Improving productivity in the determination of parameters for early *in vitro* ADME

Part 4 – Experimental details and results of plasma protein binding assay

Application Note

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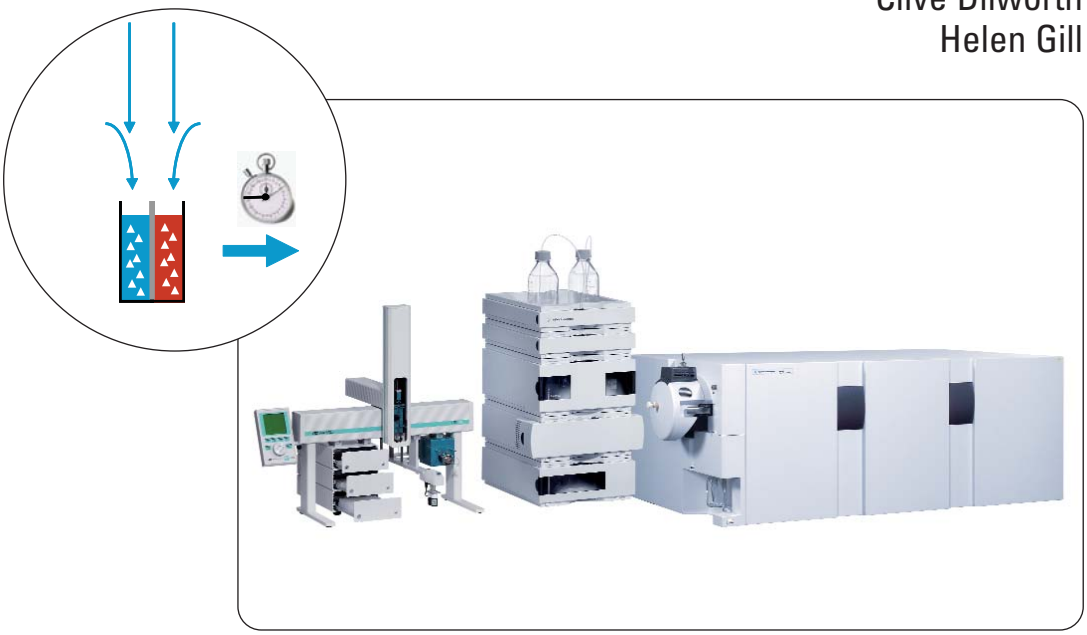

Abstract

The Application Note describes the experimental details and results of a plasma protein binding (PPB) assay. The approach and methodology of rapid resolution liquid chromatography and triple quadrupole mass spectrometry used in this study are described in a separate Application Note[1]. Experimental details and results of other typical assays performed in early ADME profiling of new hits are presented in separate publications[2,3].

Agilent Equipment:

1200 Series RRLC
6410 triple quadrupole LC/MS
CTC HTC-PAL autosampler
ZORBAX RRHT column
MassHunter workstation software

Application Area:

Drug Discovery

cyprotex

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Experimental

Method	Compound	Polarity	Precursor ion [m/z]	Product ion [m/z]	Dwell time [ms]	Fragmentor voltage [V]	C ollision energy [V]
Method_9	Diclofenac	pos	296.0	215.0	5	90	6
	Digoxin	pos	798.4	651.4	5	120	10
	Tramadol	pos	264.2	58.0	5	100	15
	Nimodipine	pos	343.1	301.0	5	120	20
	Propranolol	pos	260.1	183.0	5	120	25
	Nefazodone	pos	470.2	274.1	5	160	35
	Warfarin	pos	309.2	162.9	5	140	25
	Metoprolol	pos	268.2	74.1	5	140	25
Method_9neg (time segment 0.5 min)	Flurbiprofen	neg	199.1	199.1	50	100	0
	Metoprolol	pos	268.2	74.1	50	140	25
Method_10	Atenolol	pos	267.2	145.0	10	140	25
	Buspirone	pos	386.3	122.0	10	180	25
	Labetolol	pos	329.2	162.0	10	120	25
	Warfarin	pos	309.2	162.9	10	140	25
	Metoprolol	pos	268.2	74.1	10	140	25

Table 1
Setup of the MRM method for the PPB assay (metoprolol was used as ISTD, warfarin was used as control substance).

Plasma protein binding[4] – protocol summary

An equilibrium dialysis plate was prepared, containing two compartments separated by a semi-permeable membrane with a phosphate buffer solution on one side and the plasma on the other side (figure 1, step 1a). The test compounds were added to the equilibrium dialysis plate (step 2). After equilibration a certain fraction of the test compound was bound to the plasma proteins (step 3). The remaining free concentrations on both sides of the membrane were analyzed using calibration curves prepared in parallel (step 1b) and the LC/MS/MS method described above.

Final test compound concentration :	5 µM
Final DMSO concentration:	0.5 %
ISTD:	Metoprolol
Final ISTD concentration:	0.18 µM
Plasma:	Human, 50 % (v/v) in phosphate buffer
Phosphate buffer:	0.1 M, pH=7.4
Method:	Equilibrium dialysis
Incubation temperature:	37 °C
Incubation time:	overnight
Final Volume:	130 µL
Sample work-up:	After incubation, aliquots were removed and diluted with methanol containing internal standard. Incubation plates were centrifuged at 2500 rpm for 20 min at 4 °C to precipitate the proteins.

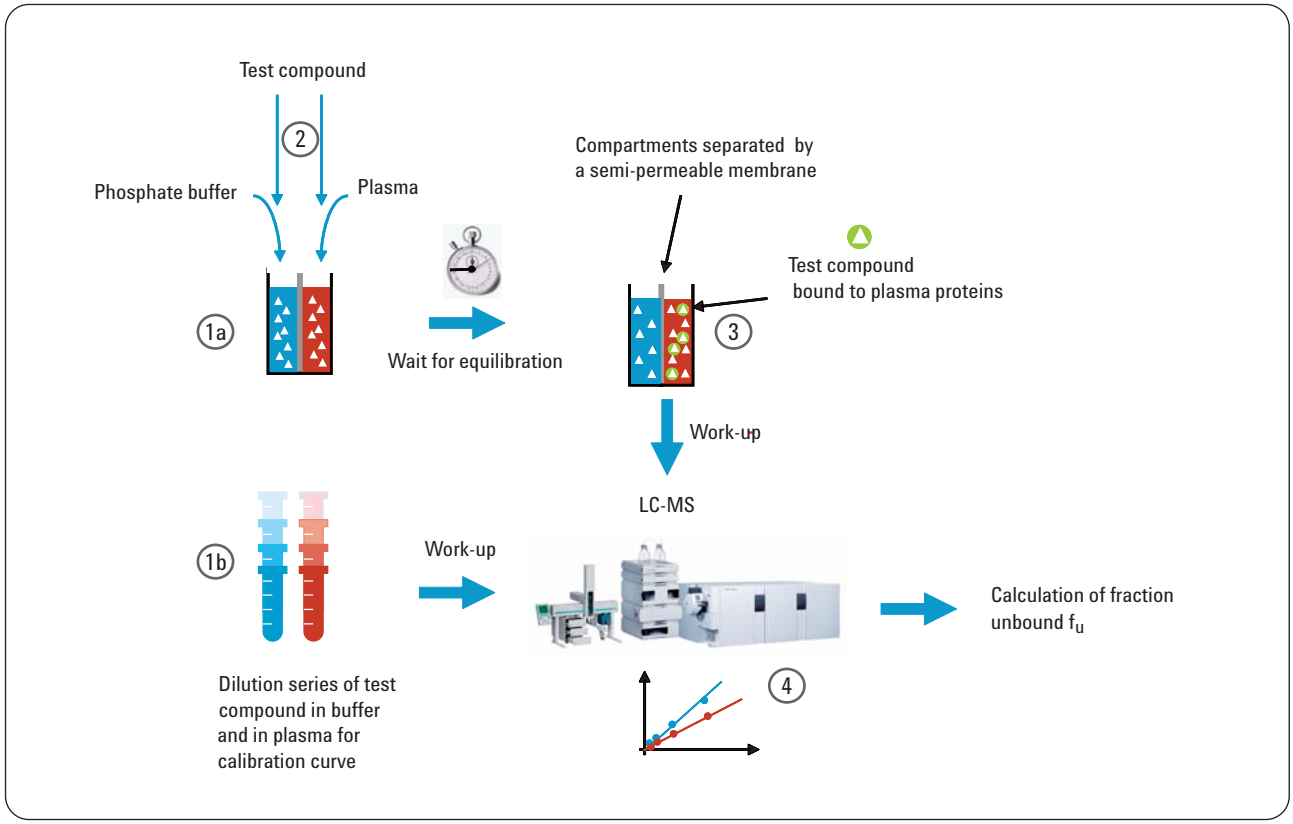

Figure 1
Schematic of the PPB assay.

Results

Compound	Fraction unbound (50 % plasma)	Fraction unbound (100 % plasma)	Recovery [%]
Atenolol	0.912	0.838	96
Buspirone	0.572	0.401	94
Diclofenac	–	–	–
Digoxin	0.792	0.655	54
Flurbiprofen	–	–	–
Labetalol	0.524	0.355	93
Nefazodone	0.063	0.033	71
Nimodipine	0.074	0.038	68
Propranolol	0.539	0.369	89
Tramadol	0.939	0.884	87

Table 2
Summary of the PPB assay results.

References

1.
Improving productivity in the determination of parameters for early in vitro ADME. Part 1 - Study approach and methodology of rapid resolution liquid. *Agilent Technologies publication number 5989-7488EN*, **2007.**

2.
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Part 2 - Experimental details and results of human liver microsome stability assay. *Agilent Technologies publication number 5989-7667EN*, **2007.**

3.
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4.
Roger E. Fessey et al. "The Role of Plasma Protein Binding in Drug Discovery", pp. 119–141 in "Pharmacokinetic Profiling in Drug Research", Ed. Bernard Testa, Stefanie D. Krämer, Heidi Wunderli-Allenspach, Gerd Folkers, *Verlag Helvetica Chemica Acta, Zürich and Wiley-VCH Weinheim*, **2006.**

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