Fast Gradient HPLC Method to Determine Compounds Binding to Human Serum Albumin. Relationships with Octanol/Water and Immobilized Artificial Membrane Lipophilicity

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ABSTRACT: A fast gradient HPLC method (cycle time 15 min) has been developed to determine Human Serum Albumin (HSA) binding of discovery compounds using chemically bonded protein stationary phases. The HSA binding values were derived from the gradient retention times that were converted to the logarithm of the equilibrium constants (log*K* HSA) using data from a calibration set of molecules. The method has been validated using literature plasma protein binding data of 68 known drug molecules. The method is fully automated, and has been used for lead optimization in more than 20 company projects. The HSA binding data obtained for more than 4000 compounds were suitable to set up global and project specific quantitative structure binding relationships that helped compound design in early drug discovery. The obtained HSA binding of known drug molecules were compared to the Immobilizd Artificial Membrane binding data (CHI IAM) obtained by our previously described HPLC-based method. The solvation equation approach has been used to characterize the normal binding ability of HSA, and this relationship shows that compound lipophilicity is a significant factor. It was found that the selectivity of the "baseline" lipophilicity governing HSA binding, membrane interaction, and octanol/water partition are very similar. However, the effect of the presence of positive or negative charges have very different effects. It was found that negatively charged compounds bind more strongly to HSA than it would be expected from the lipophilicity of the ionized species at pH 7.4. Several compounds showed stronger HSA binding than can be expected from their lipophilicity alone, and comparison between predicted and experimental binding affinity allows the identification of compounds that have good complementarities with any of the known binding sites. J Pharm Sci 92:2236–2248, 2003

Keywords: HSA binding; Immobilized Artificial Membrane; HPLC; lipophilicity

INTRODUCTION

The role of the plasma protein binding of drug molecules is still not thoroughly understood. However, strong binding (above 95%) can cause drug safety issues[1] or several adverse effects (low clearance, low brain penetration,[2] drug–drug

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interaction,[3] loss of efficacy, etc.). It has also been pointed out that not only the binding equilibrium but also the offset rate may influence the efficacy/distribution of the compound.[4] Due to the high attrition rate in the later stage of drug development, the consideration of adsorption/distribution and pharmacokinetic properties of the molecules are now taken into account at an earlier stage of the drug discovery process.[5–7] Therefore, there is a need for high throughput measurements of physical properties, membrane interaction, and plasma protein binding. It is not essential at this stage of the discovery process to provide accurate measurements of these properties. However, it is very important that the measured values provide a reproducible rank order of the compounds. This makes possible the development of structure–property relationships that help in modification of the structure without decreasing the primary activity of the molecules on a particular target. Serum albumin and α-acid–glycoprotein are the two major binders of acidic and basic drug molecules in plasma, respectively. Neutral lipophilic drug molecules can bind to both HSA and AGP as well as to other plasma proteins. The crystal structure of serum albumin has been investigated by Carter et al.[8,9] The crystallographic investigation by Curry et al.[10,11] further proved the presence of the major warfarin binding site and revealed that the conformation of the warfarin binding pocket is significantly altered by fatty acid binding. The fatty acid binding sites are long hydrophobic pockets capped by polar side chains.

Several attempts have been made to set up quantitative structure–binding relationships for HSA binding,[12,13] and they have revealed the positive contributions of lipophilicity, the presence of aromatic substituents, and presence of carboxylic acids in strong HSA binding.

Several applications of chemically bonded serum albumin on high-quality silica supports have been reported since 1990.[13–15] These methods are based on the assumption that the chemically bonded HSA retains the binding specificity and conformational mobility of the native HSA. Although it is assumed that the major binding sites of the HSA are intact, there are several nonspecific binding sites and other pharmacologically irrelevant interactions including the silica support that might contribute to the compound retention. These published methods are based on isocratic retention time and retention factor measurements. The chromatographic retention factor is directly related to the proportion of the number of molecules in the stationary phase and the mobile phase. This proportion then can be converted to % HSA bound (% HSA $= 100(k/(k+1))$. The HPLC-based methods to measure HSA binding are faster and more precise in ranking compounds (especially at a high binding region) than the traditional ultrafiltration or equilibrium dialysis methods. However, strongly bound compounds can have very long retention times (more than 30 min). We also have observed that the absolute retention time of compounds progressively decreases as the column ages; therefore, the application of relative retention times can give a more accurate measure of the binding. As HSA binding mostly affects compound activity/distribution only above 95% binding, it is important that strongly bound compounds elute within a reasonable time from the chromatographic column in a reproducible order. Therefore, we have investigated the application of a generic 2-propanol gradient during the elution of the compounds from the HSA stationary phases. In this article the validation of this method is described. For the calibration of the HSA we have used plasma protein binding data obtained from Goodman and Gilman's textbook[16] and other publications.[17,18]

An interesting QSAR investigation by Saiakhov et al.[18] revealed that although the correlation of plasma protein binding with octanol/water lipophilicity did not show good correlation for a wide range of compounds, it appeared to be an important parameter to all local QSAR relationships. They concluded that each binding site has a different lipophilicity requirement. In this article, the role of lipophilicity in the albumin binding has been investigated. The Abraham solvation equation[19] has been used to characterize the type of lipophilicity that the generalized HSA binding sites represent.

EXPERIMENTAL

Instrument

Agilent HP1100 HPLC instruments were used throughout.

HPLC Columns

Chromtech Immobilized HSA HPLC column 50 × 3 mm was purchased from Chromtech (Cheshire, UK).

Mobile Phase and Detection

The mobile phase A was 50-mM pH 7.4 ammonium acetate solution, while mobile phase B was 2-Propanol (HPLC grade, Runcorn, UK). The mobile phase flow rate was 1.8 mL/min. The column temperature was kept at 30°C. The gradient profile and run time were the same with each column, the linear gradient from 0 to 30% 2-propanol was applied from 0 to 3 min. From 3 to 10 min, the mobile phase composition was constant 30% 2-propanol and 70% 50 mM ammonium acetate. From 10 min to 10.5 min the mobile phase composition was change to 100% ammonium acetate buffer only, and remained the same until the end of the run. Each separation was stopped after 15 min.

Detection

Chromatograms were recorded at 230 and 254 nm by a diode array UV absorption detector at room temperature.

Calibration of the Protein Columns

The column performance check and the calibration have been performed before the analysis of every 96-well plate. The compounds used for the column calibrations were dissolved separately in 0.5 mg/mL concentration in 50% 2-propanol and 50% pH 7.4 ammonium acetate solution mixtures. The calibration set of compounds their literature % plasma protein binding and its linear conversion value (log*K* lit), as well as typical retention times, their logarithmic values, log*K* derived from the calibration curve and % binding data are listed in Table 1.

The literature % PPB (bound in plasma) values were converted to the linear free energy related log*K* values (logarithm of apparent affinity constant) using eq. 1.

$$\mathrm{Log}K = \log\left[\frac{\%\ \mathrm{PPB}}{101 - \%\ \mathrm{PPB}}\right] \qquad (1)$$

Note that the value of 101 was taken arbitrarily to be able to calculate a log*K* value for compounds that bind 100% to HSA. In this way by definition, the log*K* value of 100% bound compound is 2. With the assumption that binary complex is formed between the ligand and the HSA in the blood, and an excess of albumin is present compared to the concentration of drug, these log*K* values can be converted to log k_A affinity constant.[20] Supposing that the albumin concentration in plasma is around 0.6 mM, the log k_A affinity constant can be estimated by adding 3.22 to the log*K* values. This means that a drug with 99.99% HSA binding has an approximate 5.2 $(2 + 3.22)$ HSA binding affinity constant.

In a chromatographic system the retention is proportional to the ratio of the number of molecules in the stationary and mobile phases. From this ratio we can derive the % of the molecules bound to the stationary phase. Using eq. 1, the apparent log*K* values can be calculated from the percentage binding supposing the constant and large excess of free HSA in the chromatographic column. As we cannot easily determine the free HSA concentration in chromatography, we calibrate the chromatographic log*K* values with literature log*K* values derived from plasma protein binding. The logarithmic value of the gradient retention times (gtR) obtained from the HPLC experiments were plotted against the linearized values of the % PPB (i.e., log*K*). The slope and the intercept were used then to convert the gradient retention times to log*K* values for a new compound. From these log*K* values, the estimated % protein

Table 1. Calibration Set of Compounds with Their Literature and Typical Measured Chromatographic Data Obtained with the HSA Column (Literature Data Were Obtained from Ref. 16)

Compound	Literature % PPB	tR	Log*tR*	Lit log*K*	Log*K* Measured	% HSA Measured
Warfarin2	98	4.393	0.64	1.51	1.53	98.1
Nizatidine	35	0.6	−0.22	−0.28	−0.35	31.1
Bromazepam	60	1.299	0.11	0.17	0.38	71.2
Carbamazepine	75	1.48	0.17	0.46	0.50	76.8
Budesonide	88	1.826	0.26	0.83	0.70	84.2
Piroxicam	94.5	2.787	0.45	1.16	1.10	93.6
Nicardipine	95	3.768	0.58	1.20	1.38	97.0
Ketoprofen	98.7	3.916	0.59	1.63	1.42	97.3
Indomethacin	99	6.023	0.78	1.69	1.83	99.5
Diclofenac	99.8	5.94	0.77	1.92	1.81	99.5

Table 2. Literature and Measured Data of the Investigated Known Drug Molecules Used as Validation Set and Their Calculated log*P* and log*D* at pH 7.4 Values

Drug	Bound in Plasma (%)	Linearized % PPB (log*K*)	tR HSA	log*K* HSA	% HSA	CHI IAM	ACDlog*D*	Clog*P*	Back Calc* % HSA
Acetaminophen	0	−2.00	0.4	−0.79	14.0	4.0	0.34	0.494	24.88
Acyclovir	15	−0.76	0.25	−1.25	5.4	−7.0	−1.77	−2.422	3.91
Amiloride	40	−0.18	0.484	−0.61	20.0	30.7	1.87	−0.552	0.72
Amoxicillin	18	−0.66	0.435	−0.71	16.4	6.3	−2.58	−1.872	2.78
Ampicillin	18	−0.66	0.5	−0.58	21.2	6.3	−1.8	−1.204	5.97
Amrinone	45	−0.09	1.205	0.28	66.1	15.0	−0.25	−0.689	16.52
Aspirin	49	−0.03	0.713	−0.23	37.3	−1.7	−2.51	1.023	29.55
Beclomethasone	87	0.79	1.273	0.33	68.9	34.5	2.42	2.125	72.06
Betamethasone	64	0.24	0.855	−0.06	47.3	31.7	2.06	1.785	60.13
Bromazepam	60	0.17	1.262	0.32	68.5	28.5	2.41	1.703	52.09
Budesonide	88	0.83	1.591	0.55	78.7	38.6	3.24	2.905	42.55
Bumetanide	99	1.69	2.819	1.10	93.6	24.6	−0.62	3.372	80.89
Carbamazepine	74	0.44	1.48	0.69	83.8	39.2	2.67	1.98	97.98
Cefazoline	87.5	0.81	1.128	0.21	62.7	2.5	−2.89	−1.138	19.52
Ceftazidime	21	−0.58	0.3	−1.07	7.9	−5.1		−3.261	9.52
Cephalexin	14	−0.79	0.4	−0.79	14.0	−2.3	−2.52	−1.64	7.78
Chlorpheniramine	70	0.35	1.361	0.40	72.1	50.5			75.26
Chlorpromazine	97.8	1.49	3.033	1.18	94.7	61.9	1.48	5.8	97.54
Chlorpropamide	96	1.28	2.5	0.99	91.6	5.8	−0.07	2.35	68.40
Cimetidine	19	−0.64	0.5	−0.58	21.2	16.6	0.21	0.351	1.89
Cinoxacin	63	0.22	1.074	0.17	60.1	1.6	−4.61	1.502	1.14
Ciprofloxacin	40	−0.18	0.821	−0.09	45.0	47.8	−1.19	−1.146	16.83
Clonazepam	86	0.76	1.528	0.51	77.1	34.8	3.02	2.384	88.10
Clonidine	20	−0.61	0.59	−0.42	28.0	28.8	0.84	1.428	39.31
Diazepam	99	1.69	2.746	1.08	93.2	37.4	2.96	3.17	91.82
Diclofenac	99.8	1.92	5.157	1.69	99.0	33.5	0.11	4.726	96.90
Digitoxin	97	1.38	1.503	0.49	76.4	27.5	3.81	3.054	71.90
Diltiazem	78	0.53	0.961	0.06	53.9	43.4	2.12	3.647	90.15
Diphenhydramine	63	0.22	0.987	0.08	55.4	44.6	2.29	3.541	89.08
Doxepin	83	0.66	1.8	0.67	83.1	52.3	3.3	4.092	94.28
Ethosuximide	0	−2.00	0.45	−0.68	17.5	−5.4	1.13	0.395	12.16
Famotidine	17	−0.69	0.408	−0.77	14.5	15.7	−2.75	−1.196	9.97
Finasteride	90	0.91	1.444	0.45	74.7	38.9	3.24	3.013	97.57
Flumazenil	50	−0.01	0.6	−0.40	28.8	19.2	0.87	1.091	13.45
Fluoxetine	94	1.13	3.783	1.39	97.0	52.9	1.83	4.566	95.97
Flurbiprofen	99.96	1.98	7.5	2.05	100.1	26.8	0.92	3.754	97.65
Ibuprofen	99	1.69	5.991	1.84	99.5	22.8	0.77	3.679	93.99
Imipramine	90	0.91	1.8	0.67	83.1	54.1	−0.07	5.037	97.10
Indomethacin	99	0.91	5.9	1.82	99.5	32.5	−0.89	4.18	98.30
Isoniazid	0	−2.00	0.28	−1.14	6.8	−8.6	−0.89	−0.668	0.67
Ketoconazole	99.0	1.69	2.706	1.06	93.0	42.9	4.05	2.583	87.70
Ketoprofen	98.7	1.63	3.916	1.42	97.3		−0.31	2.761	92.48
Methylprednisolone	78	0.53	0.979	0.08	54.9	32.1	2.18	1.742	61.54
Metronidazole	10	−0.96	0.25	−1.25	5.4	−3.3	−0.02	−0.457	9.85
Naproxen	99.7	1.88	5.214	1.70	99.0	20.2	0.03	2.816	92.85
Nicardipine	95	1.20	2.735	1.07	93.2	45.9	5.04	5.512	94.11
Nifedipine	96	1.28	1.29	0.34	69.5	29.0	3.05	3.406	68.23
Nimodipine	98	1.51	1.711	0.62	81.4	29.0	3.94	4.144	88.63
Nitrazepam	87	0.79	1.757	0.64	82.3	33.3	2.84	2.321	78.37
Nizatidine	35	−0.28	0.49	−0.60	20.4	17.9	0.97	−0.202	3.94
Pentobarbital	51.3	0.01	0.6	−0.40	28.8	22.4	−1.92	1.419	3.58
Phenytoin	89	0.87	1.469	0.47	75.5	31.5	2.5	2.085	82.85

(*Continued*)

Table 2. (*Continued*)

Drug	Bound in Plasma (%)	Linearized % PPB (log*K*)	tR HSA	log*K* HSA	% HSA	CHI IAM	ACDlog*D*	Clog*P*	Back Calc* % HSA
Piperacillin	18.5	−0.65	0.421	−0.74	15.4	9.4	−2.14	1.697	1.58
Piroxicam	94.5	1.16	3.667	1.36	96.8	20.5	−2.89	1.888	21.83
Prednisone	75	0.59	0.716	−0.23	37.6	25.9	1.56	1.661	35.98
Propranolol	87	0.79	1.114	0.20	62.0	47.7	0.96	2.753	84.43
Quinidine	87	0.79	1.108	0.20	61.7	47.2	1.8	2.785	72.06
Ranitidine	15	−0.76	0.45	−0.68	17.5	28.0	0.24	0.63	4.22
Rifampin	89	0.87	1.53	0.51	77.2	36.0	−2.03	3.77	87.63
Spironolactone	90	0.91	1.279	0.34	69.1	36.4	3.12	2.249	86.30
Sulfamethoxazole	62	0.20	0.627	−0.36	30.9	31.6	−0.66	0.563	50.55
Terbutaline	20	−0.61	0.6	−0.40	28.8	16.8	−1.71	0.482	12.64
Tolbutamide	96	1.28	3.375	1.28	96.0		2.50	2.497	72.73
Trazodone	93	1.07	2.229	0.88	89.1	37.1	1.59	3.167	92.09
Trimethoprim	44	−0.11	0.716	−0.23	37.6	22.5	0.52	0.981	46.39
Warfarin	99	1.69	4.209	1.49	97.9	19.9	0.59	2.901	93.41
Zidovudine	20	−0.61	0.25	−1.25	5.4	1.5	−0.58	0.044	6.03
Zolpidem	92	1.01	1.407	0.43	73.6	33.9	2.35	2.826	86.84

*Bound in Plasma % obtained from ref. 16; linearized value was obtained by eq. 1; tR HSA is the measured gradient retention time; log*K* HSA was obtained from log*tR* using the calibration plot; % HSA is the measured HSA binding obtained from log*K* HSA by eq. 2; CHI IAM is the measure of compounds interaction with phospholipids and obtained using the method in ref. 25. ACDlog*D* is the calculated octanol/water distribution coefficient; clog*P* is the calculated octanol/water partition coefficient of the neutral species; Back calc % HSA was obtained from the log*K* HSA values calculated using eq. 3 and the calculated Abraham descriptors.

binding was obtained by applying eq. 2.

$$\% \text{ Binding} = \frac{101^{*}10^{\log K}}{1 + 10^{\log K}} \qquad (2)$$

The investigated sets of molecules are commercially available and were obtained from our "in house" compound store. The names of the validation set of molecules (drugs) are listed in Table 2. Table 2 also contains the literature and measured binding values as well as the calculated log*P* values (Daylight clog*P*) and log*D* values (ACDlog*D*). To characterize the generalized lipophilic binding on the HSA we have measured the log*K* values of the "Abraham set" of molecules with known molecular descriptors on the HSA column. The compounds are listed in Table 3.

Each compound was dissolved individually in a mixture of 50% 2-propanol and 50% ammonium acetate buffer solution at 0.5 mg/mL concentration. The injected volume was 3 μL. Research compounds were obtained as 10 μL 10 mM DMSO solution on 96-well plates. The DMSO solution was diluted down to 130 μL and 10 μL of the diluted sample solution was injected onto the HPLC column.

The calculated molecular descriptors of the compounds were obtained by our "in house" UNIX-based Abraham descriptor calculator. The PC version of the descriptor calculator (ABSOLV) is commercially available from Sirius Analytical Ltd. (Kent, UK). The linear regression analysis and step-wise regression calculations were carried out by JMP program package (SAS Institute).

RESULTS

Tables 2 and 3 show the measured HSA binding and the Chromatographic Hydrophobicity Indices obtained on Immobilized Artificial Membrane HPLC (CHI IAM). The CHI IAM method has been described in details in our previous publication.[21] We have included in Table 2 the calculated octanol/water partition coefficients of the unionized species (clog*P*) and distribution coefficients for all the species at pH 7.4 (ACDlog*D*), while Table 3 shows the measured octanol/water partition coefficients of the unionized species (log*P*) of the Abraham training set of compounds.

Acceptable correlation was found between the literature plasma protein binding data and the experimental HSA binding data using the fast gradient HPLC method for the validation set of compounds as is shown in Figure 1. The advantages of the described method are as follows. The analysis has a short cycle time (15 min) during which even the most strongly bound compounds can be eluted. The column is "calibrated" by nine compounds with literature plasma protein binding data that takes into account column aging and other parameters that affect the absolute value

Table 3. The "Abraham" Set of Compounds with the Abraham Molecular Descriptors and the Measured HSA Binding Data

Name	tR	log*K* HSA	% Binding	CHI IAM	Log*P*	log*D*	Calc log*K* HSA	Back Calc* % HSA
1,3,5-OH benzene	0.452	−0.71	16.42	4.6	0.16	0.16	−0.73	15.73
1,4-Dinitrobenzene	0.739	−0.31	33.04	16.5	1.47	1.47	−0.33	32.36
1-Napthol	2.87	0.79	86.83	38.9	2.84	2.84	0.67	83.21
2-Cl-phenol	1.064	−0.02	49.48	26.9	2.15	2.15	−0.08	45.91
3,4 di-Cl-phenol	4.982	1.23	95.44	42.7	3.33	3.33	1.01	91.94
3-CF3 Phenol	1.607	0.32	68.15	35.0	2.95	2.94	0.54	78.21
3-F benzoic acid	1.451	0.20	62.04	−10.3	2.15	−1.27	0.10	56.53
3-F phenol	0.613	−0.46	25.79	20.3	1.93	1.93	0.02	51.67
3-NO2 acetanilide	0.736	−0.33	32.11	22.5	1.47	1.47	−0.03	48.91
3-NO2 benzoic acid	2.011	0.46	74.94	−7.1	1.83	−1.88	0.04	52.91
3-OH benzoic acid	0.567	−0.54	22.75	−12.4	1.5	−1.86	−0.26	35.58
p-OH benzyl alcohol	0.393	−0.82	13.15	−1.0	0.49	0.49	−0.88	11.68
4-CN-phenol	1.146	0.04	52.98	16.7	1.6	1.6	−0.03	48.74
4-F benzoic acid	1.199	0.05	53.53	−10.4	2.07	−1.13	0.04	52.81
4-I-phenol	5.385	1.30	96.16	39.1	2.91	2.90	0.72	84.80
2-Nitrobenzoic acid	1.95	0.47	75.61	−12.9	1.89	−1.50	−0.05	47.51
4-Nitrophenol	2.069	0.52	77.65	15.1	1.91	1.91	0.12	57.17
4-OH-benzyl alcohol	0.379	−0.85	12.38	−1.2	0.25	0.25	−0.99	9.44
Adenine	0.42	−0.77	14.59	1.3	−0.09	−0.09	−1.05	8.30
Aldosterone	1.071	−0.01	49.79	15.7	1.08	1.08	−0.22	37.98
Aniline	0.41	−0.79	14.06	1.4	0.9	0.9	−0.70	16.79
Anisole	0.829	−0.22	37.98	20.4	2.11	2.11	−0.13	43.22
Anthracene	8.305	1.57	98.37	51.0	4.45	4.45	1.59	98.47
Barbituric acid	0.306	−1.03	8.65	−15.0	−1.47	−4.34	−2.19	0.65
Benzamide	0.42	−0.77	14.62	3.6	0.64	0.64	−0.85	12.45
Benzoic acid	0.761	−0.29	34.27	−11.7	1.87	−1.25	−0.18	40.26
Benzonitrile	0.563	−0.53	22.86	13.2	1.56	1.56	−0.44	27.06
Butalbarbital	0.528	−0.59	20.81	14.2	1.89		−0.38	29.62
Caffeine	0.415	−0.78	14.34	4.9	−0.07	−0.07	−1.50	3.13
Chlorobenzene	1.555	0.29	66.78	29.1	2.89	2.89	0.25	64.85
Cortexalone	1.705	0.36	70.55	34.6	2.52	2.52	0.53	77.91
Corticosterone	1.592	0.31	67.76	33.2	1.94	1.94	0.46	74.90
Cortisone	1.242	0.08	55.13	27.0	1.42	1.42	−0.06	47.27
Cortisone-21-acetate	1.593	0.31	67.79	33.4	2.1	2.1	0.03	51.99
Deoxycorticosterone	3.5	0.89	89.57	38.6	2.88	2.88	0.89	89.50
Dexa-Methasone	1.306	0.15	59.06	31.8	2.01	2.01	0.34	69.17
Di-benzothiophene	7.565	1.57	98.37	50.1	4.38	4.38	1.47	97.67
Di-Et pthalate	1.347	0.17	60.47	29.6	2.47	2.47	0.02	51.88
Estradiol	5.069	1.19	94.81	29.8	2.69	2.69	1.03	92.45
Hydrocortisone	1.024	−0.05	47.68	28.3	1.55	1.55	0.13	58.12
Hydroquinone	0.407	−0.80	13.90	0.1	0.59	0.59	−0.56	21.75
Ibuprofen	7.088	1.52	98.04	23.6		1.07	1.20	94.99
Indazole	0.878	−0.17	40.55	21.0	1.77	1.77	−0.05	47.32
Indomethacin	8.691	1.61	98.58	32.0	4.27	−0.89	1.66	98.84
Naphthalene	13	2.01	100.03	40.9	3.3	3.3	0.68	83.62
Penta-fluorophenol	1.352	0.15	58.94	11.1	3.23	0.77	0.33	68.60
Phenol	0.526	−0.59	20.69	10.2	1.5	1.5	−0.25	36.37
Phenylacetic acid	0.579	−0.51	23.80	−11.9	1.41	−1.56	−0.38	29.91
Procaine	0.81	−0.26	36.03	28.7	1.89	0.18	−0.29	34.01
Progesterone	4.316	1.12	93.85	43.7	3.7	3.7	1.05	92.70
Propranolol	1.972	0.44	74.25	49.0	3.37	1.19	0.78	86.62
p-Toluidine	0.611	−0.48	25.22	29.8	1.39	1.39	−0.46	26.05

(*Continued*)

Table 3. (*Continued*)

Name	tR	log*K* HSA	% Binding	CHI IAM	Log*P*	log*D*	Calc log*K* HSA	Back Calc* % HSA
Pyrene	8.507	1.67	98.88	55.5	5	5	1.98	99.95
Resorcinol	0.472	−0.68	17.56	7.1	0.8	0.76	−0.55	22.35
Salicylic acid	1.817	0.42	73.02	−3.9	2.26	−1.86	0.12	57.63
Testosterone	2.486	0.67	83.24	39.1	3.31	3.31	0.83	87.95
Theophylline	0.409	−0.79	14.01	1.5	−0.02	−0.02	−1.63	2.30

*tR HSA is the measured gradient retention time; log*K* HSA was obtained from log*tR* using the calibration plot; % HSA is the measured HSA binding obtained from log*K* HSA by eq. 2; CHI IAM is the measure of compounds interaction with phospholipids and obtained using the method in ref. 21; log*D* is the octanol/water distribution coefficient; log*P* is the octanol/water partition coefficient of the neutral species; Back calc % HSA was obtained from the log*K* HSA values using eq. 3.

of the retention time. It should be noted that the literature data relate to the plasma protein binding while our measured data refers to only the HSA binding ability of the compounds. However, the calibration set of compounds were chosen as known to bind to HSA major binding sites (warfarin site or benzodiazepine site). Although our HPLC based method measures only the HSA binding ability, it can aid compound selection at the early discovery phase. Previously published isocratic methods[13–15] can be time consuming to elute strongly bound compounds. Also, when the log*K* values are used the reproducibility of the data are dependent on the column to column reproducibility, while the calibration in our method takes care of the slight variations in the columns or HPLC systems. In addition, we have found that the results obtained with acetonitrile organic modifier used in ref. 14 did not show as good correlation with literature plasma protein binding data.

A much bigger deviation from the best fit in the middle range of the % binding values than at either ends is evident. At the early stage of discovery, where we use this method in a high throughput way to screen molecules, the results are used to identify strongly bound compounds. In addition, we would like to use the measured binding values to build structure-binding relationships that help to design compounds with reduced binding below 90–95%. Therefore, unlike the traditional ultrafiltration method, this method should provide adequately reproducible binding data at the higher range.

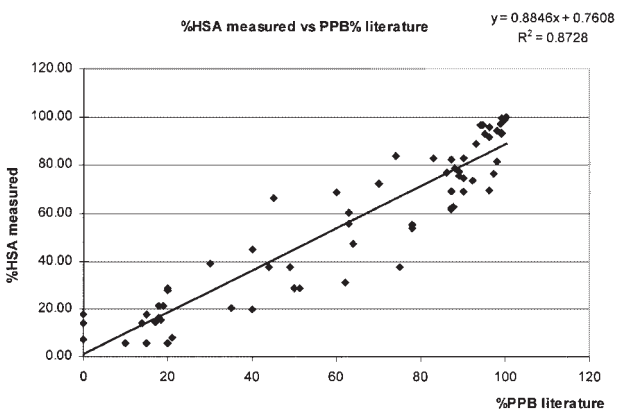

Figure 1. The plot of the literature plasma protein binding data as a function of the measured HSA binding data.

The deviation may be the result of compound's binding also to other plasma proteins. Although the investigated compounds are known to bind dominantly to HSA, we can expect that some of the basic compounds have strong binding to α-acid–glycoprotein as well.

Figure 2 shows the nonlinear relationship between log*K* and % PPB. It is important to understand that the expected error in the measurements is not constant over the range, and is not linearly related to the % binding. The error of the log*K* in the middle range, expressed in % binding, is a magnitude larger than at the high range (above 95%).

It should be noted that the big adavantage of the HPLC method, on the contrary to the ultrafiltration method, is that it is more accurate for stronger bound compounds. For example, there are several minutes differences in retention times between compounds bind to 99.2 or 99.3%.

To assess the expected experimental error at the higher % bindings we have measured 30 research compounds showing above 90% HSA binding on two batches of HSA columns by slightly altering the flow rate, and injected amount. The average measured % HSA binding values and their standard deviations are plotted in Figure 3. The error was always less than 1.5%, as it was expected.

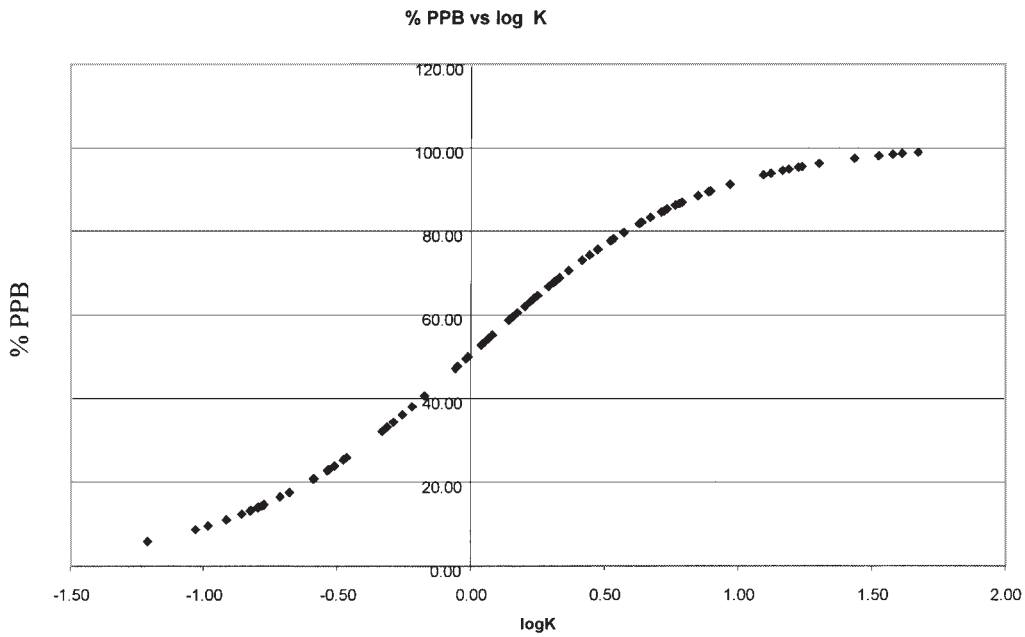

Figure 2. The relationship between % PPB and the linear free energy related value (log*K*).

DISCUSSION

To reveal the contribution of lipophilicity to the HSA binding the correlation between log*K* (HSA) values and the calculated log*P* (clog*P*, Daylight) and log*D* values (ACD Labs) was investigated. Figure 4 shows the log*K* (HSA) versus clog*P* plot, while Figure 5 shows the log*K* (HSA) versus ACD log*D* plot. Compounds have been differentiated according to their charge at pH 7.4. They were called acids if they were negatively charged and called bases if they were positively charged. The classification was based on the CHI-lipophilicity values[22] obtained by reversed-phase gradient HPLC at three buffer pHs (2, 7.4, and 10.5). Zwitterionic compounds have both positive and

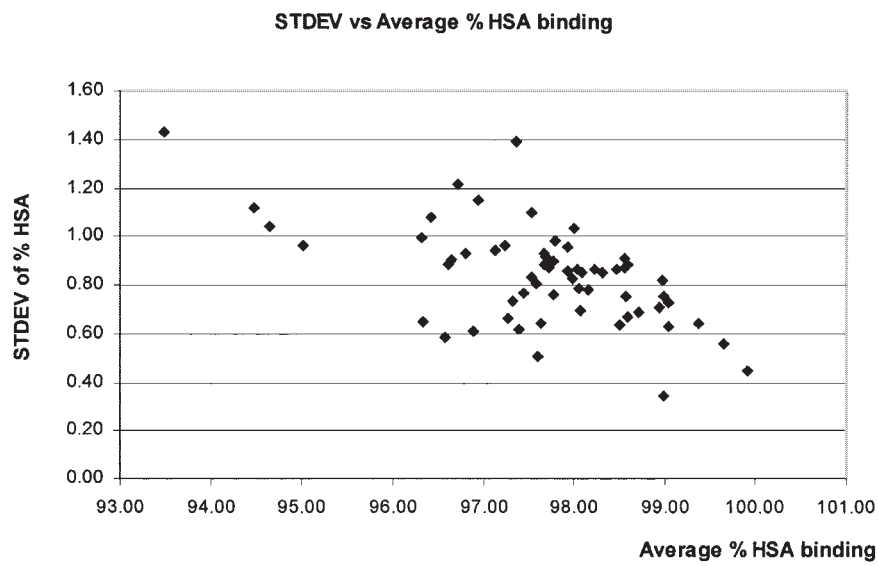

Figure 3. The average measured % HSA binding values and their experimental standard deviations obtained from six repeated measurements of 34 research compounds with % HSA binding above 90%.

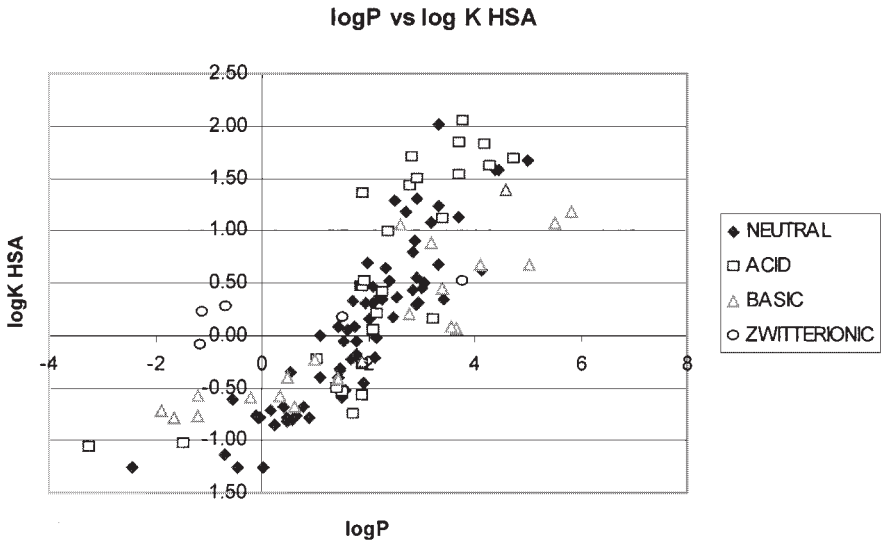

Figure 4. The plot of the measured log*K* values as a function of log*P* (octanol/water partition coefficient) values for the combined "drug set" (Table 2) and "Abraham set" (Table 3) molecules.

negative charges at pH 7.4. It is important to note that the % HSA binding values are not linear free energy related measures; therefore, the log*K* HSA values were used to set up QSPR relationships. We have used eqs. 1 and 2 to convert the percentage values to the linear free energy related log*K* values (logarithm of dissociation constant) and vice versa.

It can be seen that the ionization of the compounds does not reduce the HSA binding ability as much as it reduces the octanol/water log*D* values. The lipophilicity of the uncharged molecule (log*P*oct) better explains the observed HSA binding than the distribution coefficient (log*D* at pH 7.4), which includes the partition into octanol of both charged and uncharged species. Ionized molecules (ion pairs) are not well solvated by octanol, and have log*D* values 3–4 log units lower than the log*P* value of the unionized form, whereas HSA has charged binding sites that can accommodate ionized molecules. It can be seen that in general negatively charged compounds bind more strongly to HSA than the positively charged ones. This finding is very similar to that of described by H. Waterbeemd et al.[23] and Davis and Riley.[24] However, when the lipophilicity of the uncharged molecules is considered (clog*P*), no significant separation of acids and bases could be observed. These results clearly suggest that there is a trend between HSA binding and compound lipophilicity (expressed as the lipophilicity of uncharged molecule). The plot of clog*P* and log*K* HSA values (Figure 6) obtained for 480 research compounds from 10 different projects shows the same trend.

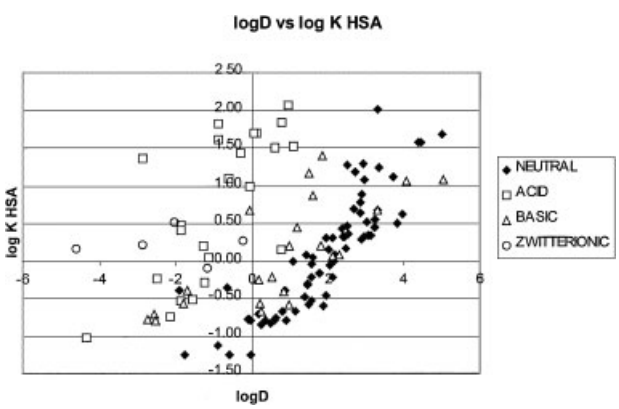

Figure 5. The plot of the measured log*K* values as a function of log*D* (octanol/water distribution coefficients at pH 7.4) values for the combined "drug set" (Table 2) and "Abraham set" (Table 3) molecules.

The Abraham solvation equation approach[19] has been applied to express the HSA binding in terms of the molecular descriptors. The following equation was obtained:

$$\log K\,(\mathrm{HSA}) = -1.28 + 0.82(\pm 0.15)^{*}E - 0.36(\pm 0.15)^{*}S + 0.18(\pm 0.14)^{*}A - 1.97\,(\pm 0.15)^{*}B + 1.62(\pm 0.21)V \quad (3)$$

$n = 52$, $r = 0.91$, $s = 0.33$, and $F = 44$

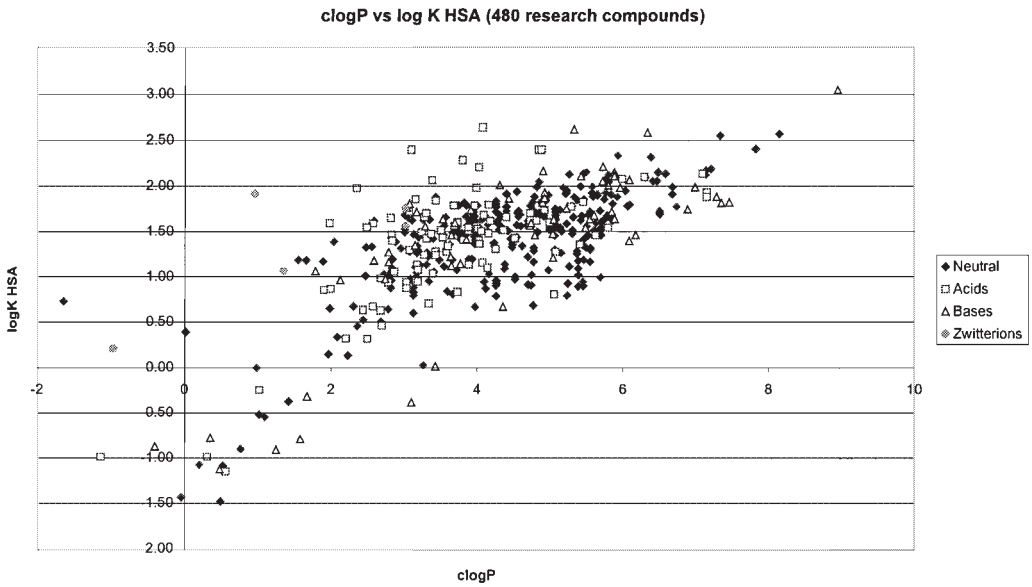

Figure 6. The plot of log*K* HSA values as a function of clog*P* values for 480 research compounds from 10 projects.

where *E* is the excess molar refraction, *S* is the dipolarity/polarisability, *A* is the H-bond acidity, *B* is H-bond basicity, and *V* is the McGowan volume, *n* is the number of compounds, *r* is the multiple regression coefficient, *s* is the root mean square error, *F* is the Fisher-test value. The molecular descriptors were obtained from UCL database.[25]

The measured and the back-calculated log*K* (HSA) values using eq. 3 are plotted in Figure 7. The correlation is surprisingly good, if we consider that the HSA binding expected to involve shape selectivity as well, unlike water/organic solvent partition. The regression coefficients of the solvation eq. 3 are very similar to that which was obtained for octanol/water log*P* and for the retention on immobilized artificial membrane column.[21]

The molecular descriptors for the drug set of molecules have been calculated using the Unix-based program developed at GlaxoWellcome.[26] Using the coefficients in eq. 3 and the calculated molecular descriptors for the drug set of molecules the log*K* values and the corresponding % HSA values have been calculated. Figure 8 shows the % HSA values (measured versus calculated) for the combined drug and Abraham set of molecules.

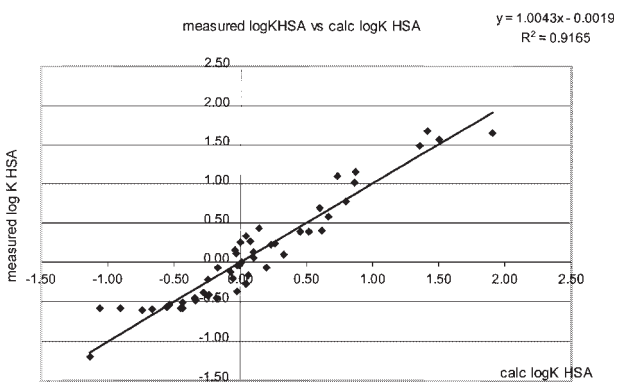

Figure 7. The plot of the measured and calculated log*K* (HSA) values for the Abraham set (Table 3) compounds.

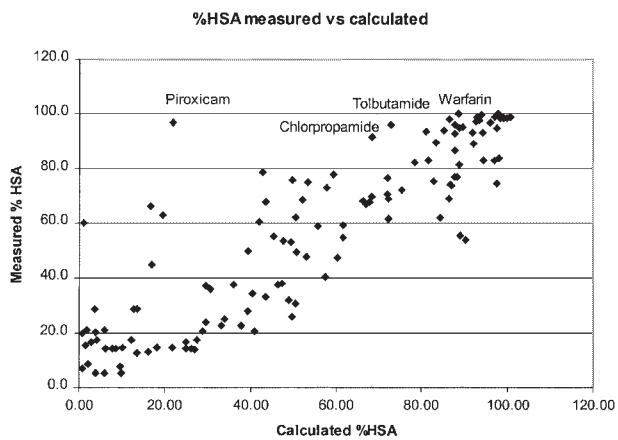

Figure 8. The measured and the calculated % HSA values for the combined drug and the Abraham set of molecules based on calculated molecular descriptors. (The calculated log*K* values were obtained from eq. 3 and then converted to % HSA bound using eq. 2).

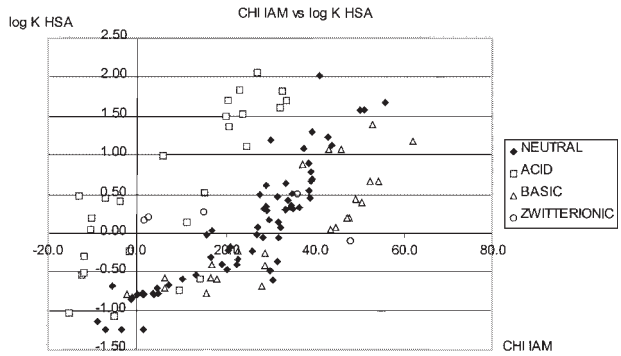

Figure 9. The plot of the CHI IAM values and the log*K* (HSA values) for the "drug set" (Table 2) and the "Abraham set" (Table 3) molecules.

It can be seen that most of the compounds bound to HSA as predicted. However, there are several compounds from the drug set that bind significantly more strongly that would have been expected only from their lipophilicity. We have observed a so-called "baseline QSAR" as it was discussed by Klopman et al.[27] In our case, it means that from the compound lipophilicity, we can confidently predict a minimum binding affinity. We can also assume that stronger binding to HSA than is expected from the lipophilicity of the compounds will sometimes occur and presumably arises when there is a particularly good "fit" between the compound and a specific binding pocket. During the drug discovery process we can use measured log*K* HSA values to identify these "unusual" high-affinity binders and identify specific structural features that characterize this behavior within a particular analog series. In our previous publication[25] we have described a similar gradient HPLC method to determin compounds interaction with Immobilized Artificial Membrane. The so-obtained Chromatographic Hydrophobicity Indices (CHI IAM) values were measured for the majority of the compounds listed in Table 2 as well. The log*K* HSA values were plotted as a function of the CHI IAM values in Figure 9. It can be seen that compounds with a certain lipophilicity bind to both membrane and HSA; however, there are several drug molecules (especially the acids) that bound more strongly to HSA that it would be expected from their membrane affinity. When we plotted the log*K* HSA values as a function of CHI IAM values for the 480 research compounds, very similar "separation" of acids, bases, and neutral compounds could be observed (Figure 9). Figures 8 and 9 reveal the different effect of ionization on the serum albumin binding and phospholipid binding. Although the presence of negative charge generally increases the albumin binding, it usually reduces the membrane affinity, and conversely, the presence of positive charge increases the membrane affinity while it has very little effect on the albumin binding. These differing effects of positive and negative charges on membrane affinity and protein binding are not modeled well by the octanol/water distribution (log*D* at pH 7.4) where both types of charges have a similar effect.

It can be concluded that while binding to HSA and to membranes is explainable to a large part by overall molecular properties and correlates with a general lipophilic parameter like log*P*, it is also

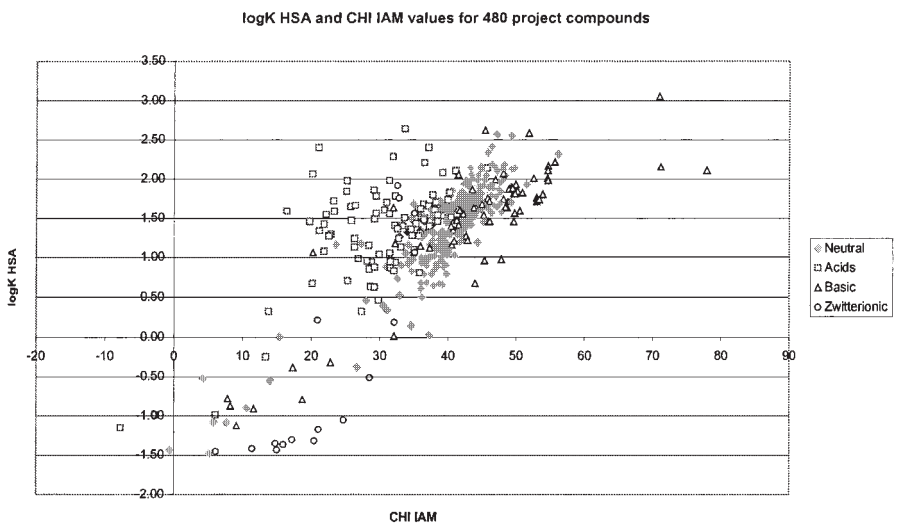

Figure 10. The plot of the log*K* HSA and CHI IAM data for 480 compounds from 10 different projects.

dependent on specific molecular recognition, such as directed hydrogen bonds, charge interactions, and steric effects relating to the filling of hydrophobic pockets. Although attempts have been made to predict this binding by comparison of pharmacophores with those of known binders,[20] it is safer to rely on measurements to detect compounds that bind more strongly than expected based on lipophilicity alone.

CONCLUSION

A fast and automated method to measure HSA binding for discovery research compounds has been described. It has been demonstrated that the method has smaller errors in the high binding region, which is the most interesting from the developability point of view. The results obtained by this method were suitable for deriving quantitative structure–property relationships. The effect of positive and negative charge on the albumin binding and membrane affinity has been revealed. We have demonstrated that the high-throughput measurements of the HSA binding and the Immobilized Artificial Membrane interaction (CHI IAM) can reveal compounds that show "unusually" strong binding to either albumin or membrane. As the drug molecules should have an optimum balance between albumin and membrane binding these methods can facilitate compound selection in the early discovery setting. Plots such as Figures 9 and 10 are useful for selecting candidates that have a good compromise between low-protein binding and high-membrane affinity, as these are both major factors in achieving good tissue penetration and increasing the duration of action.

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