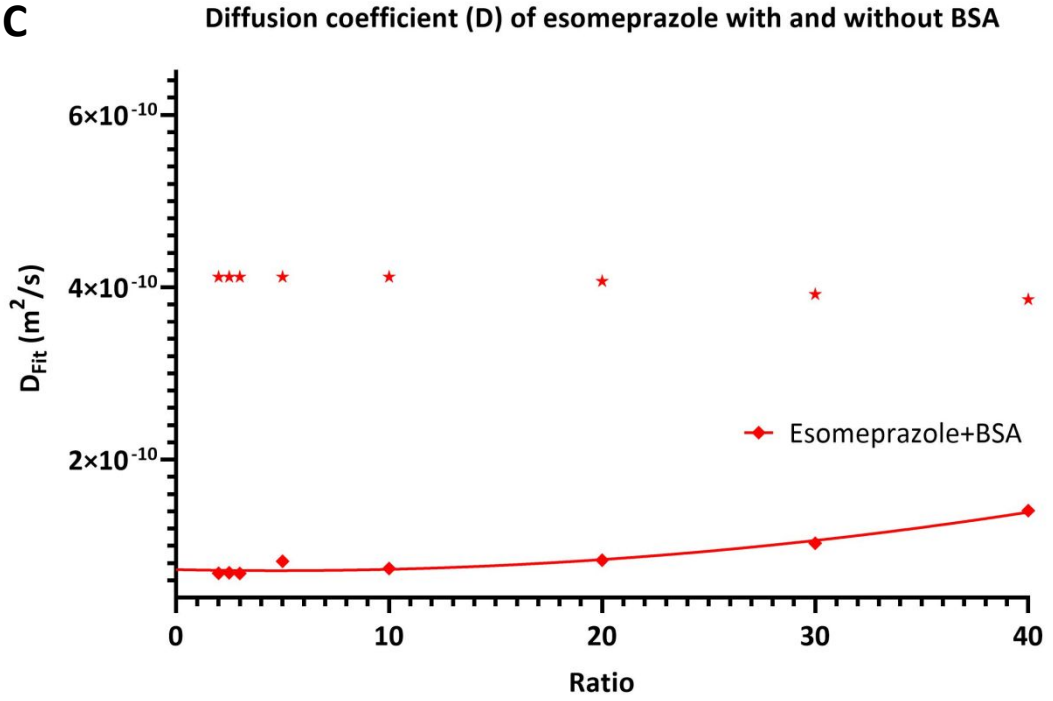
In addition to the drugs and chemical compounds mentioned above, we also investigated the binding of ribose, cimetidine and esomeprazole (as magnesium sulfate salt) to BSA (**Figure 4**). Examination of the curves confirms that esomeprazole is a high binding molecule whilst cimetidine is a low binding drug. Ribose, which appears as medium binding in comparison with the other two molecules has not been previously investigated for PPB. Interestingly, in contrast to the similarity between theophylline and caffeine, the curves for ribose and glucosamine are quite different, suggesting very different binding affinities to BSA, even though the molecules are both carbohydrate sugars.



B


Diffusion coefficient (D) of ribose, esomeprazole and cimetidine
6×10-10
4×10-10
2×10-10
DFit (m2/s)
Cimetidine+BSA
0
10
20
30
40
Ratio

C


Diffusion coefficient (D) of esomeprazole with and without BSA
6×10-10
4×10-10
2×10-10
DFit (m2/s)
Esomeprazole+BSA
0
10
20
30
40
Ratio

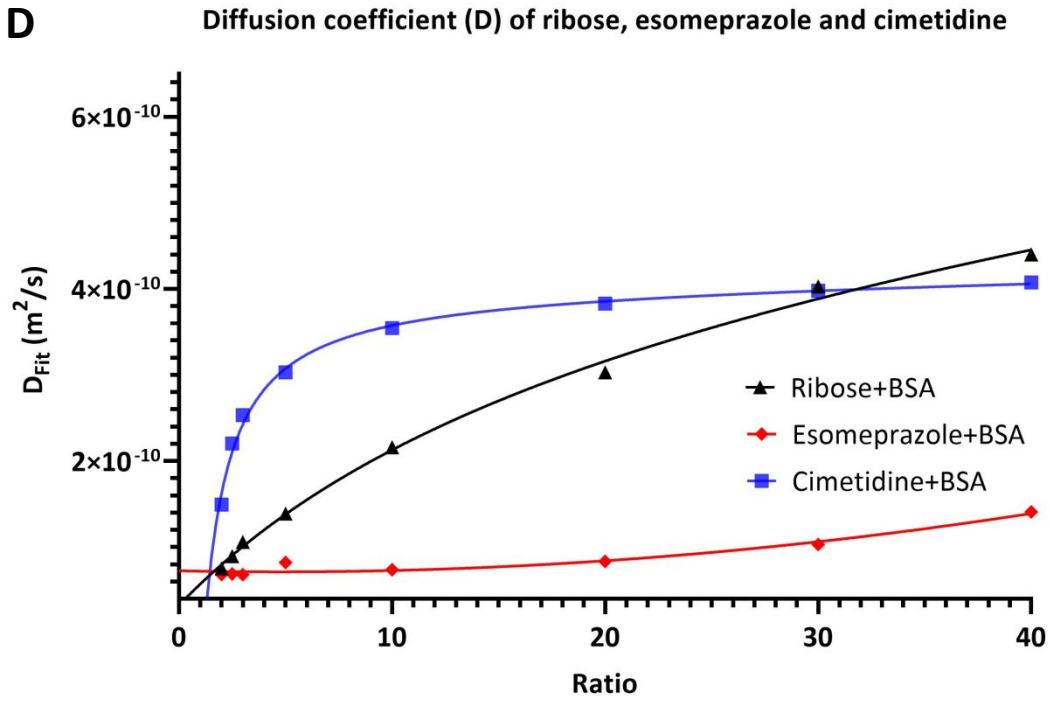


Figure 4 Differences in changes to the averaged diffusion coefficient of different molecules in the presence of BSA: (**A**) Diffusion coefficient, D, of ribose over a concentration range in the presence of BSA. (**B**) Diffusion coefficient, D, of cimetidine over a concentration range in the presence of BSA. (**C**) Diffusion coefficient, D, of esomeprazole over a concentration range in the presence of BSA. (**D**) Changes in diffusion coefficient, D, of ribose, cimetidine and esomeprazole over a concentration range in the presence of BSA. Control values for absence of BSA are not shown for clarity.

Clearly, examination of the curves provides a qualitative estimation of the binding affinities of different drug molecules to BSA. As the concentration of drug relative to a fixed concentration of BSA is increased the observed averaged diffusion coefficient for the molecule also increases approaching the value for the diffusion coefficient for the molecule in solution on its own. For molecules with low binding to BSA, in this example cimetidine, the rise to its corresponding maximum is sharper, whereas for molecules with high binding to BSA, in this example esomeprazole, the rise to its corresponding maximum is shallow.

Although the observation of fitted curves allows a qualitative measure of binding of different molecules to BSA, we wanted to quantitatively estimate the fraction of bound drug. One way to do this is to measure the relative observed diffusion coefficient (D_{obs}) to the D_{max} value at a given ratio of drug:BSA for each drug/molecule.

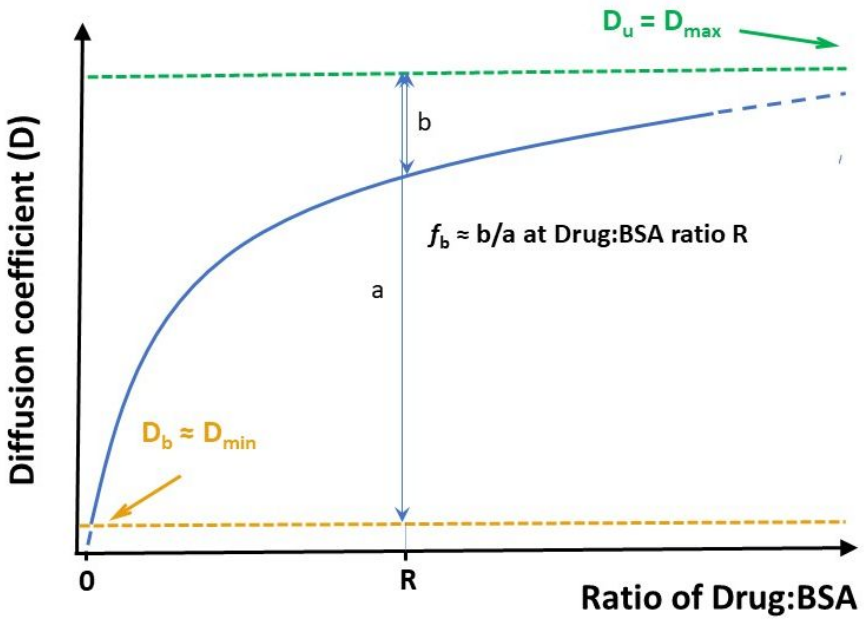


Figure 5 Estimation of the relative values of f_b at a given Drug:BSA ratio

We argued that the D_{obs} at any given drug concentration is an averaged value from all bound and unbound molecules, or $D_{obs} = (D_b \times f_u) + (D_u \times f_b)$ where D_b would be the diffusion coefficient of entirely bound drug, D_u would be the diffusion coefficient of entirely unbound drug, and f_b and f_u would be the fraction of bound and unbound drug respectively. Since $f_b + f_u = 1$, then $f_b = (D_u - D_{obs})/(D_u - D_b)$. The value of D_u is the same as the diffusion coefficient of the drug in the absence of BSA (D_{max}) which can be experimentally determined (**Figure 5**). The value of D_b however, can only be estimated. Value of D_b corresponds to when there is a large excess of BSA so that even if the drug molecule has little affinity, majority of it would be bound to BSA. However, as the ratio of Drug:BSA is reduced, it would be more difficult to discern the peaks due to the small molecule drug from protein, and hence not possible to determine diffusion coefficient. We found that for all molecules in this study, determination of diffusion coefficient was only possible at drug:BSA ratios >2.

Table 1 shows the estimated values of relative BSA binding (f_b) at 5:1, 10:1 and 20:1 ratio of drug:BSA for molecules in this study, as well as previously reported values for the whole human plasma binding of the drugs from the literature. Obviously, this qualitative

comparison carries a number of caveats. There may be differences between human and bovine serum albumin. Furthermore, albumin is only one, albeit the major component of blood proteins, and therefore, binding to other protein components of plasma can significantly change values for PPB than the ones estimated from binding to BSA. Nevertheless, as can be seen the ranking of BSA binding from our study closely mirrors the PPB binding values reported for these drugs.

Molecule	PPB reported in literature (%)	Fraction bound (f_b) to BSA (%) from this study		
		at 5:1 (%)	at 10:1 (%)	at 20:1 (%)
Diclofenac	99.5[25,27]	100	94	90
Esomeprazole	97[28]	100	98	96
Propranolol	88[25,29], 86-88[30]	79	59	51
Acetylsalicylic Acid	55[25]; <50[31]	90	84	52
Theophylline	56,[25] 40[32]	71	41	23
Caffeine	37.5[33]	73	33	29
Cimetidine	20,[34] 22.5[35]	58	34	26
D-Glucosamine	N/A	74	54	29
Ribose	N/A	89	75	64

Table 1 Comparison of the PPB values reported in the literature to the values for fraction binding to BSA (f_b) in this study.

Discussion

Poor pharmacokinetic/pharmacodynamic (PKPD) profile is one of the main reasons for the lack of *in vivo* efficacy and failure of clinical progression of drugs. [36, 37,38] PPB values have a strong association with PK/PD parameters which is the reason their evaluation is an integral part of any drug development programme. It has been recognised that during lead identification and lead optimisation steps, tools that can inform the likely levels of PPB are useful in guiding the direction of a drug discovery programme.[7,8] However, the scale and speed of lead identification and optimisation programmes dictates that methods for estimating PPB must be rapid, simple and cost-effective, in order to be able to properly inform the progression of potential drugs through preclinical stages. As the demand for

evaluation of PPB data for drug development programmes has grown, so too has an interest in the development of newer methods that meet these criteria.

Diffusion based NMR experiments were first proposed as a broad technique for measuring ligand-protein binding in 2004.[39] Since then, several studies have employed DOSY to study molecule-protein interactions. For example, changes in molecular diffusion have been used as evidence of chemical interaction between proteins and two small molecules.[40] DOSY has been shown as an excellent quantitative indicator of binding constants between small molecules,[40,41] and between small molecules and synthetic polymers.[42]

In 2015 Aroulmoji *et al*. demonstrated that DOSY studies on hyaluronate – methotrexate conjugates with bovine serum albumin produced dissociation constants of an equivalent order of magnitude to that from classical fluorescence titrations,[43] and shortly afterwards Denis-Quanguin *et al*. demonstrated that the diffusion constants between paramagnetic inorganic complexes and proteins could be easily extrapolated to monitor protein binding to tris-dipicolinate lanthanide complexes.[44] Although these are some examples where DOSY has been employed to provide binding information, NMR method traditionally used to ascertain this information is primarily via ^{1}H chemical shift changes.[44]

The work described here, builds on these earlier studies to use of DOSY to determine binding affinity of drug molecules to bovine serum albumin, as a model for human plasma proteins. Therefore, it can quickly and easily provide information that is critical to understanding PK profile of potential drugs. . The methodology is straight forward, experimentally simple to perform, and reasonably quick. As we have shown here, the method is reproducible and using binding to BSA as a model, gives quantitative estimation of plasma protein binding. The issue with solubility of test articles can be avoided since up to 3% v/v deuterated DMSO is tolerated in the experiments.

We should note here that the exact values of diffusion coefficients can be affected by temperature gradient within the sample which lead to convection[45] and sample viscosity.[46,47] There are pulse sequences that can further compensate for convection within a sample,[48] however strict control of the rate of gas flow used for temperature regulation, is required to ensure repeatability in diffusion coefficient measurements. The method provided maintains a constant concentration of protein, reducing issues with variation of viscosity between samples, which is a known concern when sampling chemical diffusion, particularly if mixed solvents are involved. In this study we have observed no significant between diffusion coefficient values were seen in 3% v/v deuterated DMSO in D_2O compared with D_2O alone. Despite these issues, methodology we have described has significant versatility and a wide scope. For example, whilst we have focused on binding to BSA as a model system representing blood plasma proteins, the methodology can also be applied, in principle to other individual proteins in plasma, to human serum albumin as a better measure of PPB binding in humans, or even to whole human plasma. Therefore, we plan to further expand the use of DOSY for estimating PPB binding of drugs and will report our results in due course.

Competing interests

The authors declare no competing interests.

Data Availability

The data generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Author Contribution

Experimental work was carried out by R.T. and T.S., R.T. D.W. and K.A. designed experiments. Manuscript was written by K.A. and all authors have reviewed the manuscript.

Acknowledgment

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Figure legends

Figure 1 (**A**) Changes in diffusion coefficient, D, of caffeine over a concentration range in the absence of BSA (blue crosses) and in the presence of BSA (black crosses). (**B**) Comparison between the diffusion coefficients of caffein/BSA mixtures in the presence or absence of 3% v/v DMSO (**C**) The maximum value for diffusion coefficient, Dmax, of a mixture of caffeine and BSA steadily rises towards the value for caffeine alone as the ratio of caffeine:BSA increases.

Figure 2 (**A**) Changes in diffusion coefficient, D, of propranolol (blue) and diclofenac (green) over a concentration range in the presence of BSA (blue crosses). Control values for absence of BSA are also shown. (**B**) The maximum value for diffusion coefficient, Dmax, of a mixture of caffeine and BSA steadily rises towards the value for diclofenac alone as the ratio of diclofenac:BSA increases.

Figure 3 (**A**). Similarity in changes in diffusion coefficient, D, of theophylline (blue) and caffeine (black) over a concentration range in the presence of BSA. (**B**) Changes in diffusion coefficient, D, of theophylline (blue), acetylsalicylic acid (black) and glucosamine over a concentration range in the presence of BSA. Control values for absence of BSA are not shown for clarity.

Figure 4 Differences in changes to the averaged diffusion coefficient of different molecules in the presence of BSA: (**A**) Diffusion coefficient, D, of ribose over a concentration range in the presence of BSA. (**B**) Diffusion coefficient, D, of cimetidine over a concentration range in the presence of BSA. (**C**) Diffusion coefficient, D, of esomeprazole over a concentration range

in the presence of BSA. (**D**) Changes in diffusion coefficient, D, of ribose, cimetidine and esomeprazole over a concentration range in the presence of BSA. Control values for absence of BSA are not shown for clarity.

Figure 5 Estimation of the relative values of f_b at a given Drug:BSA ratio.

Table 1 Comparison of the PPB values reported in the literature to the values for fraction binding to BSA (f_b) in this study.

Supporting Information

A Method for Estimation of Plasma Protein Binding Using Diffusion Ordered NMR Spectroscopy (DOSY)

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Setup of the Dosy Experiment

The DOSY spectra were collected using a Bruker Spectrospin 400 Ultrashield NMR spectrophotometer operating at 400 MHz, with samples maintained at 298.2 K via a condensed gas feed (400 lph). ^{1}H DOSY NMR measurements were set up with 16 gradient scans with 16 N repeat samples across each gradient. All measurements were initiated using Bruker TopSpin (version 3.7) with IconNMR automation. The Bruker pulse sequence ledbpgp2s (LED with bipolar gradient pulse pair, 2 spoil gradients) without modification (**Figure S1**) was used to acquire date.

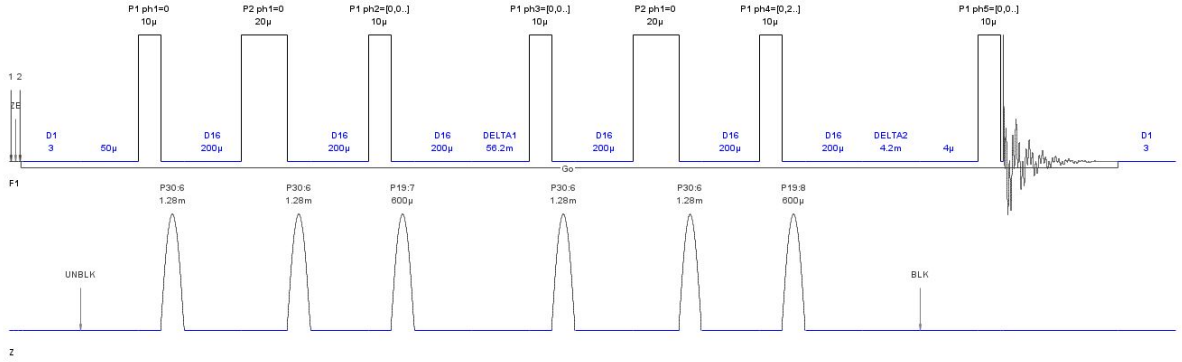


Figure S1: Pulse sequence ledbpgp2s used in DOSY experiments

*Reference: Wu, D.H.; Chen, A.D.; Johnson, C.S. (**1995**) "An Improved Diffusion-Ordered Spectroscopy Experiment Incorporating Bipolar-Gradient Pulses" J. Magn. Reson. Ser. A 115(2), 260-264.*

Full Summary of Diffusion NMR experiments and Exemplar Raw Data

^{1}H NMR of Caffeine, BSA and mixtures thereof produces a variety of peaks visible between 1 and 8 ppm which were of relevance to the study as shown in **Figure S2**.

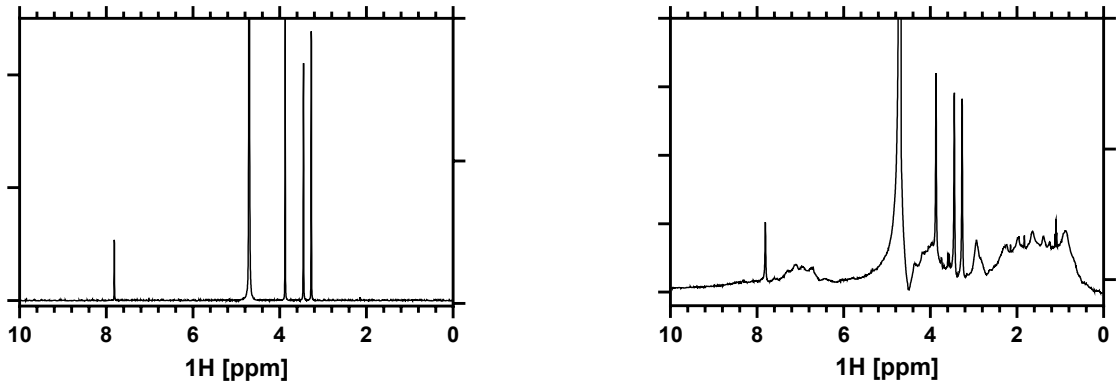


Figure S2 – ^{1}H NMR of pure Caffeine (left) and a Caffeine : BSA 3 :1 ratio (right)

BSA, Caffeine, and mixtures thereof were analysed by DOSY NMR and found to diffuse at a rate of 6.54 x 10^{-11} m^2 /S and 6.19 x 10^{-10} m^2 /S respectively (fitting to peaks δ 1.63 and δ 3.87 with 16 gradient steps as shown in **Figures S3** and **S4**).

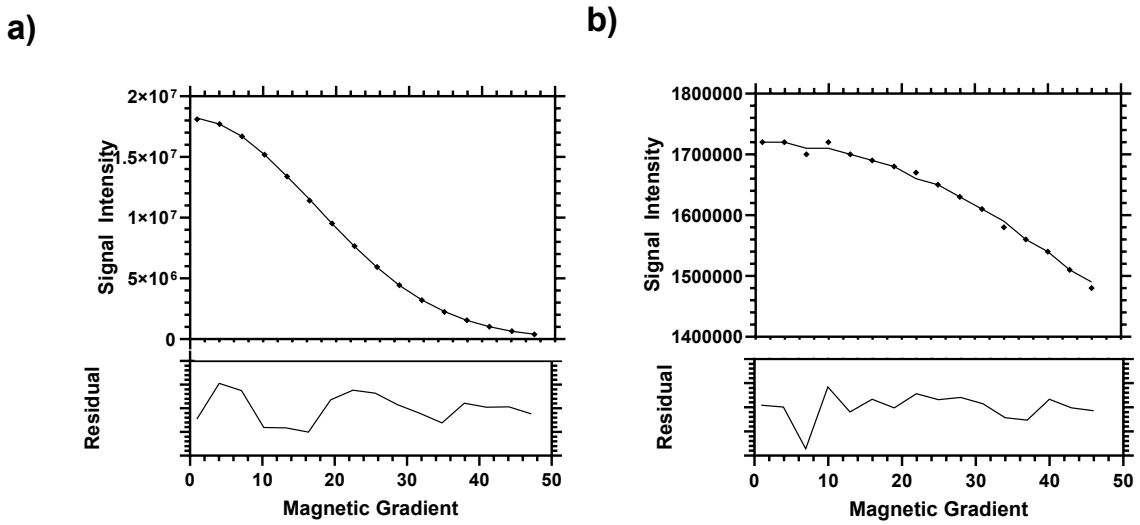


Figure S3 – Diffusion Decay of Pure Compounds. A) Caffeine: Diffusion decay (peaks = raw data, line = applied fit and std. deviation residuals below) of pure Caffeine in D_2O δ 3.87 pm. γ 26752 rad/(s*Gauss), δ 0.0015800 s and Δ 0.059900 s. Calculated D = 6.19E-10 ± 3.010e-12 m^2/s. b) BSA: Diffusion decay (peaks = raw data, line = applied fit and std. deviation residuals below) of pure BSA in D_2O δ 1.63 pm. γ 26752 rad/(s*Gauss), δ 0.0015800 s and Δ 0.059900 s. Calculated D = 6.54E-11 ± 2.846e-12 m^2/s

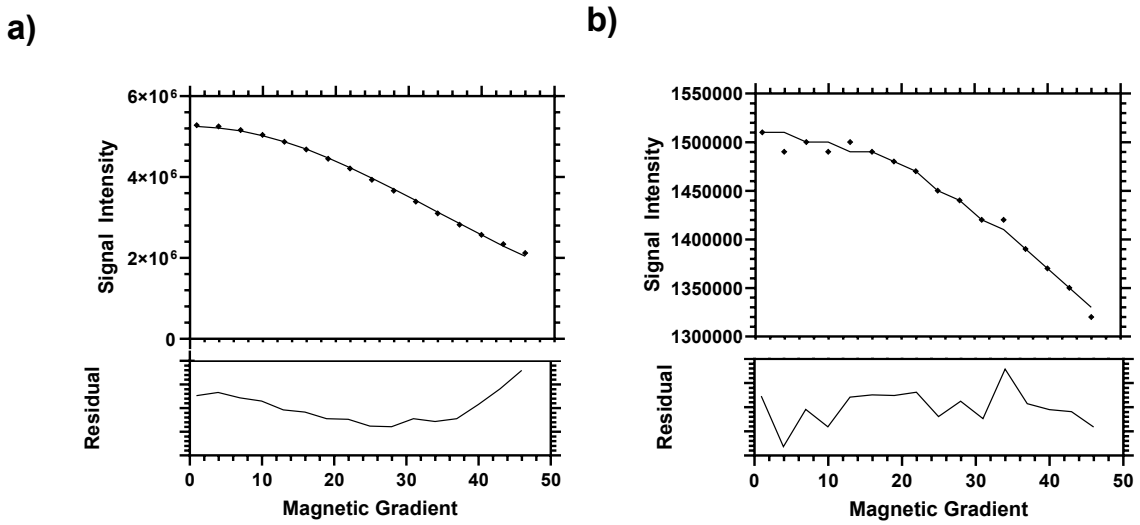


Figure S4 – Diffusion Decay of Caffeine and BSA in a 3 : 1 Ratio. A) Caffeine: Diffusion decay (peaks = raw data, line = applied fit and std. deviation residuals below) of Caffeine in 3 :1 BSA ratio δ 3.87 pm. γ 26752 rad/(s*Gauss), δ 0.0015800 s and Δ 0.059900 s. Calculated D = 5.25E-10 ± 8.416e-12 m^2/s. b) BSA: Diffusion decay (peaks = raw data, line = applied fit and std. deviation residuals below) of BSA in 3 :1 mixture δ 1.64 pm. γ 26752 rad/(s*Gauss), δ 0.0015800 s and Δ 0.059900 s. Calculated D = 5.62E-11 ±4.114e-12 m^2/s

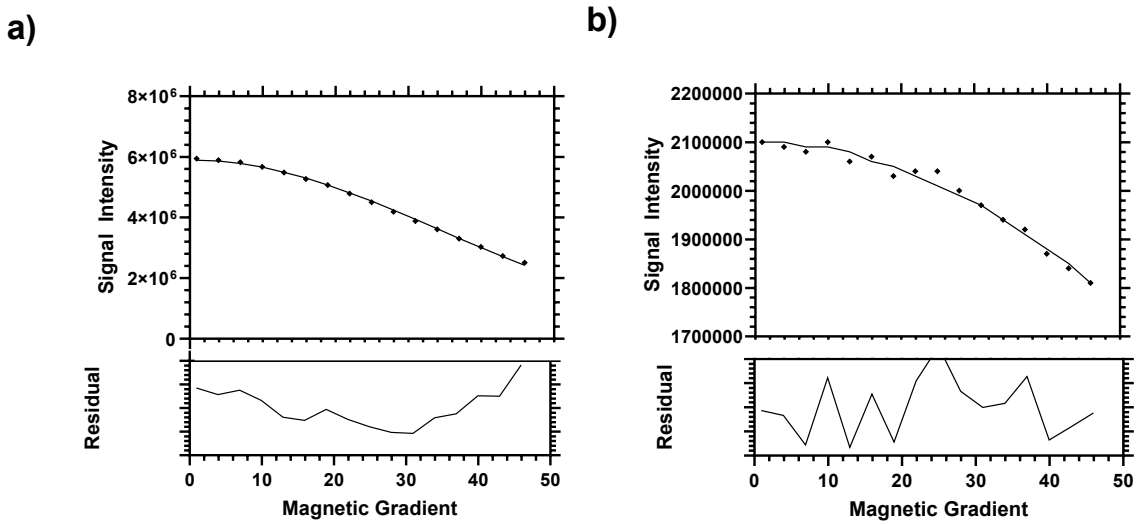
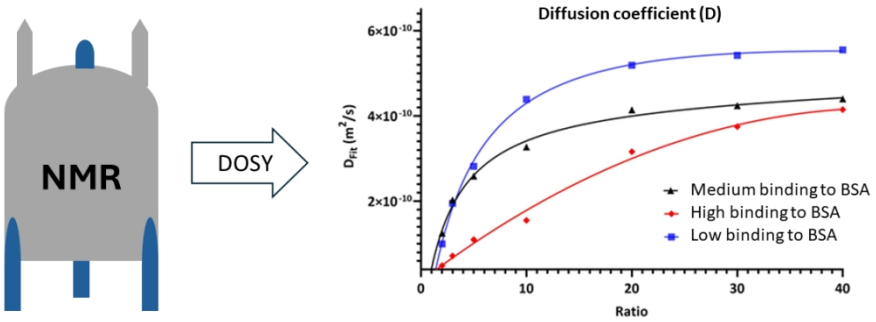


Figure S4 – Diffusion Decay of Caffeine and BSA in a 10 : 1 Ratio. A) Caffeine: Diffusion decay (peaks = raw data, line = applied fit and std. deviation residuals below) of Caffeine in 10 :1 BSA ratio δ 3.87 pm. γ 26752 rad/(s*Gauss), δ 0.0015800 s and Δ 0.059900 s. Calculated D = 4.08E-10 ± 8.865e-12 m^2/s. b) BSA: Diffusion decay (peaks = raw data, line = applied fit and std. deviation residuals below) of BSA in 10 :1 mixture δ 1.64 pm. γ 26752 rad/(s*Gauss), δ 0.0015800 s and Δ 0.059900 s. Calculated D = 6.69E-11 ± 4.9633e-12 m^2/s



We describes a fast and simple method for quantitative determination of plasma protein binding of drug molecules using diffusion ordered spectroscopy (DOSY).

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