Application Note

TRANSIL® Brain Absorption Assay with Mithras LB 940

Hinnerk Boriss[1], Bettina Felletschin[2]

Introduction

The blood-brain barrier (BBB) separates circulating blood from the cerebrospinal fluid (CSF) maintained by the choroid plexus in the central nervous system (CNS). This "barrier" results from the selectivity of the tight junctions between endothelial cells in CNS vessels that restricts the passage of solutes.

In the central nervous system (CNS) early assessment of compound availability is essential for CNS drugs and useful for optimizing the toxicity profile of non-CNS drugs. Designing pharmaceutical agents so that they pass the blood-brain barrier and are freely available to interact with receptors is one of the great challenges in CNS drug development.

The TRANSIL® Brain Absorption assay kit has been developed to help overcome this hurdle. Since the TRANSIL® Brain Absorption Kit is a fast high-throughput format assay, it can also be used to assess brain penetration of any non-CNS drug in secondary screening. Hence, the assay helps to screen for compounds which are less likely to cause undesirable side effects in brain.

With increasing lipophilicity compounds tend to penetrate the brain more easily. However, since brain dry mass is mostly lipids, their unspecific binding in brain increases as well. This in turn, decreases the compounds' free fraction in brain. Hence, to determine the extent of brain penetration it is not only important to know how much compound enters the brain, but also how large the fraction of a compound is, that is freely available in brain. As brain membrane affinity correlates strongly with the brain free fraction estimate from dialysis against brain homogenate, TRANSIL® Brain Absorption assay provides an accurate estimate of the brain free fraction.

Assay Principle

TRANSIL® Brain Absorption assay plates are ready-to-use. They are delivered with assay buffer and TRANSIL® beads with reconstituted porcine brain lipid membranes (figure 1).

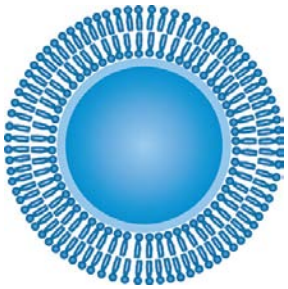

Figure 1: Illustration of a TRANSIL® Brain Absorption Bead with a single lipid bilayer reconstituted from porcine brain lipids

[1] Sovicell GMBH, Leipzig, Germany, contact@sovicell.com
[2] BERTHOLD TECHNOLOGIES, Bad Wildbad, Germany, Bettina.Felletschin@Berthold.com

The assay principle consists of simply adding a fixed concentration of compound to the microplate wells with pre-dispensed TRANSIL® beads in increasing concentrations (increasing membrane surface area).
The TRANSIL® plate is thawed and test compounds are added. After short mixing and incubation for 2 minutes at room temperature TRANSIL® beads are easily separated by low-speed centrifugation. This allows the quantification of the remaining compound concentration in the supernatant.
The measured brain membrane affinity is used directly to predict the brain free fraction. The brain-to-plasma distribution coefficient is predicted based on the measured membrane affinity, the compounds' calculated polar surface area, and the plasma protein binding.
The kit directly assesses compound's affinity for the brain membrane. Membrane affinity is related to permeation (rate) and blood-to-brain absorption of a drug (extent). Also, the kit is used to predict the brain free fraction. In combination with plasma protein binding data and polar surface area the model predicts the brain-to-plasma distribution coefficent logBB.

Mithras

The Mithras LB 940 is a multimode plate reader with a unique optical design (DOPS – Dedicated Optical Path System) to ensure optimized performance for the detection technologies implemented. These are

- luminescence
- BRET/BRET²
- Fluorescence
- FRET
- UV/VIS absorbance
- fluorescence polarization
- AlphaScreen®
- TRF
- HTRF®

In addition options like reagent injectors, temperature control and cooled PMT detection units are available.

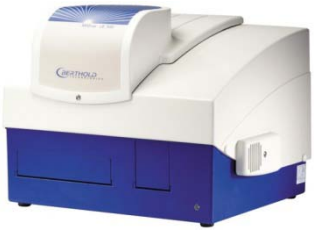

Figure 2: Mithras LB 940 multimode reader

Assay Protocol

The TRANSIL® Brain Absorption assay kit comes ready-to-use. Test items (table 1) were prepared in a 10x stock solution and 45 µL of this solution were transferred to each well of the assay plate. The final assay concentration of each compound was 50 µM.

Test compounds	Assay concentration
Carbamazepine	50 µM
Cimetidine	50 µM
Ranitidine	50 µM
Chlorpromazine	50 µM
Diclofenac	50 µM
Imipramine	50 µM
Propranolol	50 µM
Theophylline	50 µM
Chlorambucil	50 µM
Tolbutamide	50 µM

Table 1: Test compounds and final assay concentration

Samples were mixed 8 times by aspiration and dispension of the TRANSIL®/sample suspension. After incubating the assay plate for 2 minutes at room temperature, the plate was centrifuged to separate the TRANSIL® beads from the suspension. 100 µL of each supernatant were transferred to a 96 well half-area UV plate for quantification of the remaining drug concentration in the

Mithras reader. Extinction was measured at 240, 260, and 280 nm. The wavelength with the highest signal-to-noise ratio was selected for quantifying the interaction of the test drugs with the TRANSIL® beads. Linear models for predicting the brain tissue binding, brain-to-plasma distribution and brain availability were used as provided in Sovicell's software.

The compound classification using the rate and extent estimates from the TRANSIL® Brain Absorption kit yields a high performing classification of CNS+ and CNS- compounds.

Instrument Settings

Mithras LB 940 is operated through the PC software MikroWin 2000 which also can serve as a data evaluation tool. The Mithras LB 940 reader must be equipped with the filters 240 nm, 260 nm and 280 nm.

In the measurement sequence window all three measurements can be selected and will be automatically processed sequentially.

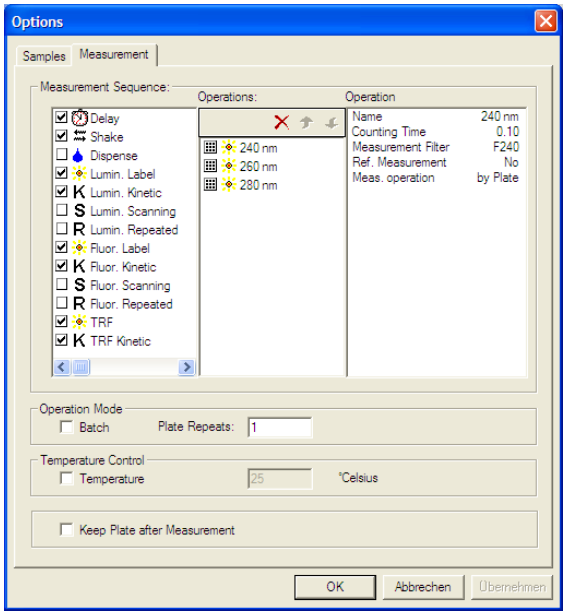

Figure 3: Operation sequence

Results

The wavelength with the highest signal-to-noise ratio (see table 2) was selected and used for quantifying the interaction of the test drugs with the TRANSIL® beads.

The compounds' affinity to brain membranes, brain tissue binding (fu brain), the brain-to-plasma distribution coefficent, and the availability of compounds in brain to interact with drug receptors are displayed in table 3.

Test compounds	Wavelength with highest S/N ratio [nm]
Carbamazepine	240
Cimetidine	280
Ranitidine	280
Chlorpromazine	280
Diclofenac	240
Imipramine	260
Propranolol	240
Theophylline	260
Chlorambucil	260
Tolbutamide	280

Table 2: Wavelength with the highest signal-to-noise ratio

The compound classification chart in figure 4 shows the classification by rate and extent. The classifier (black line) correctly classified 87% of the training set data. In the test set used for this study, we found only ranitidine marginally classified as CNS drug, while it is a highly potent CNS drug. All other drugs were correctly classified.

Conclusion

The TRANSIL® Brain Absorption Kit is perfectly suited for screening of drug candidate availability in the CNS in combination with the Berthold Mithras microplate reader.

Compound	Affinity to brain membranes (logMA brain)	Brain tissue binding (fu brain)	Availability of compounds to interact	Distribution coefficient total drug between brain and plasma (log BB)	Drug Type
Carbamazepine	1,69	0,15	0,11	-0,14	CNS
Cimetidine	4,33	0,00	0,02	1,65	CNS
Ranitidine	2,15	0,06	0,02	-0,22	CNS
Chlorpromazine	2,71	0,02	0,19	1,07	CNS
Diclofenac	2,96	0,01	0,04	0,58	non CNS + CNS side effect
Imipramine	1,53	0,22	0,09	-0,37	non CNS + CNS side effect
Propranolol	1,61	0,18	0,06	-0,52	non CNS, no CNS side effect
Theophylline	1,47	0,25	0,03	-0,95	non CNS, no CNS side effect
Chlorambucil	2,25	0,04	0,01	-0,51	non CNS, no CNS side effect
Tolbutamide	0,94	0,80	0,06	-1,10	non CNS, no CNS side effect

Table 3: Results summary of the TRANSIL® Brain Absorption assay. The assay determines the compounds' affinity to brain membranes and derives the brain tissue binding (fu brain), the brain-to-plasma distribution coefficent, and the availability of compounds in brain to interact with drug receptors. These parameters are used to predict whether a drug has CNS drug like properties or whether it is likely to exhibit CNS side effects.

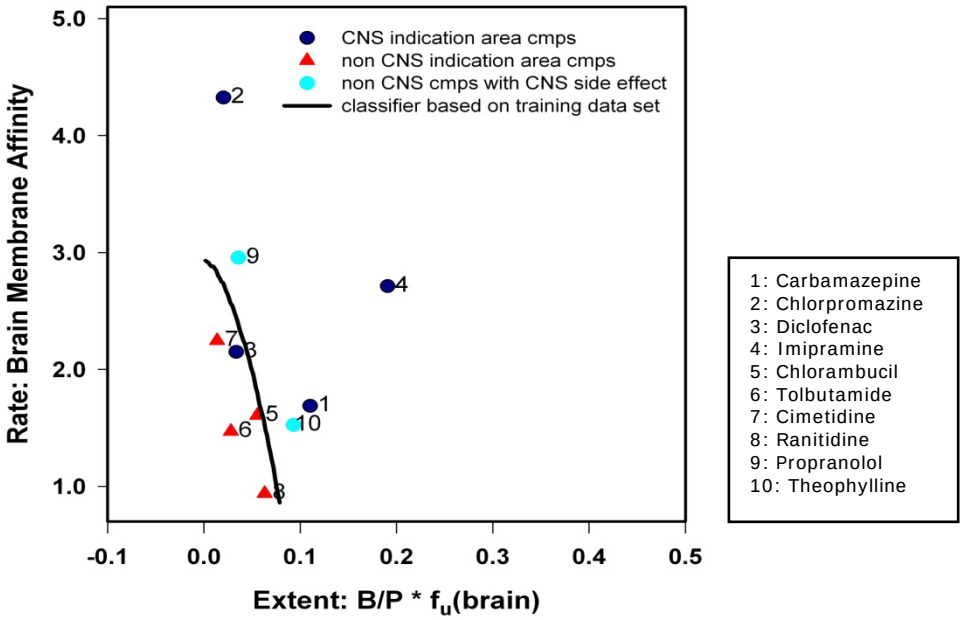

Figure 4: Compound Classification chart by rate and extent based on the measurements with the TRANSIL® Brain absorption kit. The classifier (black line) was calculated from a training set of 60 drugs and classified 87% of the training set correctly. It separates drugs that are not available in brain (red triangles) from drugs available in brain (light and dark blue dots). The drugs available in brain have either CNS drug like properties (dark blue dots) or likely to exhibit CNS side effects (light blue dots).

Material

- TRANSIL® Brain Absorption Kit, Plate, 96 Well (TMP-0110-0096, Sovicell)
- 96-well plates, half well, transparent, UV (Corning Costar)
- Mithras LB 940 multimode reader (Berthold Technologies)
- Filter 240nm (55598) (Berthold Technologies)
- Filter 260nm (55595) (Berthold Technologies)
- Filter 280nm (55594) (Berthold Technologies)
- Centrifuge for microplates
- Pipette/Liquid Handler

Literature

Boriss, H (2010): Brain availability is the key parameter for optimizing the permeability of central nervous system drugs. *Drug Discovery* 7:57-60.

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Loidl-Stahlofen, A. et al. (2001): Multilamellar liposomes and solid-supported lipid membranes (TRANSIL): Screening of lipid-water partitioning towards a high-throughput scale. *Pharmaceutical Research* 18 (12):1782-1788