Optimisation of a high throughput *in vitro* assay to determine the extent of brain tissue binding and the fraction unbound of potential CNS drugs

Vanessa R. Portman, Helen M. Hetherington, Michelle Ward
Sygnature Discovery, BioCity, Pennyfoot Street, Nottingham, NG1 1GR, United Kingdom

As a result of the increase in total population in recent years, the demand for novel therapeutics is growing; in particular the ageing population ensures neurological disorders are becoming more prevalent. The goal of this work was to develop a robust *in vitro* brain homogenate binding (BHB) assay and corresponding positive controls to be used in future early drug discovery research regarding the CNS. The assay adopts the "free drug hypothesis" mindset and is used to identify an approximate value for the fraction unbound of a test compound in the cerebrospinal fluid (CSF) once across the blood-brain barrier (BBB).

Current understanding of drug distribution in the brain

Once compounds cross the BBB it is only the free concentration of the compound in the ISF (and CSF) which is available for its intended purpose. CNS drugs must have limited non-specific binding and a sufficient fraction unbound ($f_{u,brain}$) to be effective. The $f_{u,brain}$ value of a compound is the proportion of the initial concentration administered which is unbound once over the BBB; in other words it is the concentration of the drug in the CSF. A schematic diagram of the various regions of the CNS and transfer across the BBB is shown in Figure 1. The double-headed arrows depict both passive and active transfer across the various regions of the brain and blood.
Various assays can be used to identify the different PK parameters regarding the CNS; these are summarised in Table 1.

Table 1. Summary of concentration parameters identified via various experiments.

In vivo PK	*In vitro* PPB	*In vitro* BHB
Brain/plasma ratio	$f_{u,plasma}$	$f_{u,brain}$
$C_{total,brain}$	$C_{u,plasma}$	$C_{u,brain}$
$C_{total,plasma}$		

CSF = cerebrospinal fluid
BCSFB = blood-cerebrospinal fluid barrier
ICF = intracellular fluid
ISF = interstitial fluid
BBB = blood-brain barrier
$f_{u,brain}$ = fraction unbound in brain
$f_{u,plasma}$ = fraction unbound in plasma
$c_{u,brain}$ = concentration unbound in brain
$c_{u,plasma}$ = concentration unbound in plasma

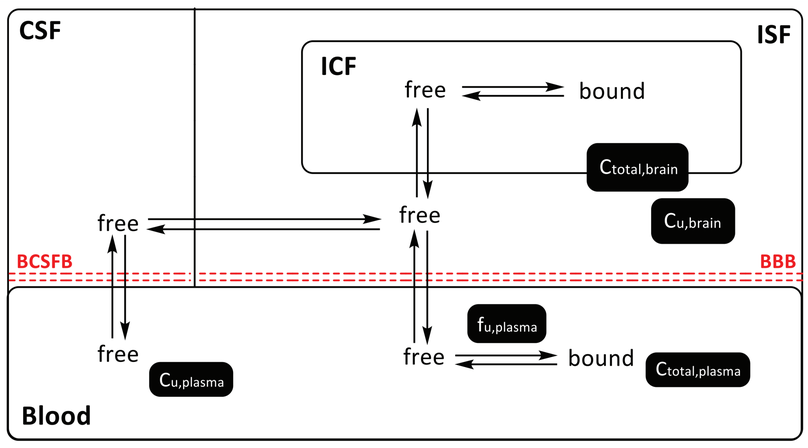
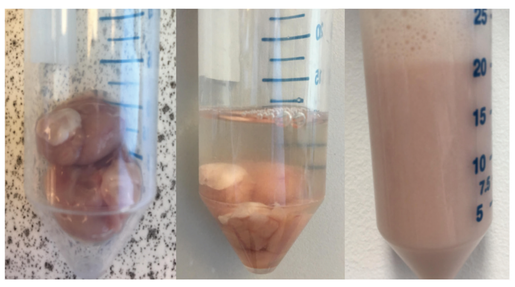


Figure 1: Schematic diagram of the various parameters identifiable for a compound in the CNS.

Experimental

The BHB assay involves a compound distribution equilibrium between a buffer (CSF surrogate) compartment and a brain homogenate compartment over a certain time period. Once-frozen rat brains were used; these were homogenised fresh for each assay.* Three dilution factors for the brain homogenate were investigated, in addition to incubation time and rotation rate of rapid equilibrium dialysis (RED) plate used during incubation, so as to identify optimum assay conditions. Various potential control compounds were also researched and the four which generated the most consistent data were chosen as positive controls for future assays.
The equations used for drug distribution quantification are shown below;



$$\text{Percentage bound (\%)} = (1 - f_{u,brain}) \times 100$$

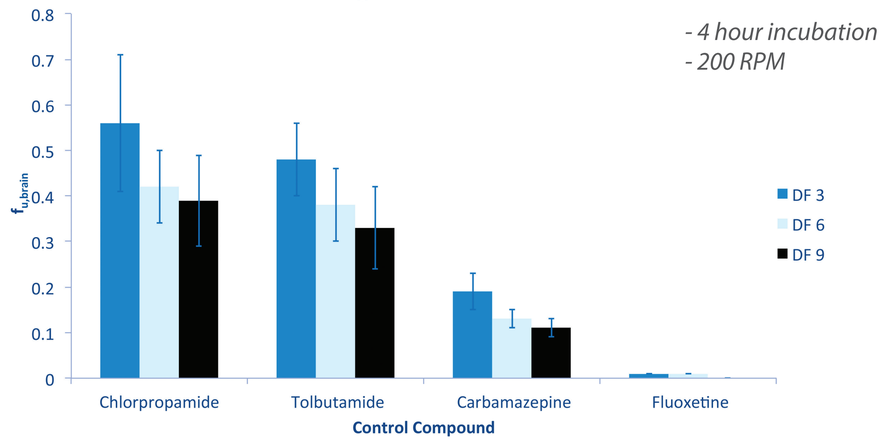
$$f_{u,meas} = \frac{C_{bu}}{C_{br}}$$

$$f_{u,brain} = \frac{1/x}{\left(\frac{1}{f_{u,meas}} - 1\right) + \frac{1}{x}}$$

C_{bu} = concentration in the buffer (calculated using calibration curve)
C_{br} = concentration in the brain (calculated using calibration curve)
$f_{u,brain}$ = fraction unbound in the brain
$f_{u,meas}$ = fraction unbound in the brain (measured)
x = dilution factor (3 in initial control compound selection)

*It has previously been investigated and determined that there is no species dependence in BHB and rat brains are suitable replacements when studying potential drugs for human consumption.[5]

Dilution Factor Change



- DF 9 chosen

Potential future work:

- Re-run compounds with low literature $f_{u,brain}$ values using modified dilution factor.

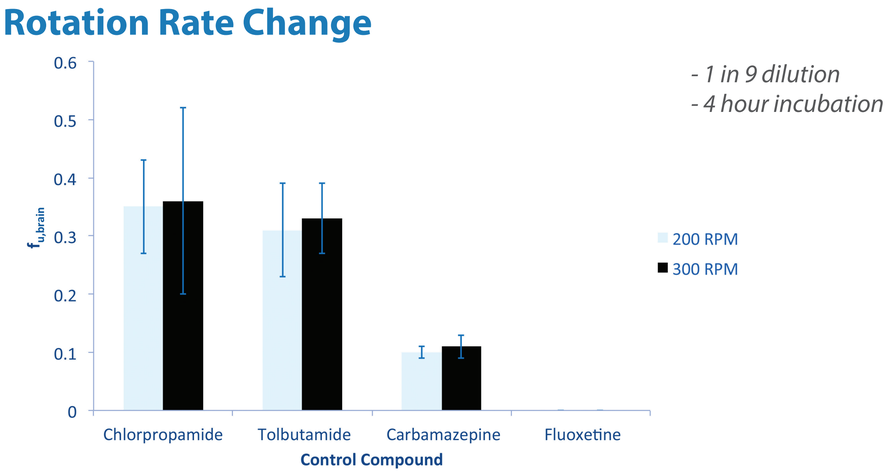
Control Compound Selection

- Four controls were chosen so as to cover a larger therapeutic window

- 1 in 3 dilution
- 4 hour incubation
- 200 RPM

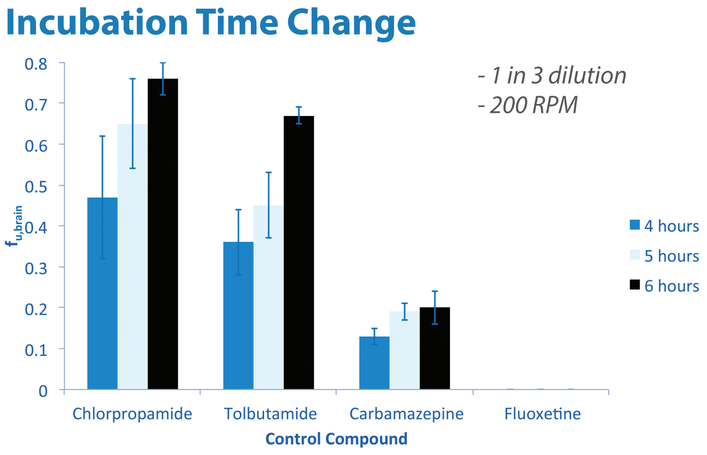
Control Compound	Num. of repeats	Av. binding (%)	Binding St. Dev (%)	Av. $f_{u,brain}$	$f_{u,brain}$ St. Dev	Literature $f_{u,brain}$
Propranolol	6	97.18	0.47	0.03	0.01	0.036 ± 0.009[2]
Phenytoin	6	92.41	5.25	0.08	0.05	0.134 ± 0.088[1]
Paroxetine	6	99.74	0.15	0.003	0.001	0.006 ± 0.005[1]
Diazepam	9	94.64	1.75	0.05	0.02	0.0500 ± 0.0120[3]
Sulpiride	12	21.51	15.57	0.78	0.16	0.6000 ± 0.1700[3]
Fluoxetine	15	99.31	0.35	0.008	0.004	0.00094 ± 0.00050[2]
Clozapine	15	98.61	0.46	0.01	0.004	0.0094 ±0.0003[3]
Carbamazepine	18	83.07	0.04	0.17	0.04	0.124 ± 0.014[1]
Metoclopramide	24	52.78	9.72	0.47	0.10	0.145 ± 0.045[1]
Tolbutamide	24	49.20	12.68	0.51	0.13	0.8443[4]
Caffeine	24	21.75	12.19	0.78	0.12	0.5000 ± 0.1180[3]
Chlorpropamide	30	39.34	10.51	0.61	0.10	0.668 ± 0.000[1]

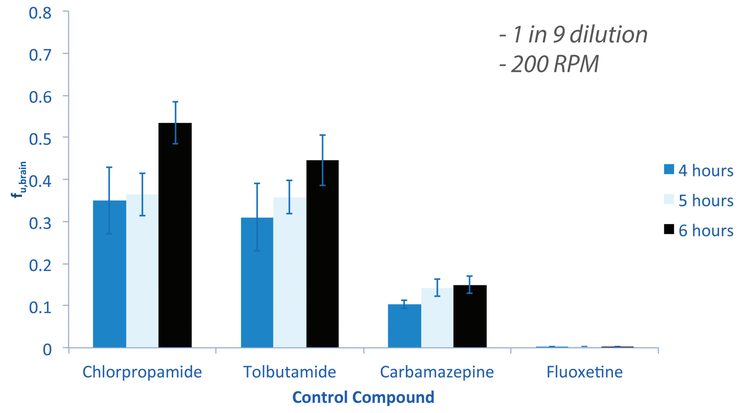
Rotation Rate Change



- 200 RPM chosen due to generating a narrower range of results

Incubation Time Change





- 4 hours chosen for consistency and practicality
- 1 in 9 dilution proved to narrow the range of results
- More outliers identified in lower dilution and longer incubation period

Summary

- Successfully optimised a high throughput assay for drug distribution within the brain
- 4 positive control compounds identified;
  - Chlorpropamide (highest distribution)
  - Tolbutamide
  - Carbamazepine
  - Fluoxetine (lowest distribution)
- Optimum conditions were:
  - 1 in 9 dilution
  - 4 hour incubation
  - 200 RPM
- Protocol completed with COSHH assessment and currently in use

References

[1]L. Di, J. P. Umland, G. Chang, Y. Huang, Z. Lin, D. O. Scott, M. D. Troutman, T. E. Liston, Drug Metabolism and Disposition, 2011, **39**, 1270-1277.
[2]X. Liu, B. J. Smith, C. Chen, E. Callegari, S. L. Becker, X. Chen, J. Cianfrogna, A. C. Doran, S. D. Doran, S. D. Doran, J. P. Gibbsm N. Hosea, J. Liu, F. R. Nelson, M. A. Szewc, J. Van Deusen, The Journal of Pharmacology and Experimental Therapeutics, 2005, **313**, 3, 1254-1262.
[3]M. Culot, A. Fabulas-da Costa, E. Sevin, E. Szorath, S. Martinsson, M. Renftel, Y. Hongmei, R. Cecchelli, S. Lundquist, PLOS One, 2013, **8**, 12, 1-11.
[4]R. Longhi, S. Corbioli, S. Fontana, F. Vinco, S. Braggio, L. Helmdach, J. Schiller, H. Boriss, Drug Metabolism and Disposition, 2011, **39**, 312-321.
[5]L. Di, J. P. Umland, G. Chang, Y. Huang, Z. Lin, D. O. Scott, M. D. Troutman, T. E. Liston, Drug Metabolism and Disposition, 2011, **39**, 1270-1277.

Enabling Success

sygnaturediscovery.com

www.sygnaturediscovery.com
busdev@sygnaturediscovery.com

Contact us

@SygnatureDiscov
www.linkedin.com/company/sygnature-discovery