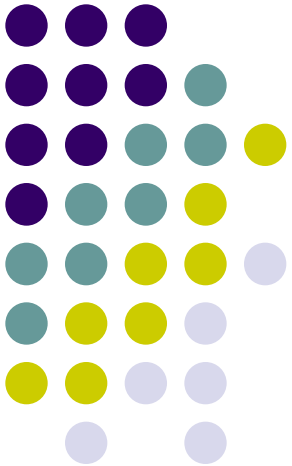
Application of bio-mimetic HPLC for Protein Binding Measurements in Drug Discovery



Klara Valko

Physicochemical Characterisation

Analytical Chemistry

Molecular Discovery Research

GlaxoSmithKline

Stevenage, UK

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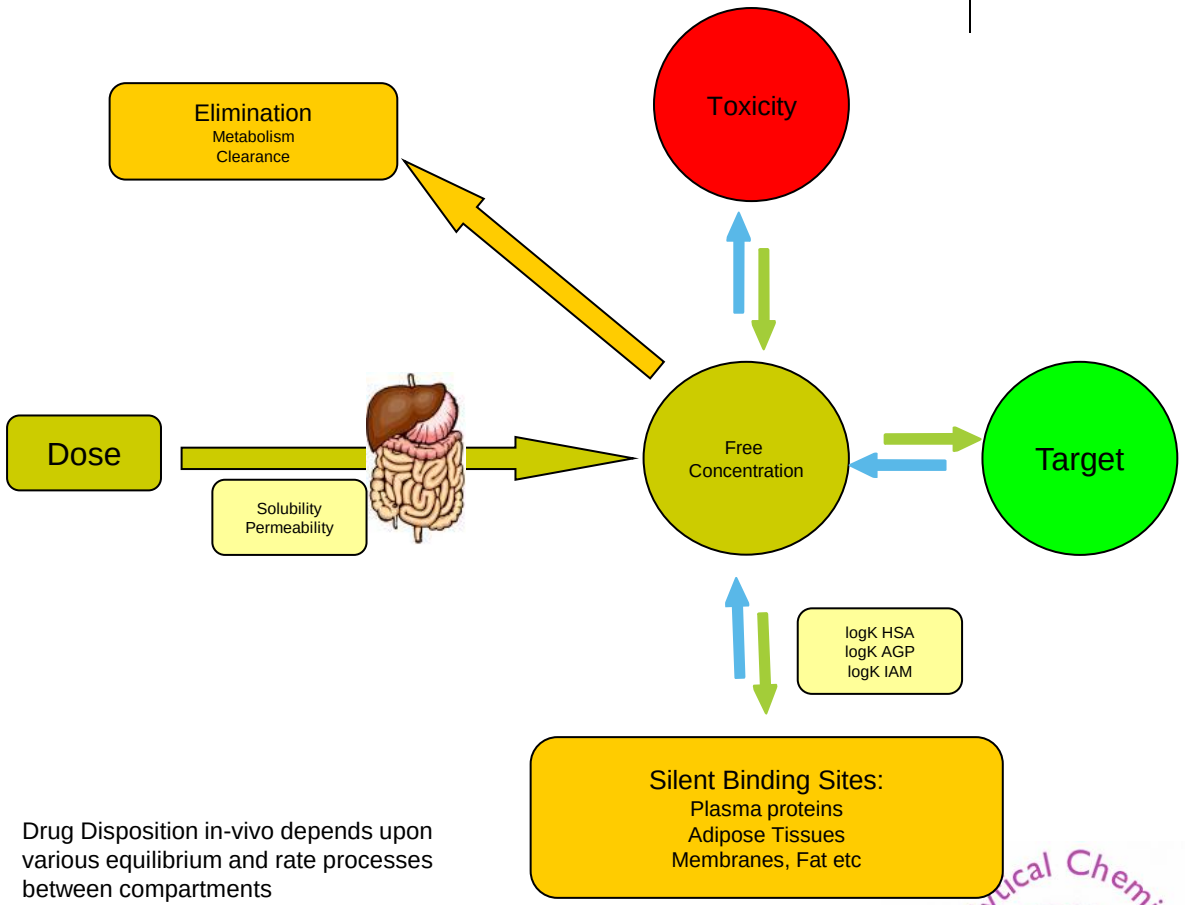
Why do we need plasma protein binding measurements in early drug discovery?

In vitro activity does not necessarily imply that the compound will be active and non-toxic in vivo!

Only free drug can exert pharmacological activity (toxicity)

The free drug concentration depends on compounds binding to plasma proteins and tissues.



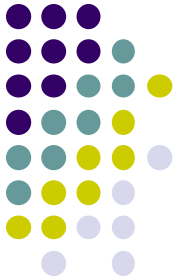
Drug Disposition in-vivo depends upon various equilibrium and rate processes between compartments

G. L. Trainor: The importance of plasma protein binding in drug discovery, Expert Opin. Drug Disc. (2007) 2(1) 51-64

Our strategy in lead optimisation support

- Measure compounds binding to major plasma constituents (HSA, AGP)
- Establish structure – binding property relationship
- Compare binding with lipophilicity in order to reveal potential strong/specific binding
- Estimate the total fraction bound in plasma/blood
- Put these data into various models (for example volume of distribution, CNS penetration) to estimate the effect of plasma protein binding on in vivo distribution of the compounds
- Identify which binding property should be changed to achieve better in vivo profile and how to change the structure to achieve that

Plasma constituents that might affect total plasma protein binding

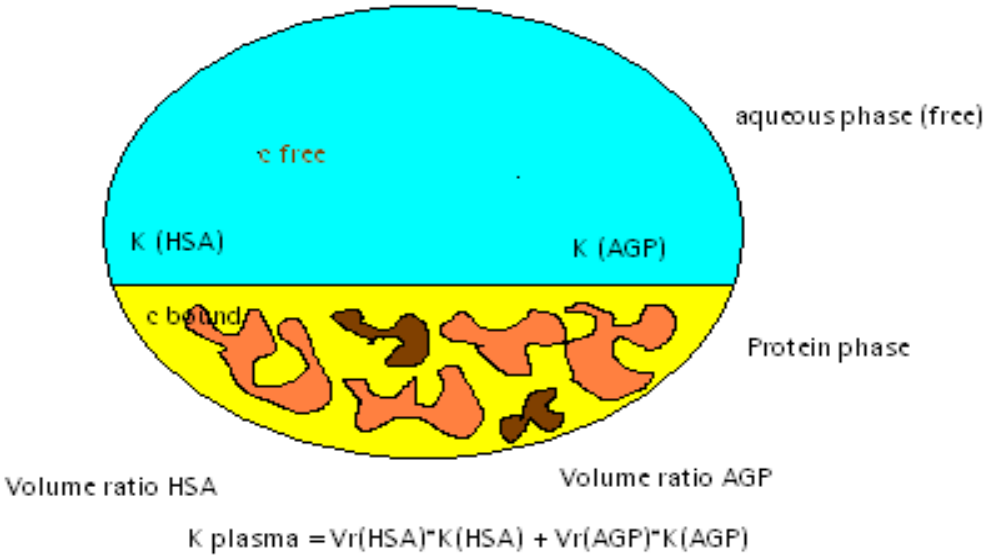


Plasma contains:

- serum albumin (HSA) (60%) (40 -75 g/L)
- alpha-1-acidglycoprotein (AGP) (1.5%) (0.7 g/L)
- globulins (alpha, beta, gamma)
- lipoproteins
- fibrinogen
- non-proteins (fatty acids, urea, creatinine, ammonium salts, amino acids, hormones, etc)

Estimating overall plasma protein binding from the individual binding data

K plasma = Vr(HSA)*K(HSA) + Vr (AGP)*K(AGP) + Vr (other proteins)*K(other proteins)



K plasma =

%PPB

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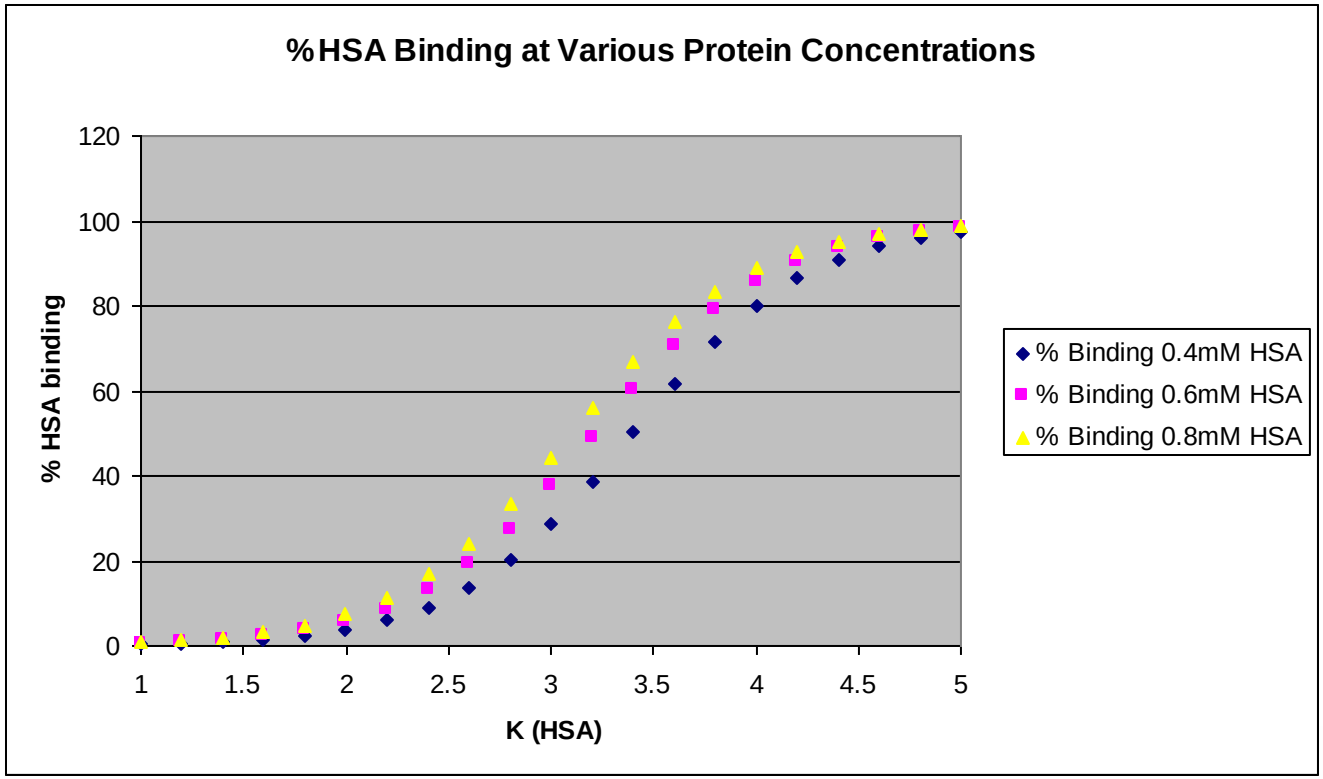
100 - %PPB

Vr = volume ratio (physiological variation !)

K(protein) = partition coefficient

How % bound relates to K (association constants)?

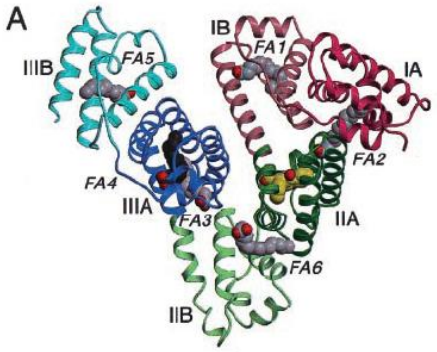


% bound

K

Bio-mimetic HPLC stationary phases to measure protein and phospholipid binding

- Immobilised human serum albumin (HSA)
- Immobilised alpha-1-acid-glicoprotein (AGP)
- Immobilised artificial membrane (IAM)



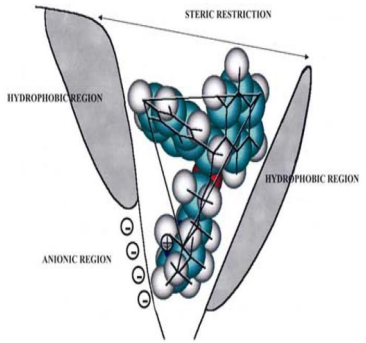
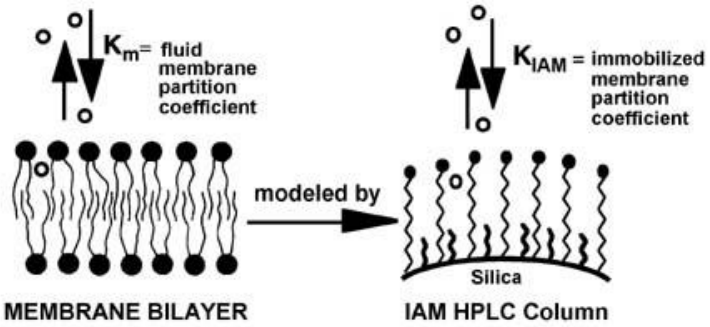


Fig. (8). Hypothetical mode of binding of basic drugs by AGP [400].



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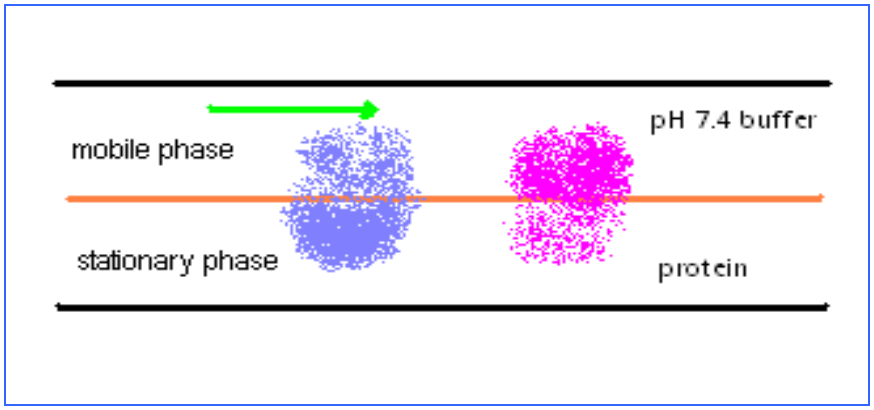
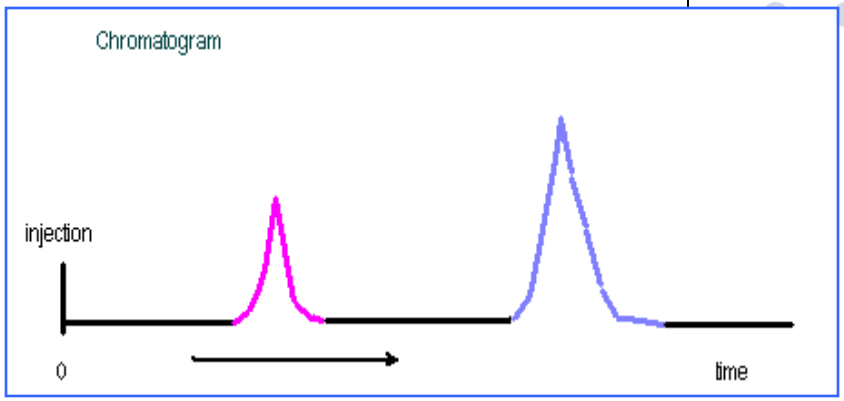
The principles of using HPLC retention on a protein column to measure protein binding





- Different compounds travel at different speeds in the chromatographic system.
- The differential migration depends on the interaction of compounds between the mobile and stationary phase.

- **k (retention factor) can be obtained from retention time and it relates to the partition coefficient of the compounds between the mobile and stationary phases.**

$$k = \frac{\text{the number of moles in the stationary phase}}{\text{number of moles in the mobile phase}} \quad \text{also } k = \frac{t_R - t_0}{t_0} \quad \text{and} \quad \log k = \log K + \log(Vs/Vm)$$

Calibrated retention times on bio-mimetic stationary phases are converted to binding constants

$$\text{Log } K = a^{*}\log t_R + b$$

$$\% = 100^{*}10^{\log K}/(1+10^{\log K})$$

a and b constants are established using the binding data of a calibration set of compounds obtained by other methodology (equilibrium dialysis or ultra-filtration)

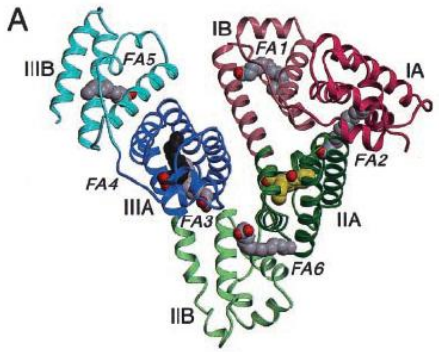
HPLC based HSA and AGP binding measurements

- Column Chiral HSA/AGP 50 x 3 mm (Chromtech Ltd)
- Mobile phase:

  (A) 50 mM ammonium acetate buffer pH 7.4

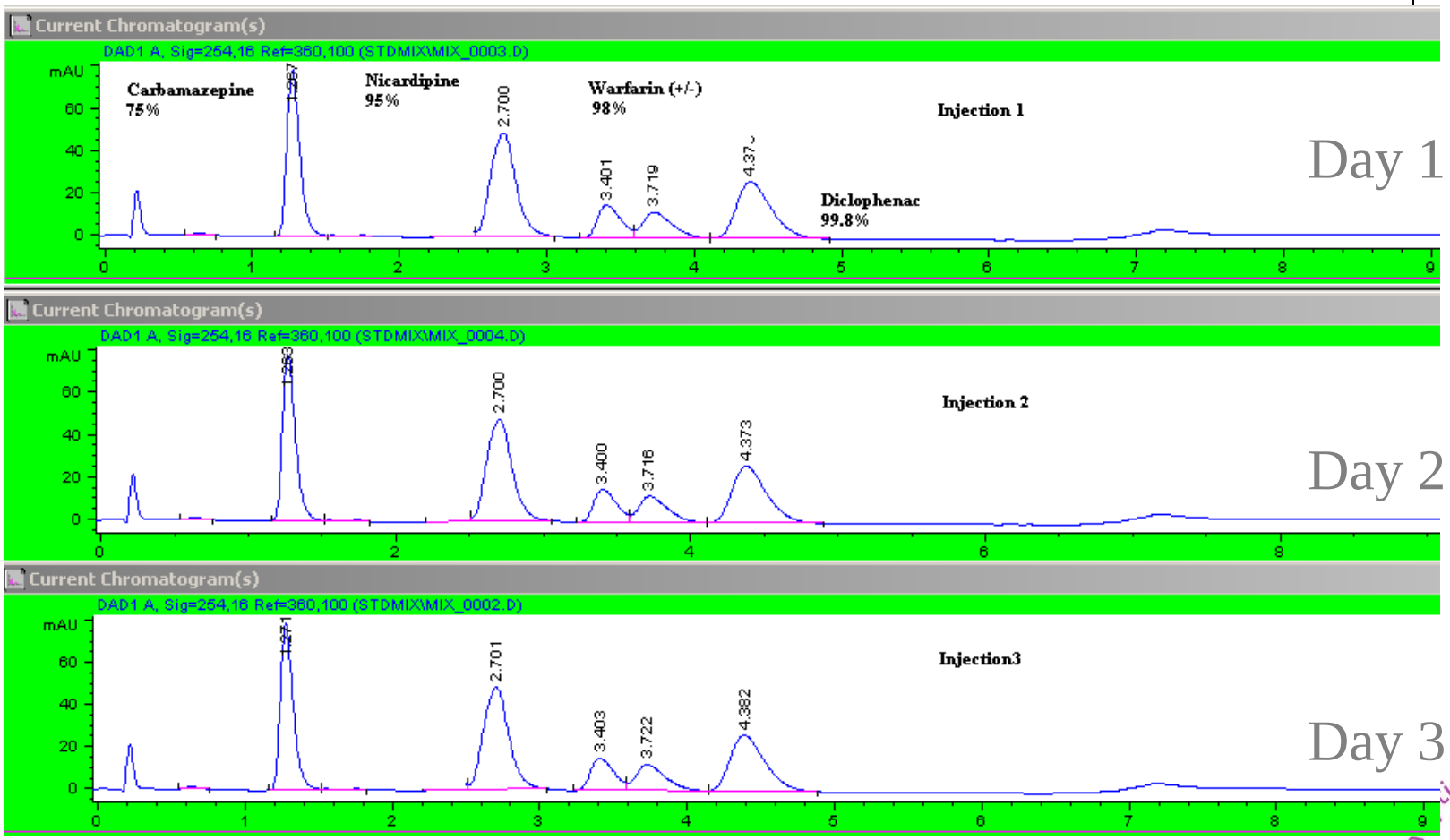
  (B) propan-2-ol

  - **Flow rate: 1.8 ml/min**
  - **Run time: 6 min**



Calibration of the HSA – HPLC system

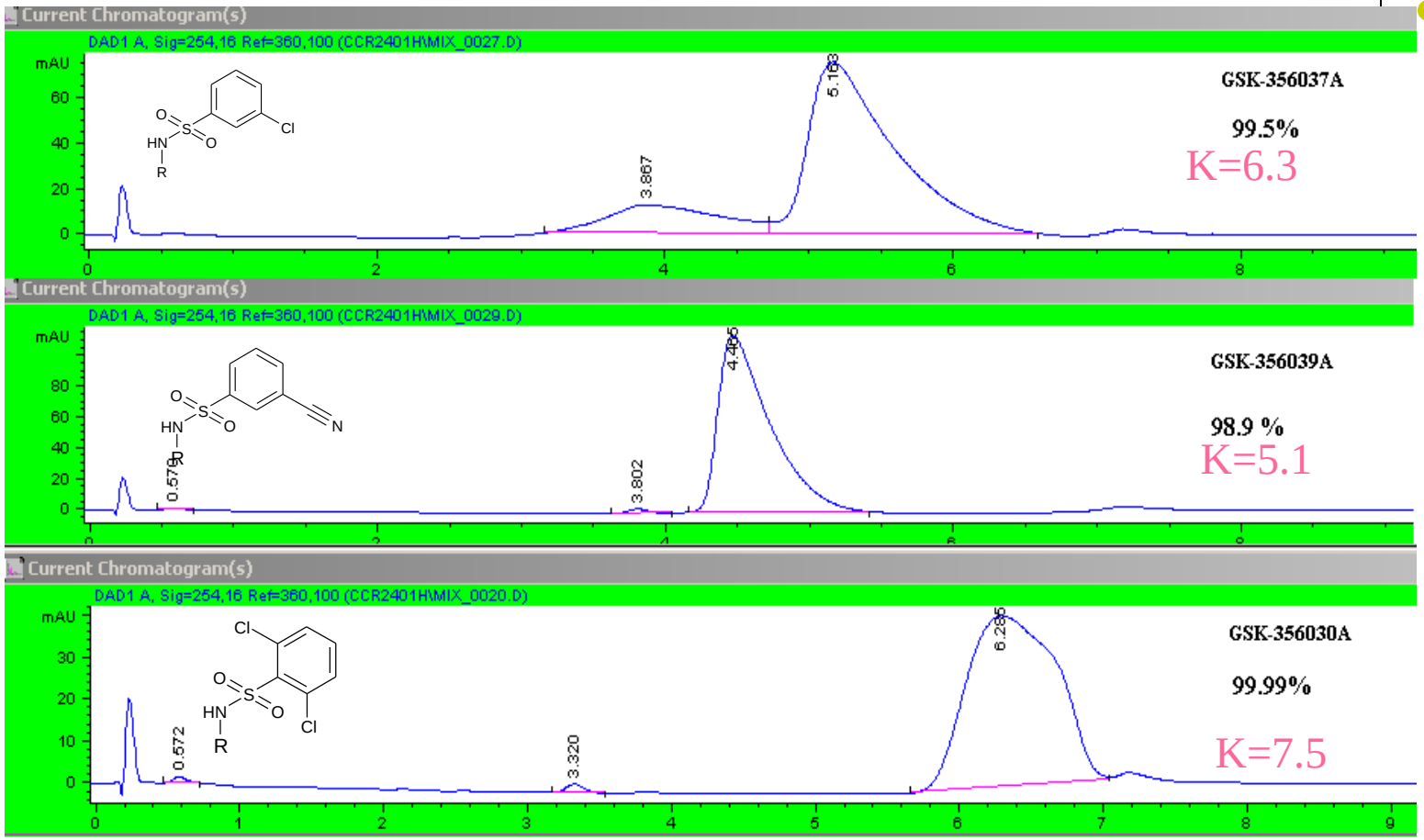
K. Valko, S. Nunhuck, C. Bevan, MH Abraham, D. Reynolds: Fast gradient HPLC method to determine compounds binding to human serum albumin. Relationships with octanol/water and immobilised artificial membrane lipophilicity, J. Pharm. Sci. 2003 (92), 2236-2248



1.6 3.3 4.5 6.8 8.0

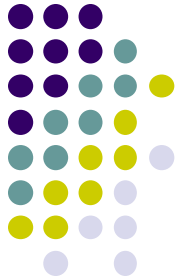
75% 95% 98% 99.8% 99.9999 %

Differentiating between closely related highly bound compounds



We are measuring the bound proportion and not the free, so the accuracy increases with higher binding.

Advantages of the HPLC column technology



- Fully automated based only on retention time measurement.
- Reproducible and robust because of using a calibration set of compounds.
- Fast (10 min cycle time), small amount of sample is needed (10 ul 10 mM DMSO solution).
- Data can be used to set up local and global structure - binding relationships, thus help the design process.

K. Valko, J. Chromatogr. 1037 (2004) 299-310

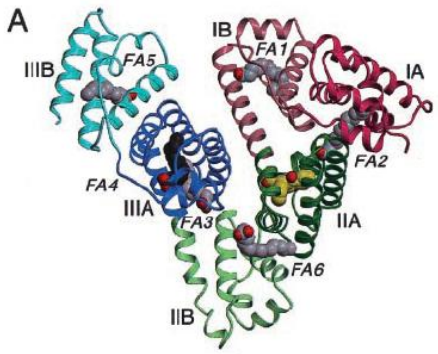
HPLC based HSA binding data were suitable to build SAR for compounds above 95% bound (PPAR example)

Examples:

-CH2-CH2-CH2-CH3 %HSA >98

-CH2-CH2-O-CH2-CH3 % HSA ~95%

-CH2-CH2-CH2-O-CH3 % HSA ~ 92%



General rules to reduce high binding:

- **Reduce lipophilicity (introduce polar group)**
- **Eliminate negative charge if possible**
- **Introduce basic functionality (+ charge)**

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Hypothetical mode of binding of basic drugs by AGP

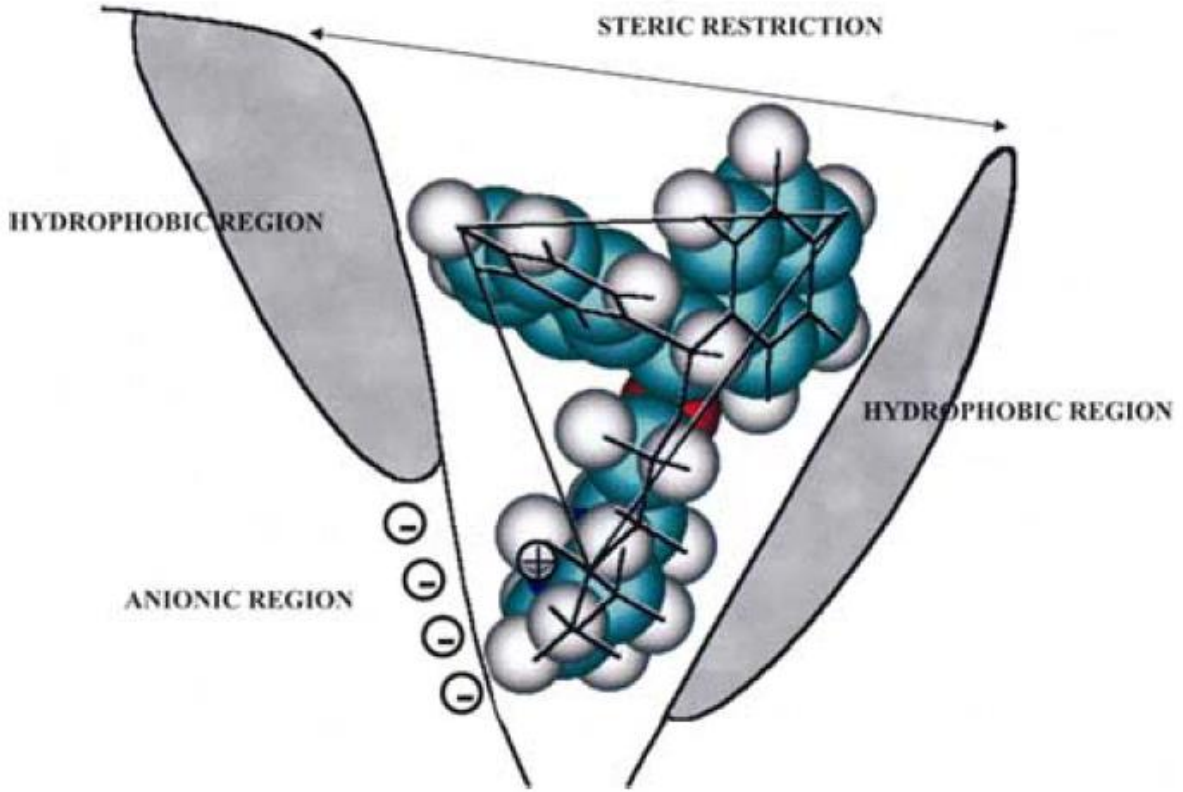
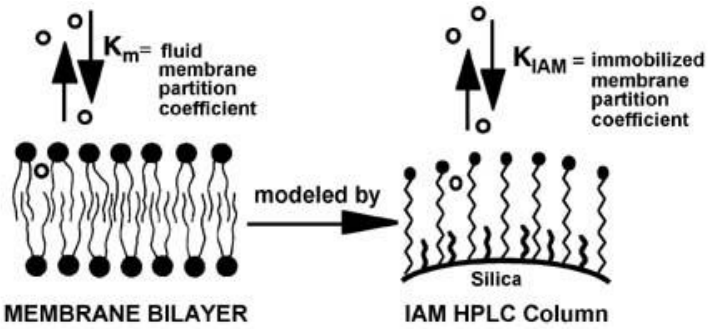


Fig. (8). Hypothetical mode of binding of basic drugs by AGP [400].

A. Nasal, D. Siluk, R. Kaliszan: Chromatographic Retention Parameters in Medicinal Chemistry and Molecular Pharmacology; Curr. Med. Chem. 2003, 10 381-426

Chromatographic Hydrophobicity Index obtained on IAM

- 3 min (0-70% AcN) gradient was applied using Immobilised Artificial Membrane (IAM) column.
- Injecting the test mix the CHI values could be determined on the model compounds.
- Higher value of CHI IAM means stronger interaction with phospholipids.



K. Valko, C. M. Du, C. Bevan, D. Reynolds, M. H. Abraham,

J. Pharm. Sci., 89 (8) (2000)

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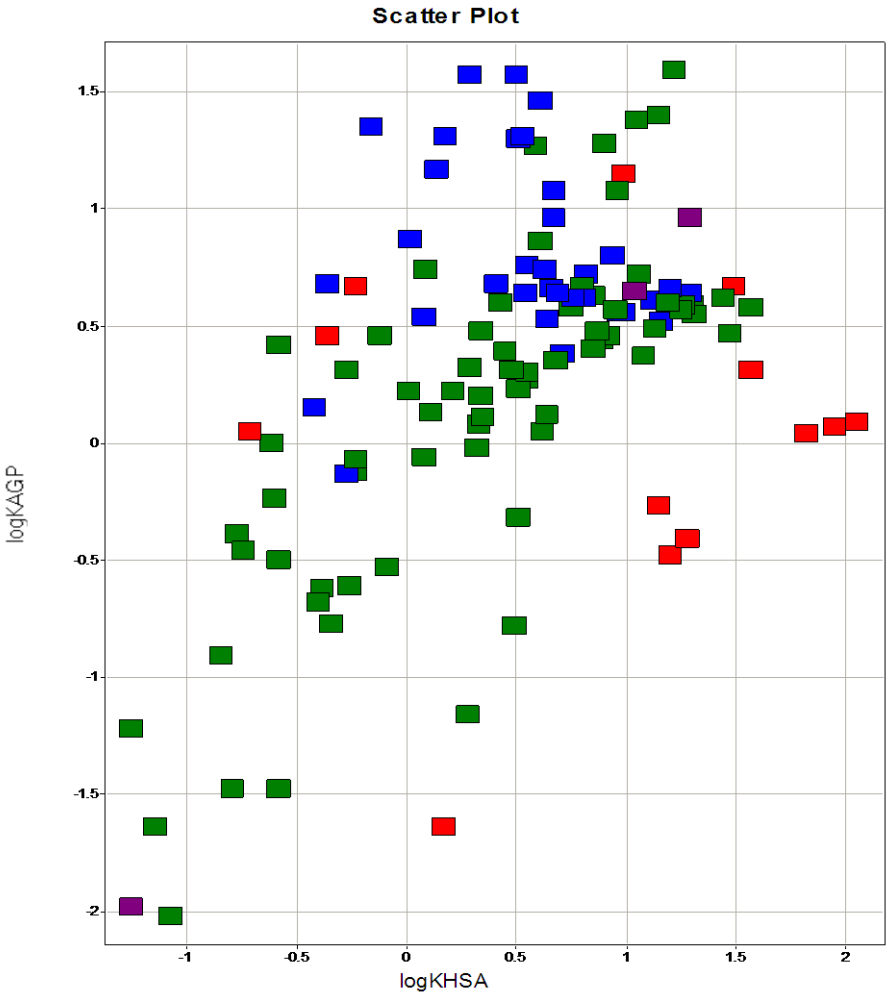
There is no correlation between HSA and AGP binding!



However, there is a general trend that HSA binds the negatively charged compound more strongly.

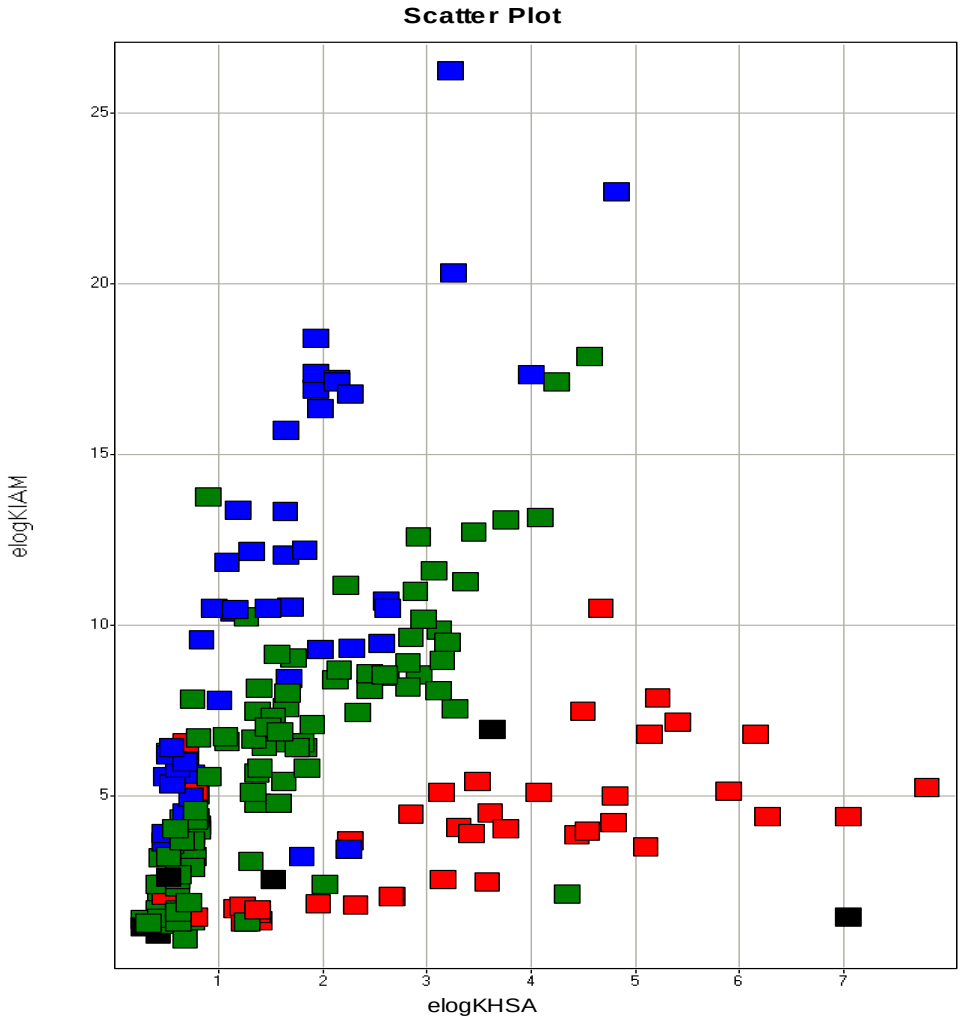
There are strong AGP binders among positively charged compounds.

Red: negatively charged

Green: neutral

Blue: positively charged

IAM binds positively charged compounds and HSA binds negatively charged compounds

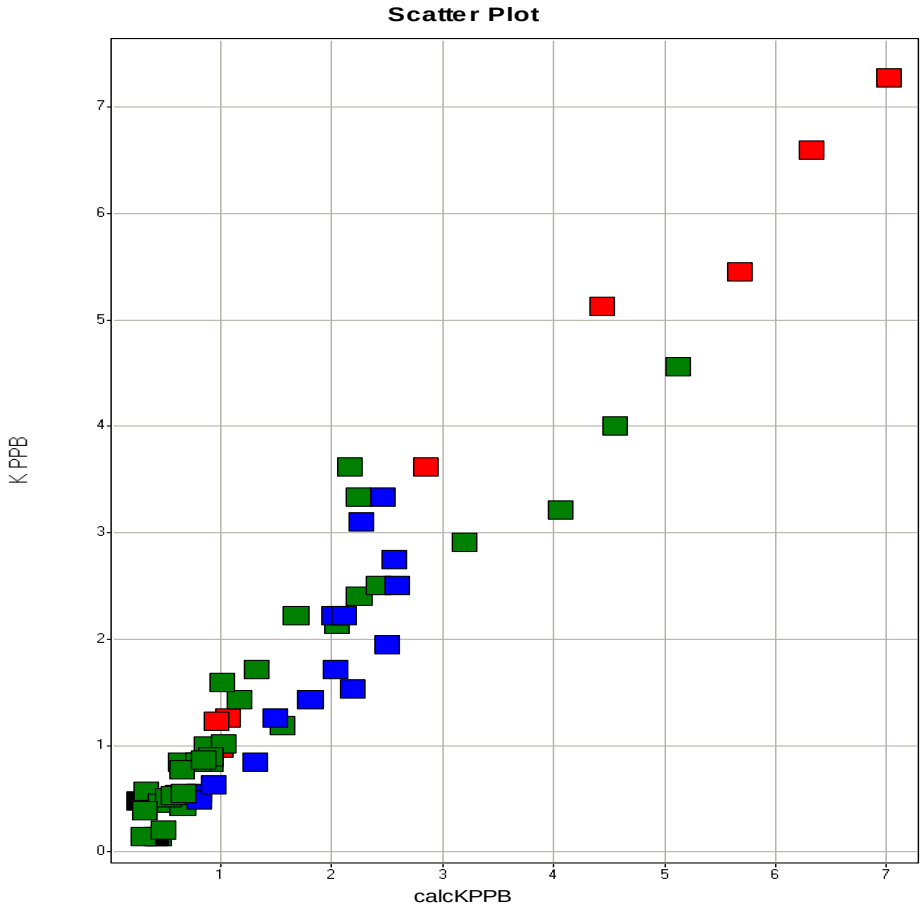


Our mechanistic model shows the importance of HSA and IAM binding in modelling volume of distribution.

Both HSA binding and membrane partition can be related to lipophilicity of the compounds.

However the presence of positive or negative charge makes a significant difference between the two types of binding.

Estimating total plasma binding from HSA and AGP binding measurements



Red: acids
Blue:bases
Green: neutrals
Black: zwitterions

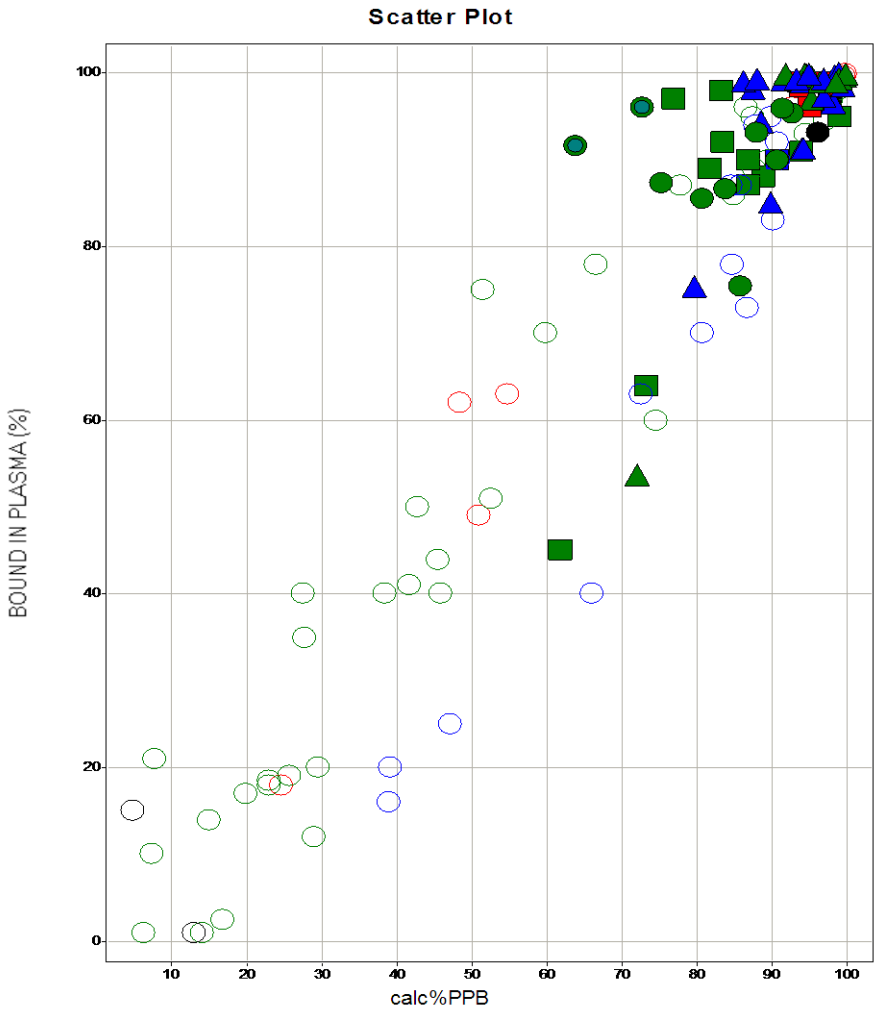
Log K PPB=0.87*logK HSA +0.17*logK AGP +0.063*cMR -0.273

n=55 r=0.92 s=0.36 F=92

Comparison of the plasma protein binding data obtained by the HPLC based method with the traditional ultra-dialysis method

Dialysis

% PPB



Red: acids

Blue:bases

Green: neutrals

Black: zwitterionic

Empty circle: training set

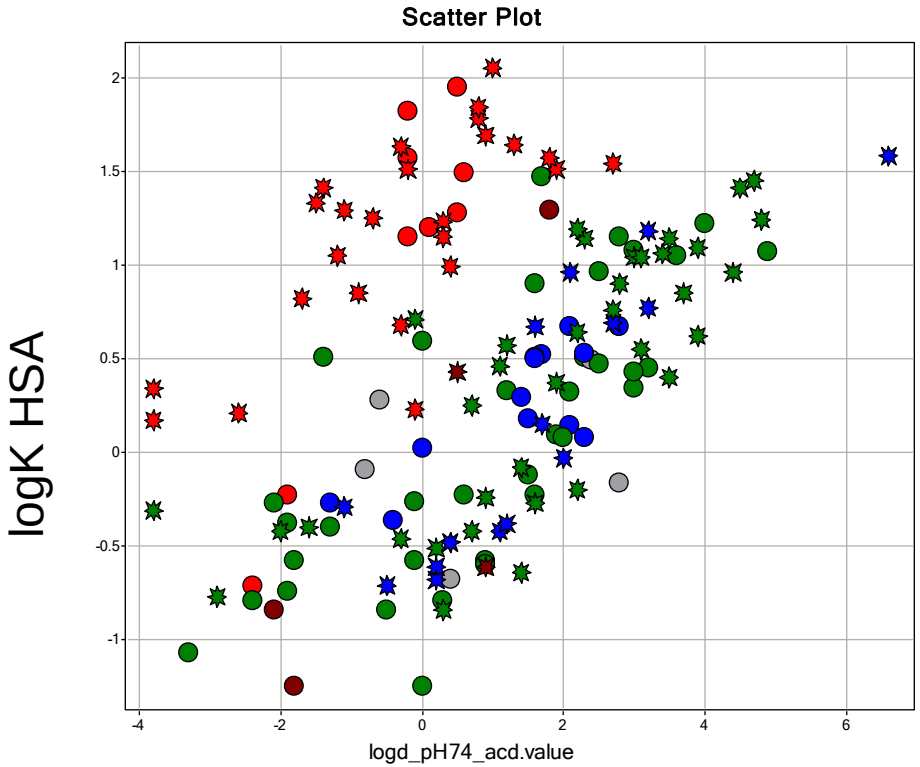
Square: drug test set

Triangle: test set (measured by Sara Coggon)

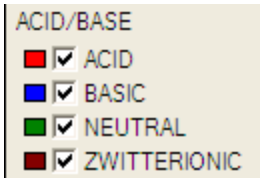
Full circle: CCR3 compounds

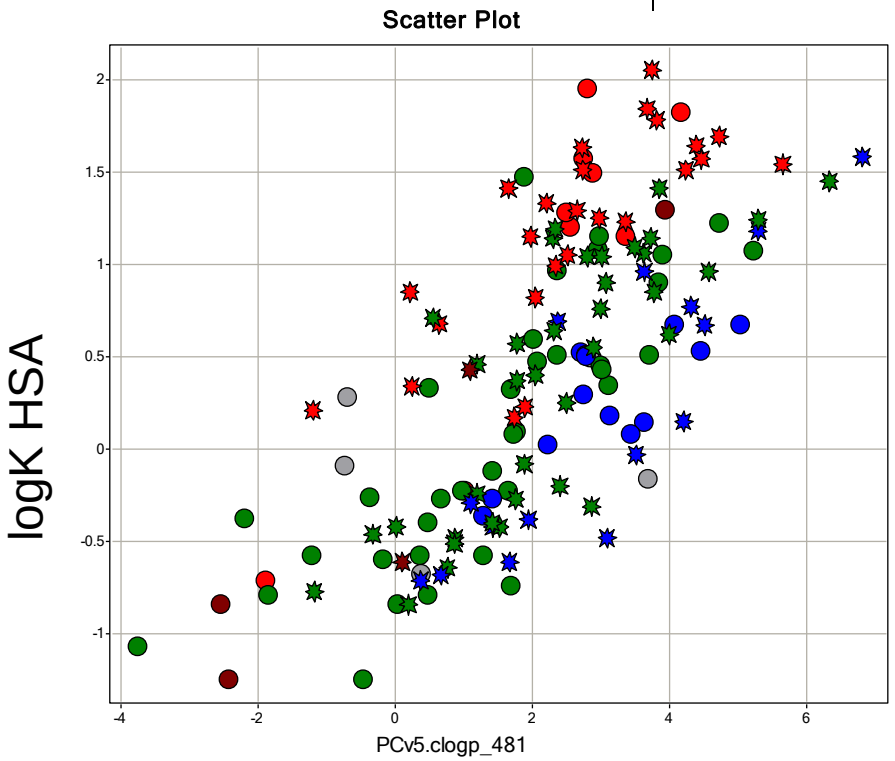
HPLC

Are we just measuring another lipophilicity by HSA binding?



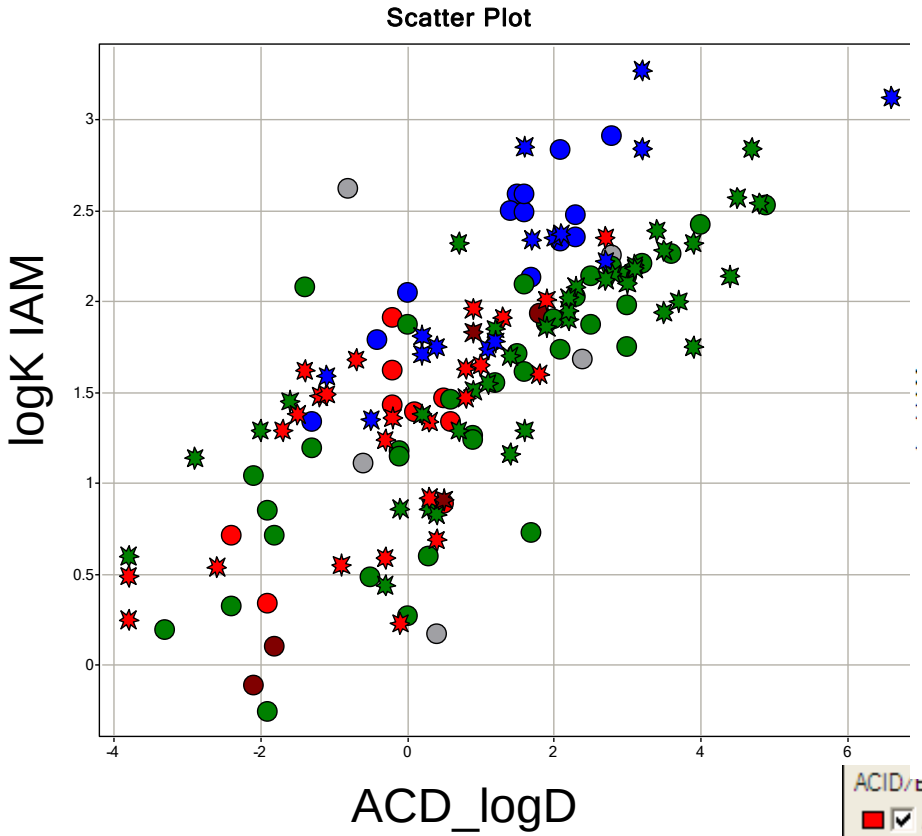
ACD_logD

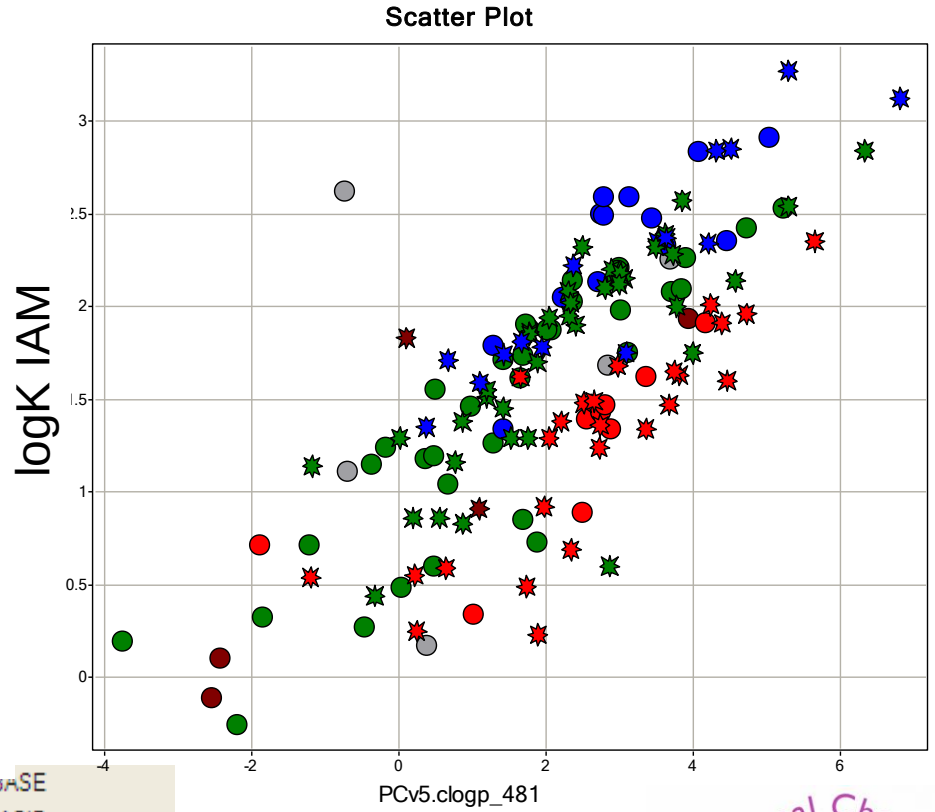




clogP

Are we just measuring another lipophilicity by IAM binding?





Klara Valko
Rovinj 2009

How can we use the HPLC based HSA binding data for estimating in vivo distribution of compounds?

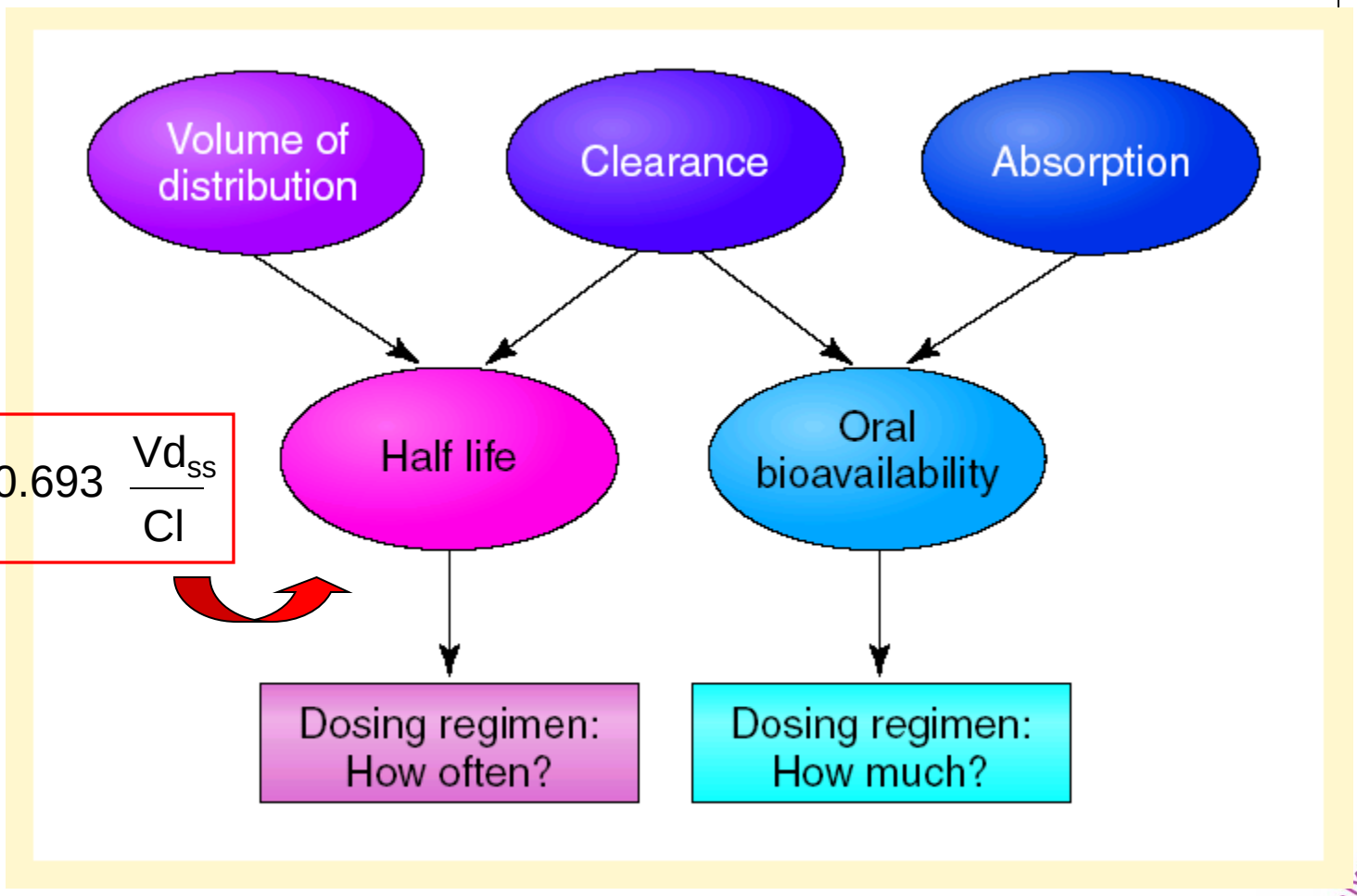
Modelling Volume of Distribution

- Volume of distribution (Vd) is a theoretical concept that relates the administered dose with the steady state concentration present in the circulatory system (C).

$$Vd_{ss} = \frac{Dose}{C} \quad [\text{ litre; or litre/kg }]$$

Vd_{ss} = volume of distribution at steady-state

Why VD is important?



$$t_{1/2} = 0.693 \frac{Vd_{ss}}{Cl}$$

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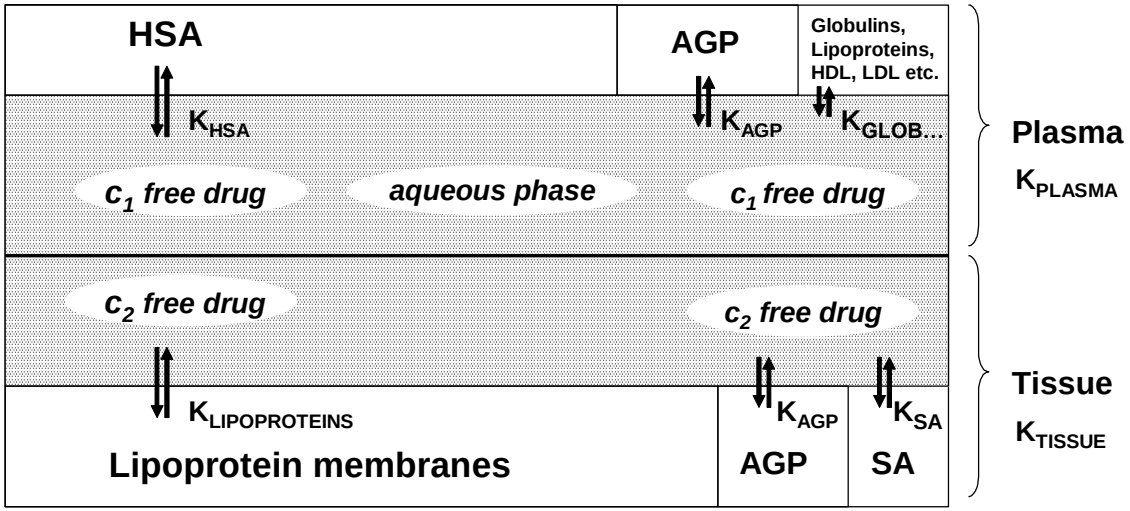
Modelling volume of distribution with plasma protein binding and membrane partition data

K (plasma proteins/free)

K (HSA) + K(AGP) + K(globulins)



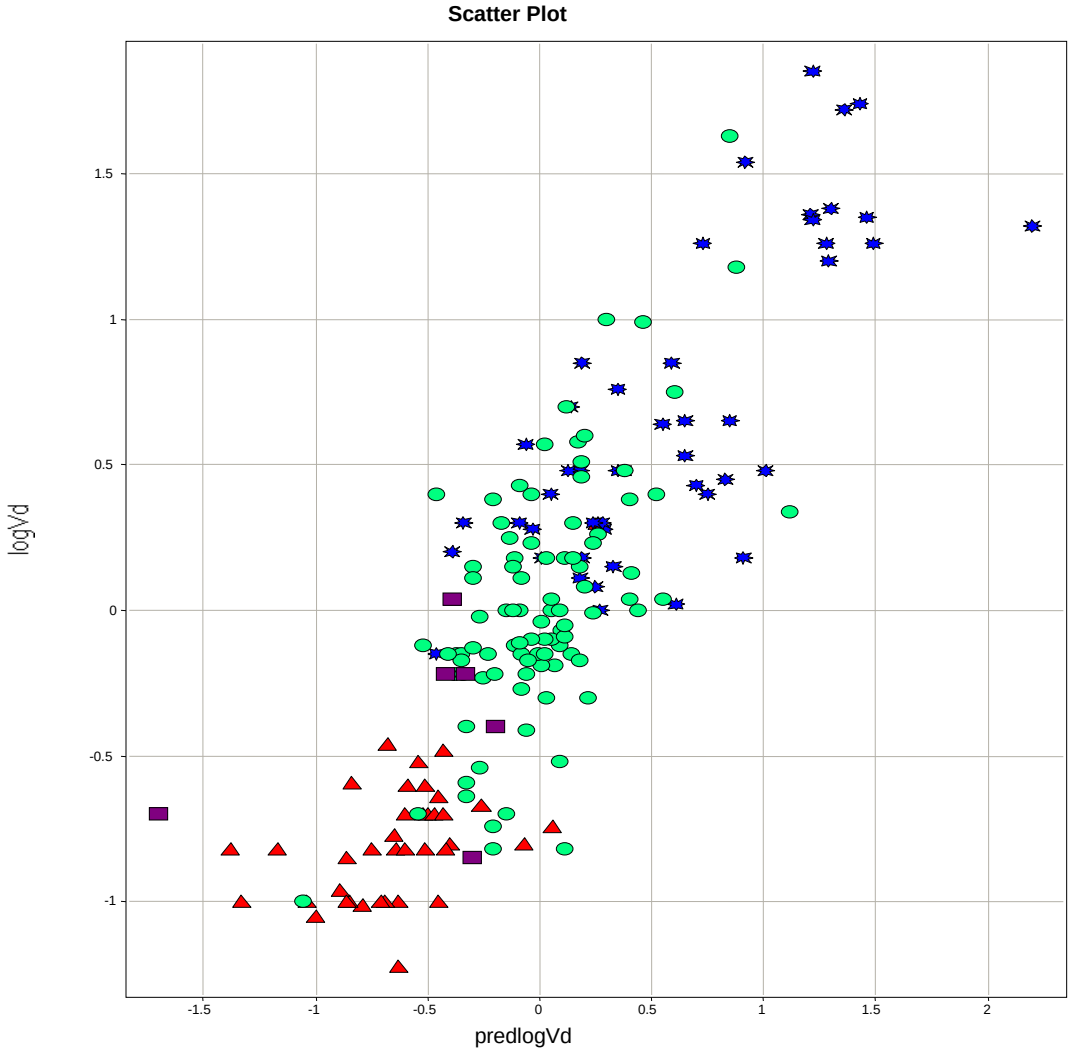
Free drug concentration in equilibrium $c_1 = c_2$

. (IAM)

K (tissue /free)

Vd_{ss}= K(plasma/free)/K(tissue/free)

Model for Volume of distribution from HPLC data (HSA and IAM binding)



logVd= 0.131*logK(IAM) - 0.2672*logK(HSA) -0.305

n=130 r=0.88 s=0.31

Plot shows training and test set together!

F. Hollosy, K. Valko, A, Hersey, S. Nunhuck, G. Keri, C. Bevan, J. Med Chem. 2006, 49, 6958-6971

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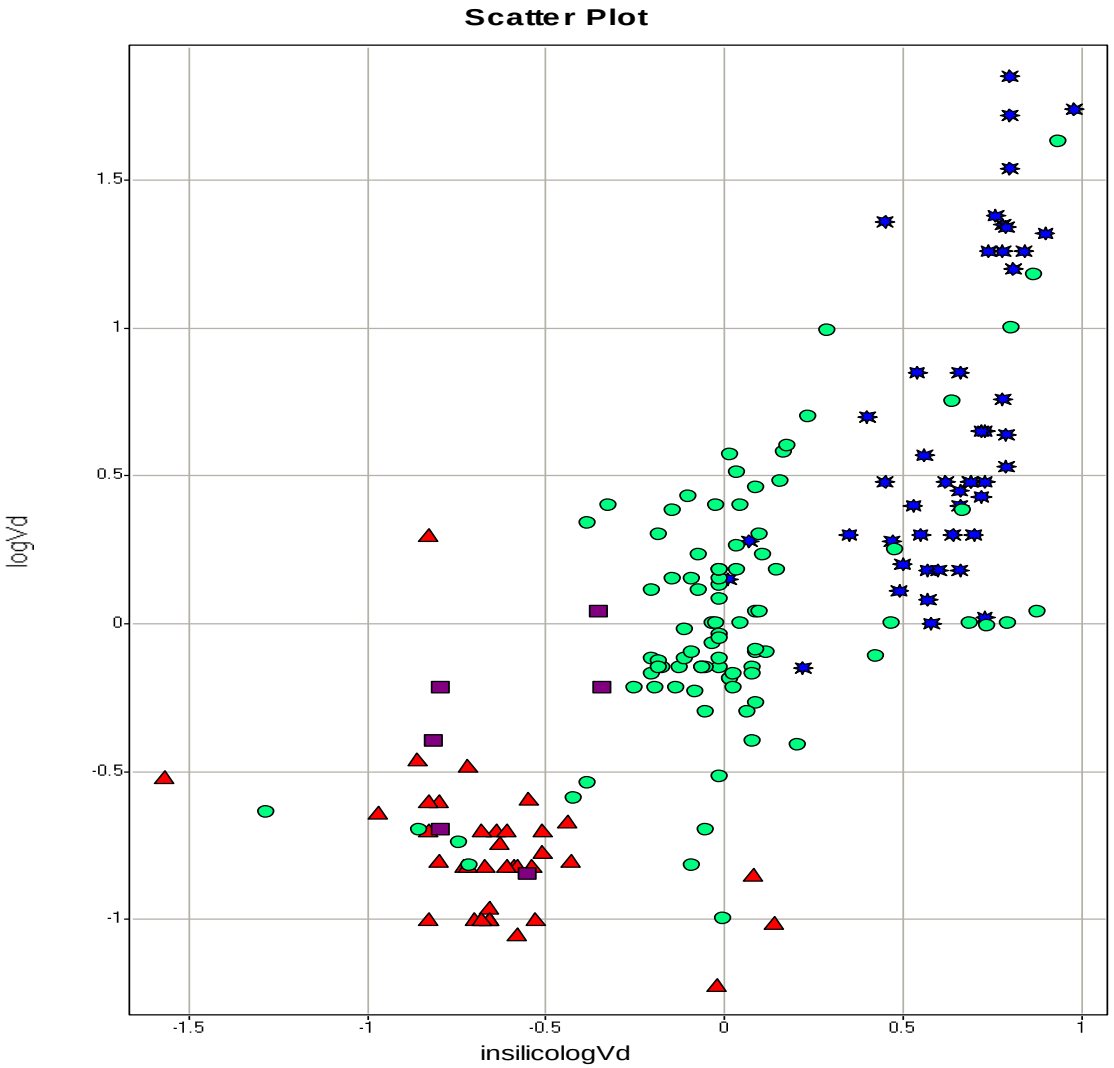
Model for Volume of distribution from in silico calculated data

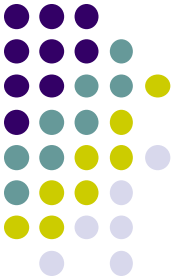


logVd= 0.0789clogP + 0.588*posChg - 0.731negChg- 0.147

n=118 r=0.80 s=0.41

The application of the HPLC based bio mimetic binding data can improve the prediction of in vivo compound distribution in comparison to purely in silico calculations.

Estimating unbound volume of distribution (Vdu) from HSA and IAM binding

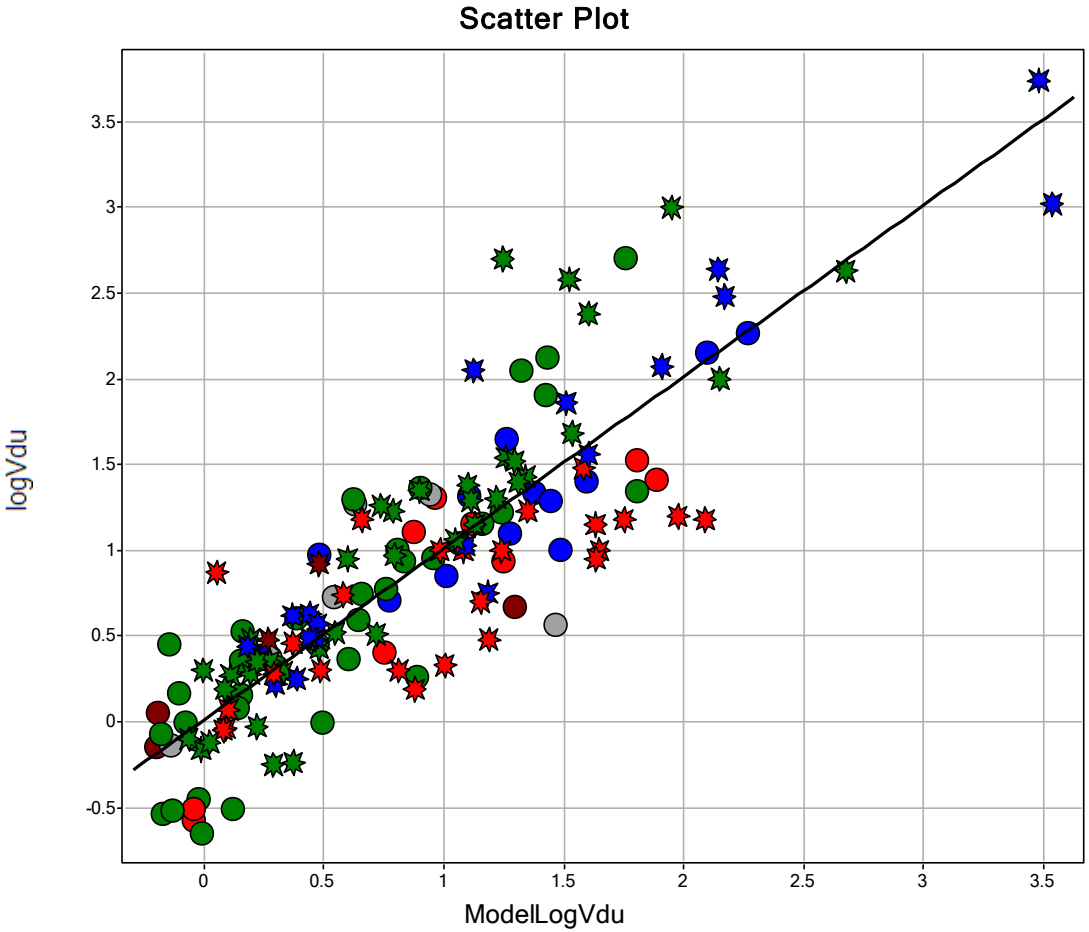


Vdu = Dose/ free plasma concentration

Estimating the proportion of the dose and free plasma concentration without in vivo measurements has a great impact on the lead optimisation. It enables us to select candidates that requires the lowest possible dose for the highest possible free plasma concentration and consequently the highest receptor occupancy.

Candidates with low Vdu have the highest probability for showing efficacy with low dose and without side effects in clinic.

Estimated and measured human clinical Vdu for 150 known drug molecules



Circle: training set
Star: test set

Green: not charged
Blue: positively charged
Red: negatively charged

logVdu = 0.24*logKHSA +0.12*logKIAM -0.4

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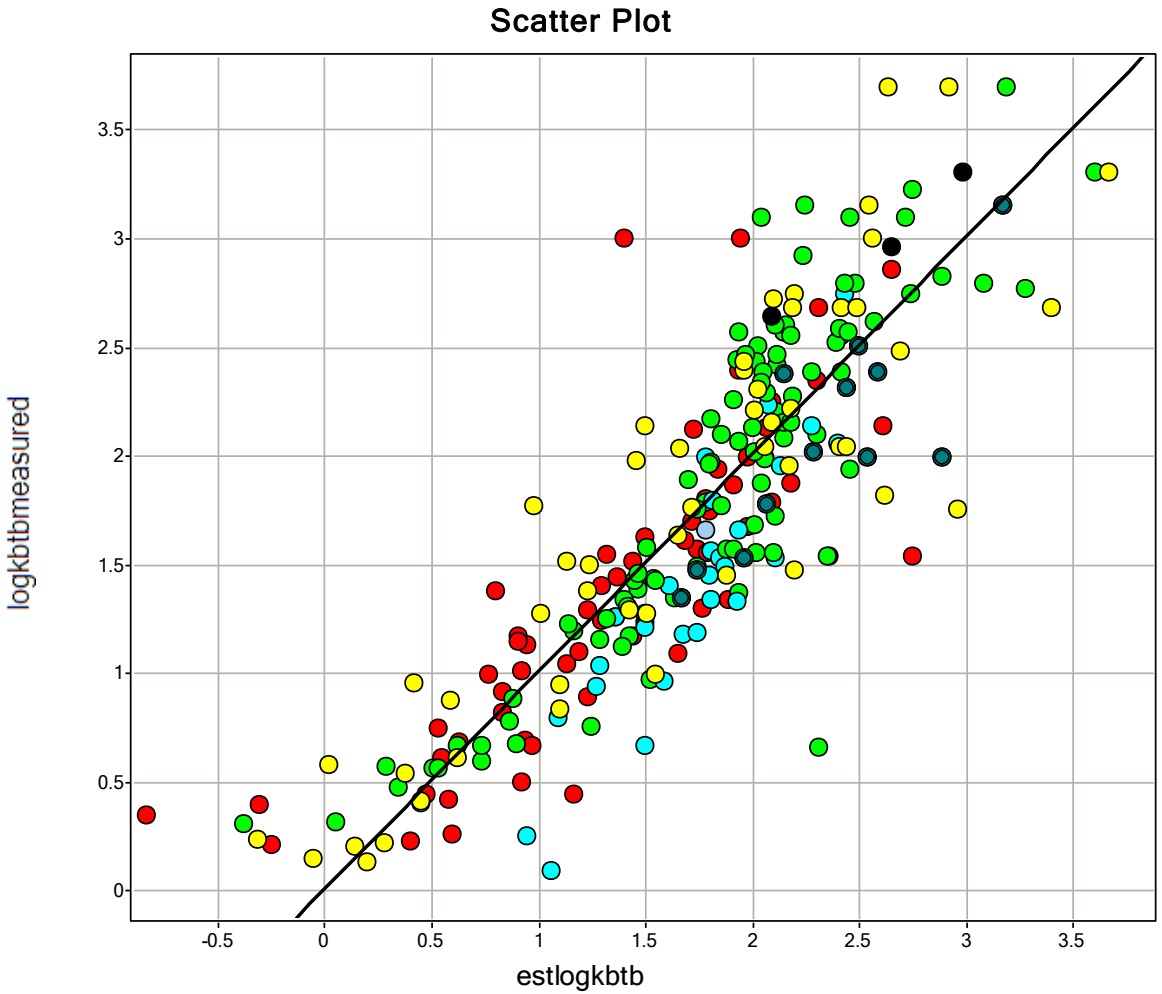
Physchem Forum 8 2010

Model for brain tissue binding (BTB, log k BTB)



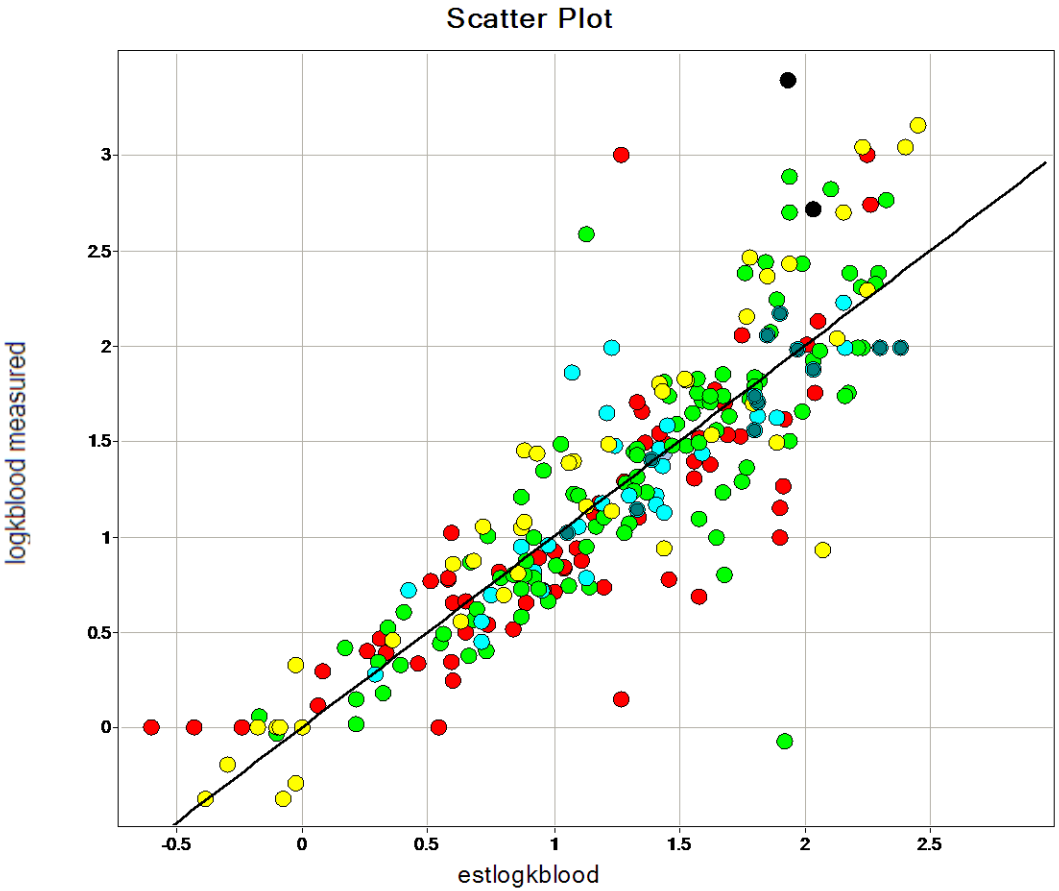
Different colours are from different projects. Red compounds showed strong specific AGP binding.

Plot shows test and training sets together. Line obtained only from the training set of compounds.

logkBTB=1.29(+/-0.10)*logk(IAM)+1.03(+/-0.10)*logk(HSA) – 2.37

n=135 r^2=0.76 s=0.35 F=212

Model for fraction bound in blood (log k blood)



Different colours are from different projects. Red compounds showed strong specific AGP binding.

Plot shows test and training sets together. Line obtained only from the training set of compounds.

logkblood=0.14*CHIlogD7.4+0.46*logK(HSA) +0.43*logkAGP –0.72

n=133 r^2=0.79 s=0.29 F=164

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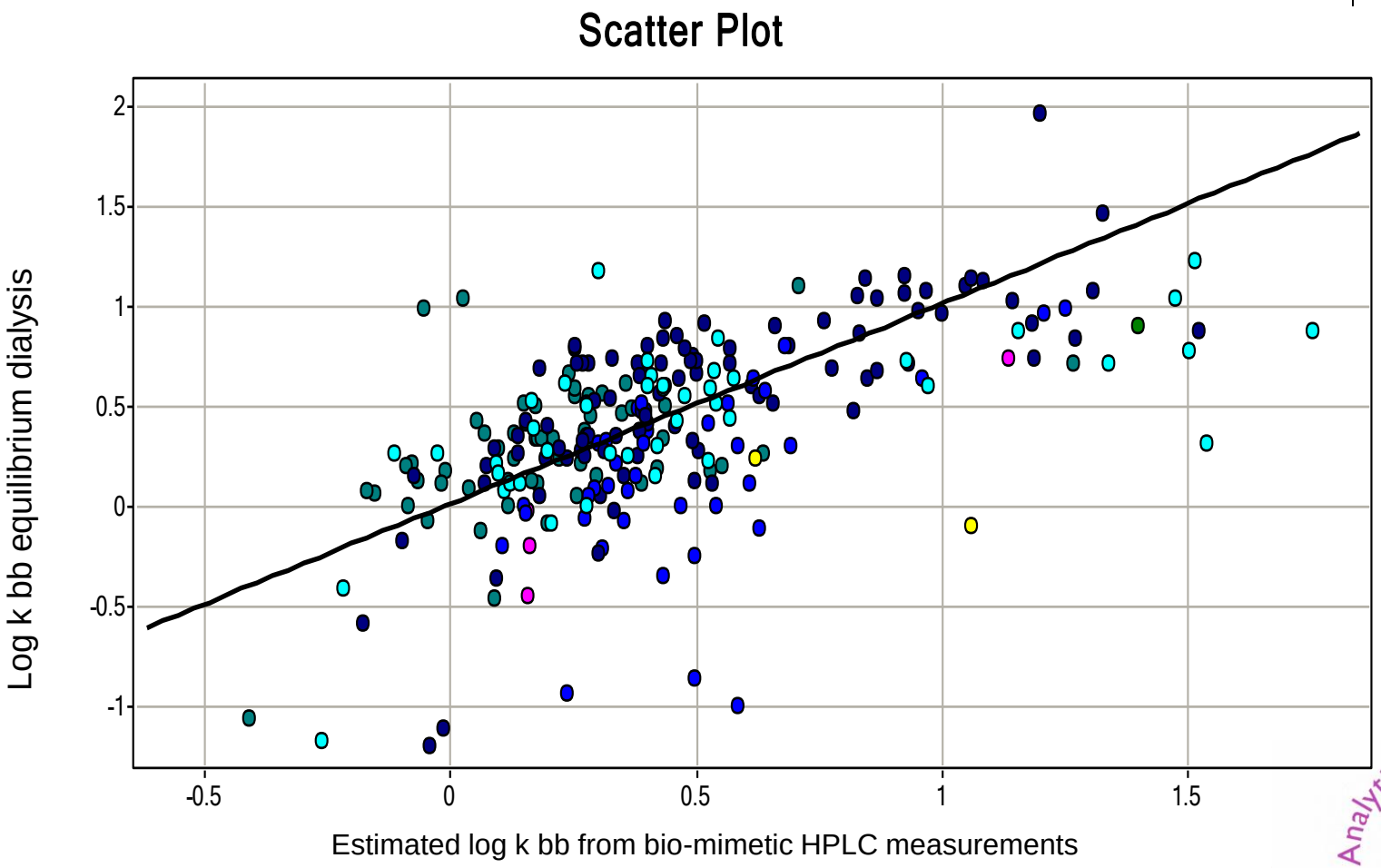
Comparison of brain to blood ratio (k_{bb}) estimated from HPLC based measurements and from in vitro binding measurements



$k_{bb} = fu_{blood}/fu_{brain}$

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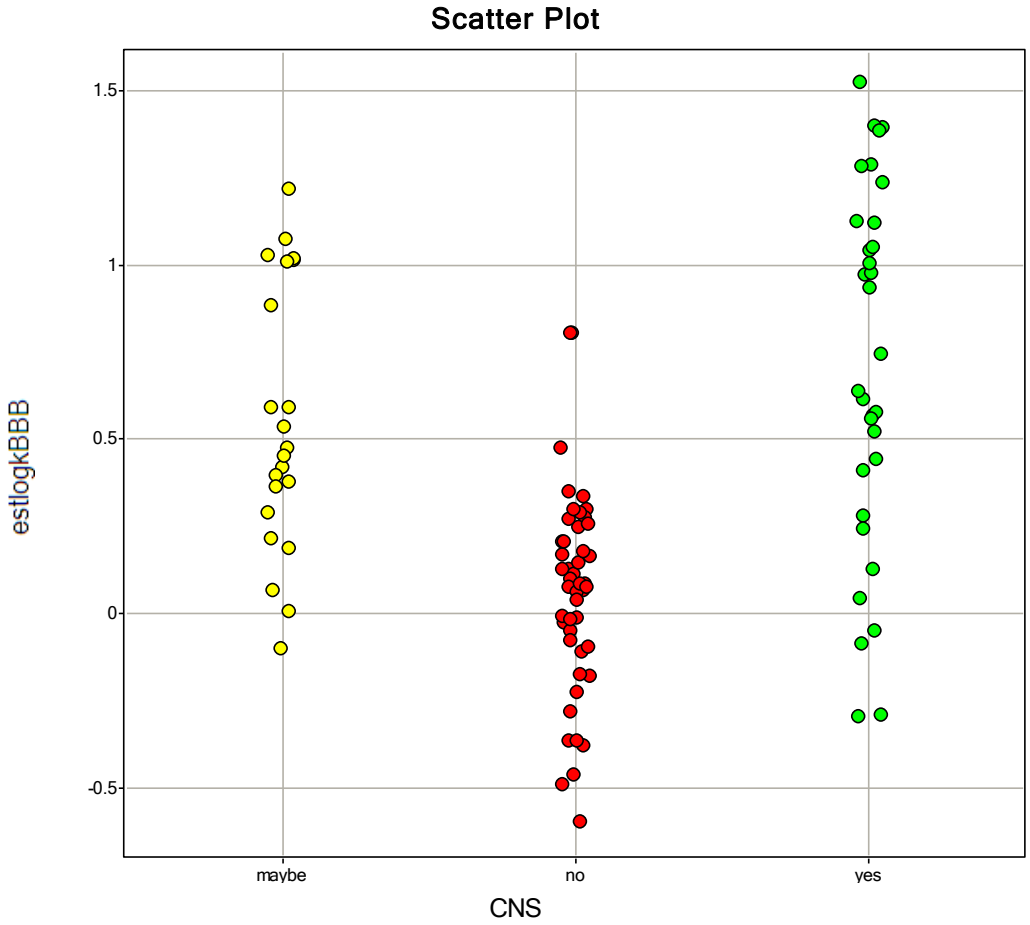
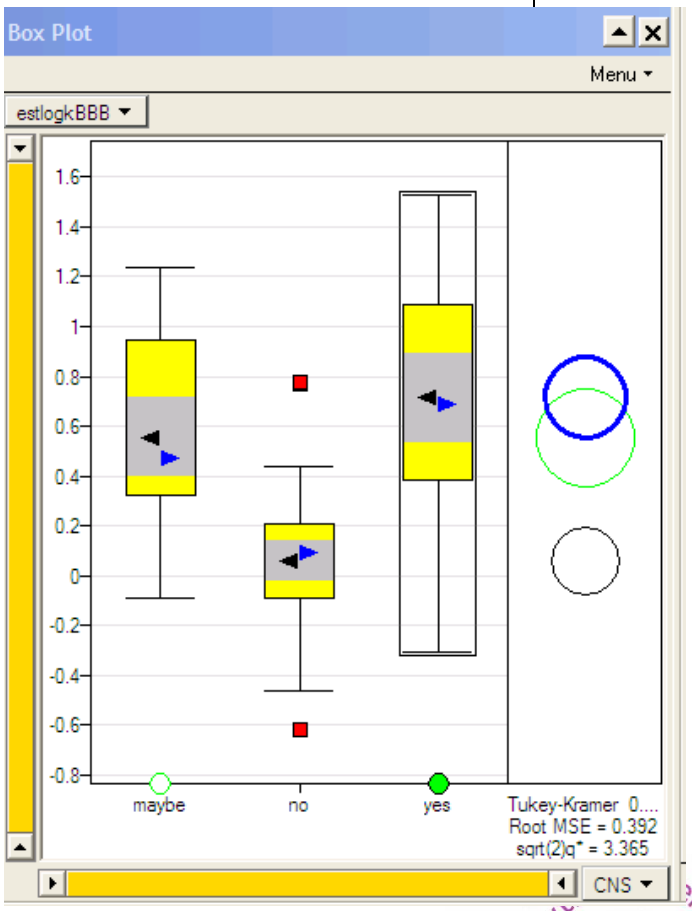
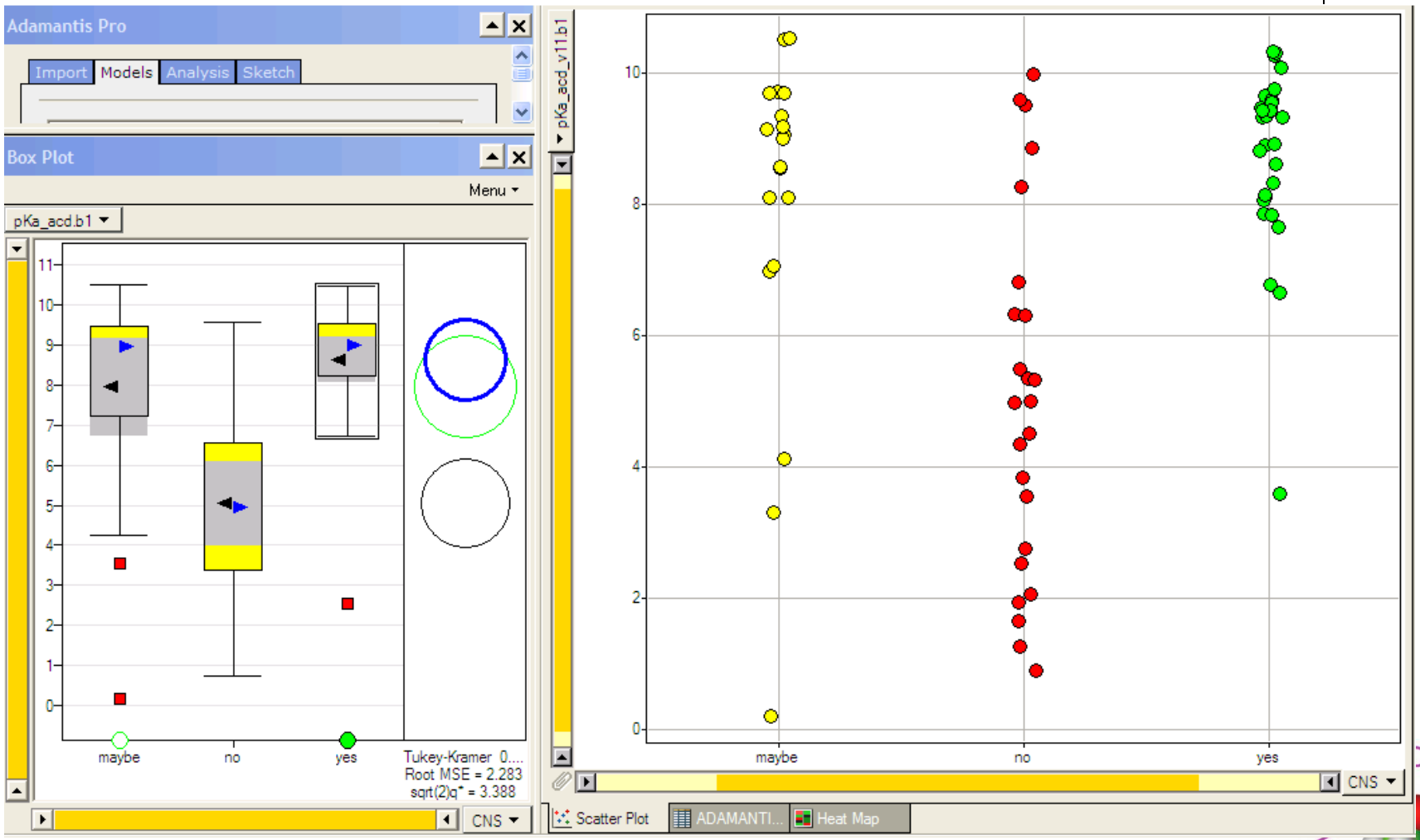
Estimates of log k bb shows good discrimination to the CNS penetrant drugs



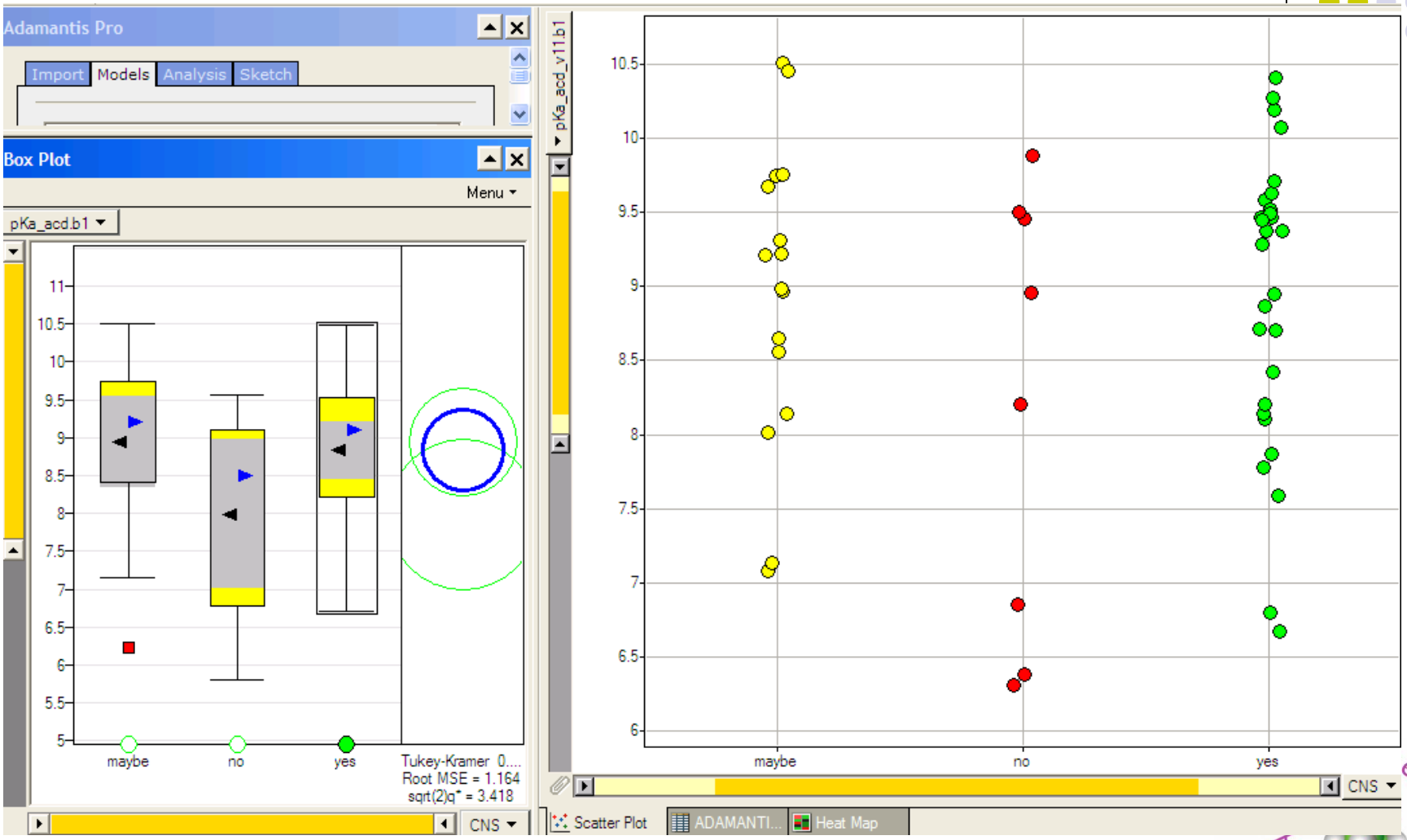


Green (yes): CNS targeted drugs; Yellow (maybe): none CNS targeted but CNS side effects; red (no): no indication for CNS penetration

Calculated basic pKa also discriminates between the CNS and non-CNS drugs

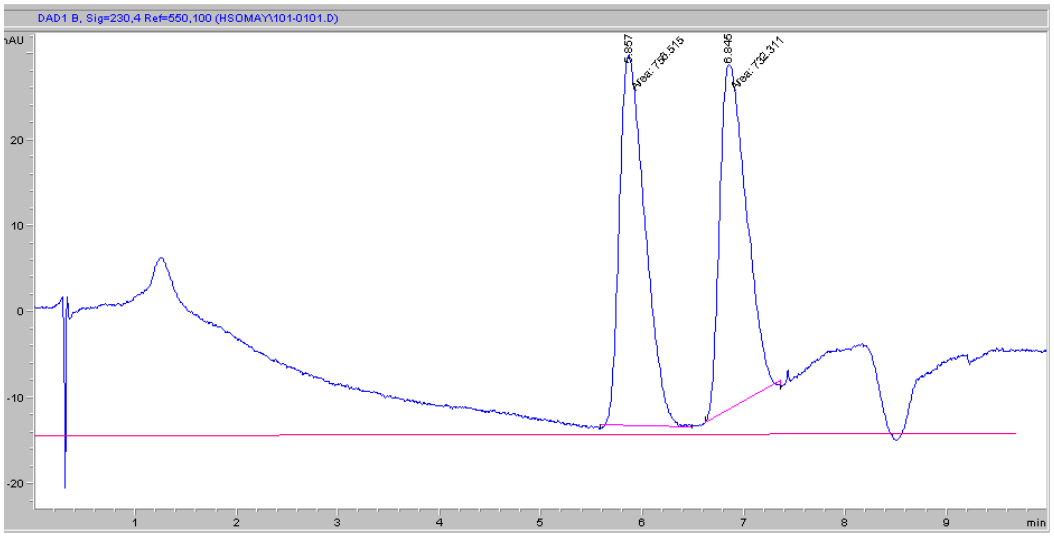


However, considering the basic pKa only above 6, no discrimination of CNS activity



Chiral separation occurs frequently during the HSA and AGP binding measurements of racemic mixtures

We can report different % binding data for the two optical isomers. Consequently, the total plasma protein binding, volume of distribution, free drug concentration, brain tissue binding, etc will be also different for the two enantiomers.



Separation of the R and S Warfarin

The bio-mimetic HPLC based binding data can be used to estimate the different PK/PD behaviour of enantiomers.

Physchem Forum 2010
Klara Valko

Analytical Chemistry
MOLECULAR DISCOVERY RESEARCH
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Conclusions

- HPLC method offers binding measurements to individual proteins.

- The HPLC based method can reproducibly differentiate between very strong binders and between some enantiomeric compounds.

- Structure -binding relationships can be set up for very high binders to help modifying structure to reduce binding. The structure – binding relationships for HSA and AGP are very different.

- The individual protein binding data can be used to simulate physiological (pathological) variation of total plasma protein binding.

- A model has been established and validated that allows estimation of the total plasma protein binding from HSA and AGP binding.

- The protein binding data together with the membrane partition data (K IAM) can be used to predict the in vivo volume of distribution (free tissue and plasma concentration) of compounds.

- The bio-mimetic protein and phospholipid binding data can be used for estimating brain tissue binding, and CNS penetration of the compounds too.

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UP Group

Box 1. Theoretical basis for the 'Free Drug Principle'.

- (a) Drug binding to target and albumin is rapid and reversible
- (b) All drug-binding components are at steady-state equilibrium
- (c)

$$[albumin]_{total} \gg [drug]_{total} \gg [target]_{total}$$

- (d)

$$K_{target} = \frac{[target]_{free}[drug]_{free}}{[target \bullet drug]}$$

- (e)

$$K_{albumin} = \frac{[albumin]_{free}[drug]_{free}}{[albumin \bullet drug]}$$

- (f)

$$[drug]_{total} = [drug]_{free} + [drug \bullet albumin] + \cancel{[drug \bullet target]}$$

- (g)

$$[albumin]_{total} = [albumin]_{free} + \cancel{[drug \bullet albumin]}$$

- (h)

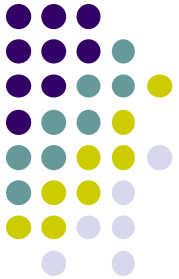
$$[drug]_{free} = \frac{K_{albumin}[drug]_{total}}{K_{albumin} + [albumin]_{total}}$$

- (i)

$$Free\ fraction\ (fu) = \frac{K_{albumin}}{K_{albumin} + [albumin]_{total}}$$

- (j)

$$\frac{[target \bullet drug]}{[target]_{free}} = \frac{[drug]_{free}}{K_{target}} = \frac{fu\ [drug]_{total}}{K_{target}}$$



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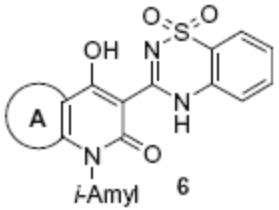
Synthesis and biological activity of heteroaryl 3-(1,1-dioxo-2*H*-(1,2,4)-benzothiadizin-3-yl)-4-hydroxy-2(1*H*)-quinolinone derivatives as hepatitis C virus NS5B polymerase inhibitors

Rosanna Tedesco [a,*], Deping Chai [a], Michael G. Darcy [a], Dashyant Dhanak [a], Duke M. Fitch [a], Adam Gates [b], Victor K. Johnston [b], Richard M. Keenan [a], Juili Lin-Goerke [b], Robert T. Sarisky [b], Antony N. Shaw [a], Klara L. Valko [c], Kenneth J. Wiggall [a], Michael N. Zimmerman [a], Kevin J. Duffy [a]

[a] *Department of Medicinal Chemistry, GlaxoSmithKline Pharmaceuticals, Collegeville, PA 19426, USA*
[b] *Department of Virology, GlaxoSmithKline Pharmaceuticals, Collegeville, PA 19426, USA*
[c] *Department of Analytical Sciences, GlaxoSmithKline Pharmaceuticals, Stevenage, Herts, SG1 2NY, UK*

Table 1
Heterocyclic pyrimidinediones



6

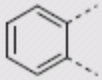
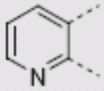
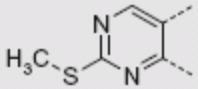
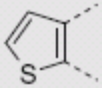
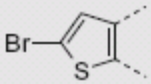
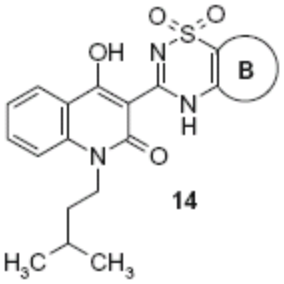
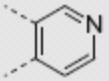
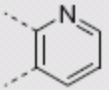
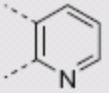
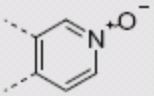
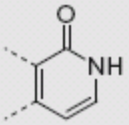
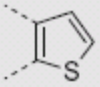
Compd	A	RdRp[a] IC_{50} (nM)	Replicon[a] IC_{50} (nM)	HSA (% bound)
6a		32	417	>99.9
6b		212	1359	>99.9
6c		>30,000	–	–
6d		48	942	>99.9
6e		132	1164	>99.9
6f		1168	–	>99.9

Table 2
Heterocyclic thiadiazines



14

Compd	B	RdRp IC_{50} (nM)	Replicon IC_{50} (nM)	HSA (% bound)
14a		20	226	99.0
14b		31	743	98.7
14c		51	1681	–
14d		18	2377	97.7
14e		16	13,679	–
14f		20	2887	99.5