

Fast Biomimetic High-Performance Liquid Chromatography Method for Evaluating Human Plasma Protein Binding

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Abstract

The unbound fraction (fu) of drugs in plasma plays an essential role in elucidating pharmacokinetic behaviors. Traditional plasma protein binding (PPB) studies primarily determine fu using plasma-based experiments, such as equilibrium dialysis, ultracentrifugation, or ultrafiltration. However, these methods face challenges in accurately determining fu for highly lipophilic compounds, those with significant non-specific binding, or compounds that are unstable in plasma. To address these issues, this study developed a biomimetic high-performance liquid chromatography (HPLC) method to evaluate human plasma protein binding. This method employs a stationary phase containing human serum albumin^[1] (HSA) to mimic the biological environment of drug-protein binding. The chromatographic retention behavior of compounds on the biomimetic stationary phase reveals their affinity for albumin. This innovative approach offers a rapid and high-throughput strategy for assessing plasma protein binding of small molecules and peptides, which holds significant implications for efficacy and safety evaluations in drug discovery.

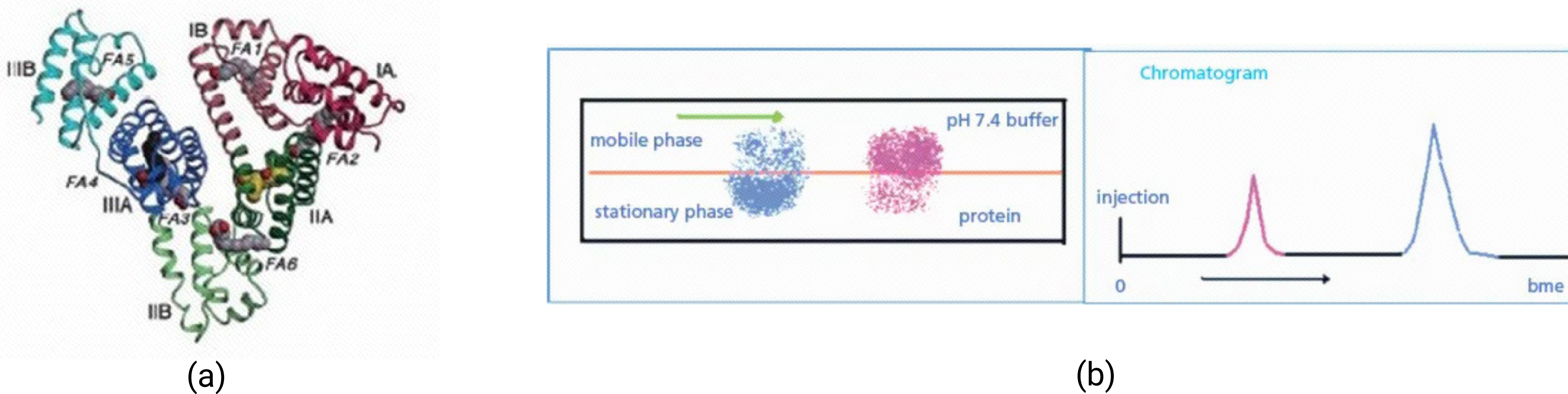


Figure 1: (a) The structure of human serum albumin. (b) The principles of using HPLC retention on a HSA column to measure protein binding^[1]: the differential migration of compounds in a chromatographic system occurs because they travel at different speeds due to interactions between the mobile and stationary phases

Method

Experiments

Compounds were prepared at 2 μM concentration in 50 mM ammonium acetate solution and 2-propanol solution mixture (v : v, 50 : 50), mixed well and then analyzed using LC-MS/MS with an HSA column, the gradient retention times (t_R) of the analytes were detected.

LC-MS/MS Method

Table 1. LC condition and MS parameter	
LC System	Shimadzu LC-40D X3
Mass Spectrometer	SCIEX API 4000
Electrospray ionization	ESI
Column	CHIRAL PAK HSA 50 × 4 mm (Daicel Inc.)
Mobile Phase A	50 mM NH ₄ OAc in Water (pH 7.4)
Mobile Phase B	2-Propanol
MS Scan Mode	MRM
Run Time	15 min

Data Processing

- The literature %PPB (%bound in human plasma) values of 10 calibration compounds were converted to the linear free-energy-related log K values using Equation 1^[2].
- A linear regression equation was established using linearized %PPB (log K) as the y-axis and log t_R as the x-axis (log K = a log t_R + b).
- The slope and intercept of the calibration curve were then used to convert the retention times of the tested 36 compounds into log K values.
- These log K values were applied to Equation 2^[2] to obtain HSA %binding values, which subsequently used to estimate the overall human plasma protein binding for the tested compound.

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

Equation.2 $\text{HSA \%binding} = \left[\frac{101 \cdot 10^{\log K}}{1 + 10^{\log K}}\right]$

Conclusion

- Biomimetic chromatography method can effectively evaluate plasma protein binding by testing the %unbound values of 36 compounds, 81% of the compounds were within a two-fold range of the values from ultracentrifugation, which offers complementary approach for determining the plasma protein binding.
- No plasma and no incubation are required for biomimetic chromatography, significantly reducing costs and turnaround time.
- Throughput for biomimetic chromatography in this study was approximately 4 times faster than that of equilibrium dialysis.

References

[1]. Valkó K. Biomimetic Chromatography to Accelerate Drug Discovery: Part I. LCGC North America.2018, 36(6), 397-405.
[2]. Valkó K, Nunhuck SB, Hill AP. Estimating unbound volume of distribution and tissue binding by *in vitro* HPLC-based human serum albumin and immobilized artificial membrane-binding measurements. Journal of Pharmaceutical Sciences. 2011, 100(3), 849-862.
[3]. Lombardo F, Berellini G, Obach RS. Trend Analysis of a Database of Intravenous Pharmacokinetic Parameters in Humans for 1352 Drug Compounds. Drug Metab Dispos. 2018, 46(11), 1466-1477.
[4]. Valkó K, Nunhuck S, Bevan C, Abraham MH, Reynolds DP. Fast gradient HPLC method to determine compounds binding to human serum albumin. Relationships with octanol/water and immobilized artificial membrane lipophilicity. Journal of Pharmaceutical Sciences. 2003, 92(11), 2236-2248.

Result

Chromatogram

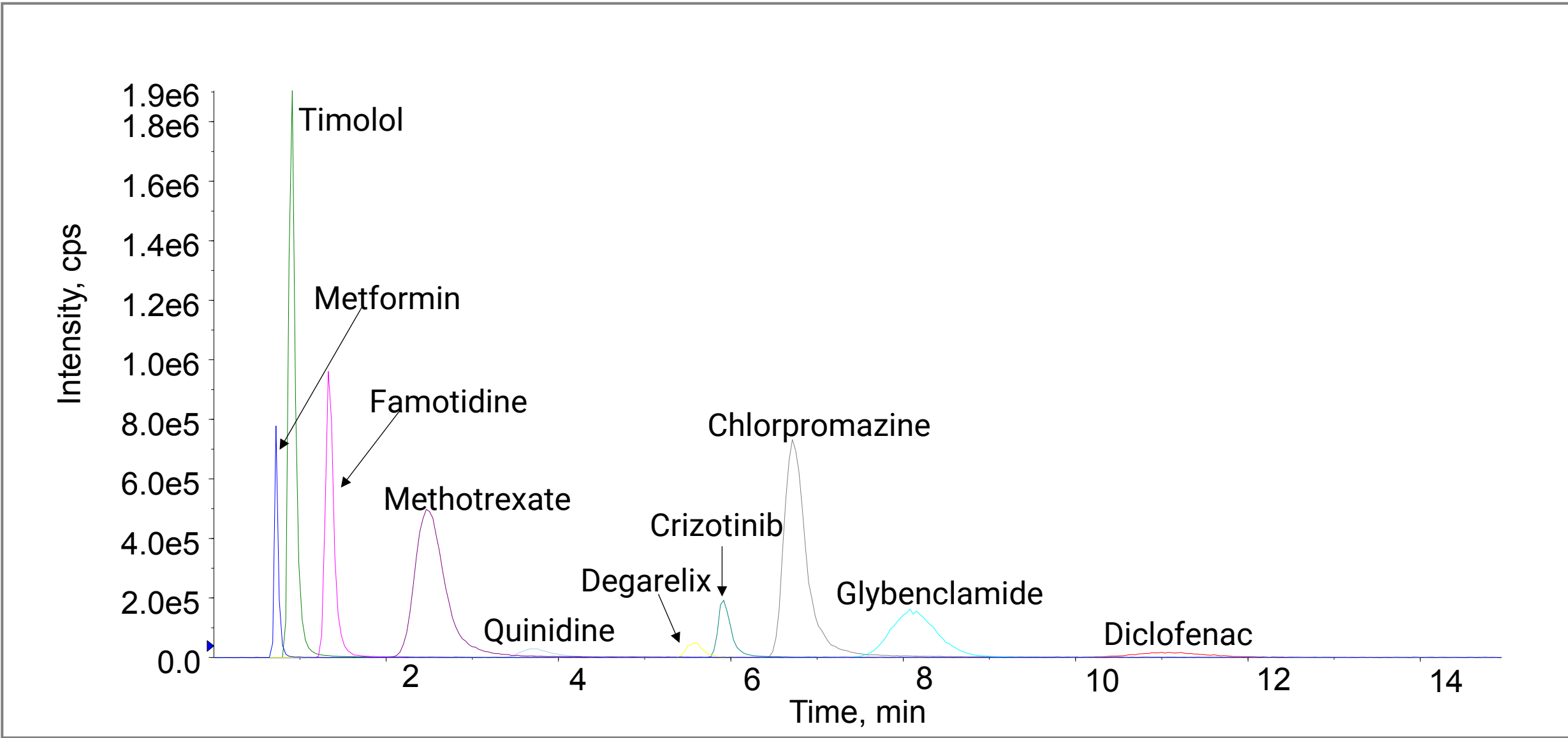


Figure 2: Chromatogram of 10 calibration compounds

Calibration Curve

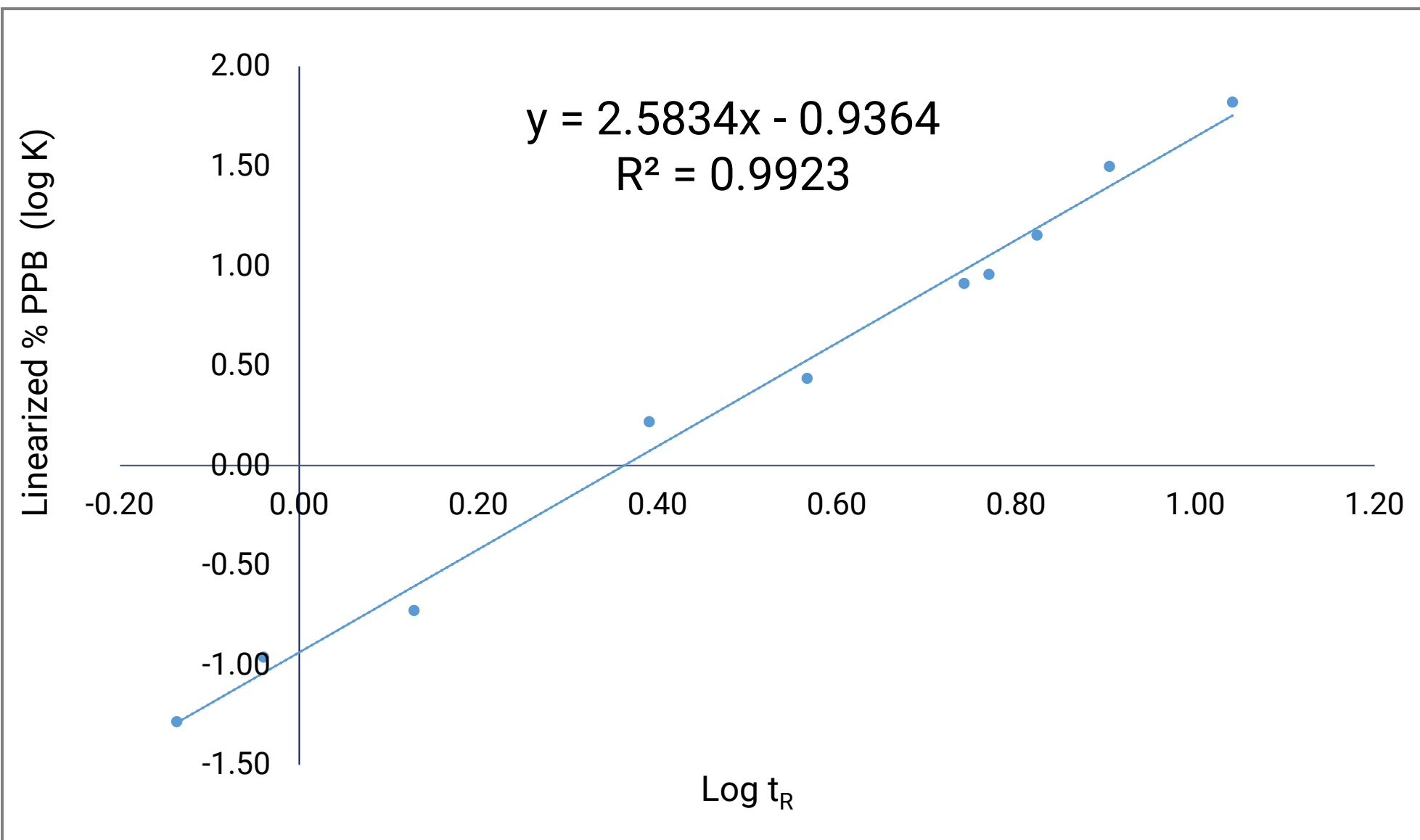


Figure 3: Calibration curve for log_{t_R} and linearized% PPB (log K) of 10 calibration compounds

Results of 36 Compounds

Compound	t _R (min)	Log t _R	Log K	%HSA Measured	%Unbound -HSA Measured	%Unbound-Literature ^[2-4]	Compound	t _R (min)	Log t _R	Log K	%HSA Measured	%Unbound-HSA Measured	%Unbound-Literature ^[2-4]
Antipyrine	0.73	-0.14	-1.29	4.9	95.1	93.0	Cyclo-sporine	5.33	0.73	0.94	90.6	9.4	6.8
Metformin	0.73	-0.14	-1.29	4.9	95.1	95.0	Imipramine ^a	5.47	0.74	0.97	91.2	8.8	0.5
Timolol	0.91	-0.04	-1.04	8.4	91.6	90.0	Degarelix	5.52	0.74	0.98	91.4	8.6	10.0
Aspirin	0.99	0.00	-0.95	10.2	89.8	51.0	Nicardipine	5.55	0.74	0.99	91.6	8.4	5.0
Eptifibatide	1.15	0.06	-0.78	14.4	85.6	75.0	Clozapine	5.60	0.75	1.00	91.8	8.2	5.5
Zonisamide	1.20	0.08	-0.73	15.8	84.2	60.0	Cetorelix	5.68	0.75	1.01	92.1	7.9	14.0
Famotidine	1.34	0.13	-0.61	20.0	80.0	84.0	Daptomycin	5.71	0.76	1.02	92.2	7.8	8.3
Pravastatin	1.64	0.21	-0.38	29.6	70.4	50.0	Itraconazole ^a	5.72	0.76	1.02	92.2	7.8	0.2
Dexamethasone	2.44	0.39	0.06	54.2	45.8	32.0	Crizotinib	5.88	0.77	1.05	92.8	7.2	9.0
Methotrexate	2.46	0.39	0.07	54.8	45.2	37.0	Nafarelin ^a	6.00	0.78	1.07	93.1	6.9	20.0
Paxalisib ^a	2.46	0.39	0.07	54.8	45.2	19.0	Probenecid	6.03	0.78	1.08	93.2	6.8	13.0
Carbamazepine	2.66	0.42	0.16	59.8	40.2	25.0	Tepotinib ^a	6.04	0.78	1.08	93.3	6.7	2.0
Labetalol	3.00	0.48	0.30	67.1	32.9	50.0	Imatinib	6.30	0.80	1.13	94.0	6.0	5.0
Propranolol	3.56	0.55	0.49	76.2	23.8	13.0	Chlorpromazine	6.66	0.82	1.19	94.9	5.1	5.6
Quinidine	3.69	0.57	0.53	77.9	22.1	26.0	Glybenclamide	8.01	0.90	1.40	97.1	2.9	2.1
Verapamil	4.30	0.63	0.70	84.2	15.8	9.3	Ketoprofen	10.71	1.03	1.72	99.1	0.9	0.8
Artesunate	4.36	0.64	0.72	84.7	15.3	25.0	Diclofenac	11.00	1.04	1.75	99.3	0.7	0.5
Octreotide Acetate ^a	4.63	0.67	0.78	86.7	13.3	35.0	Icatibant ^a	11.47	1.06	1.80	99.4	0.6	56.0

a: The measured %unbound is outside a 2-fold range of the literature value

Biomimetic Chromatography vs Equilibrium Dialysis Assay

Biomimetic Chromatography		Equilibrium Dialysis
Matrix Cost	No need (column only)	0.8 mL/compound/species
Incubation	No need	4-6 hr/assay
Time/Assay (including analysis)	30 min/cassette (10 compounds in one method)	6-8 hr, 32 compounds/HTD plate (dialysis → supernatant collection → LC-MS/MS analysis)
Throughput	400 compounds/day	96 compounds/day
Binding Range	5-99.5%	0-99.9%

