Application Note

Automated TRANSIL® HSA Binding Kit on CyBi®-Well vario

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Abstract

This application note describes the HTS set-up of the ADME/Tox determination of human serum albumin binding using the TRANSIL® HSA Binding Kit and the CyBi®-Well vario with the 384/25 µL head.

Introduction

After intestinal absorption a test compound enters the blood stream and is subject to interactions with serum proteins. This binding to serum proteins may limit the bioavailability of a compound and can influence the distribution of the compound in the human body. Thus, the quantification of protein binding properties of compounds is an important early screening step in the drug discovery process and is of fundamental interest for estimation of safety margins. For this quantification there is a requirement for automation and high performance liquid handling.

NIMBUS Biotechnology provides human serum albumin, rat serum albumin and alpha-1-acid glycoprotein covalently immobilized in random orientation on an inert, soft and non-interfering surface. This prevents non-specific interaction of the compound with the immobilization matrix and ensures the free accessibility of all binding sites (figure 1).

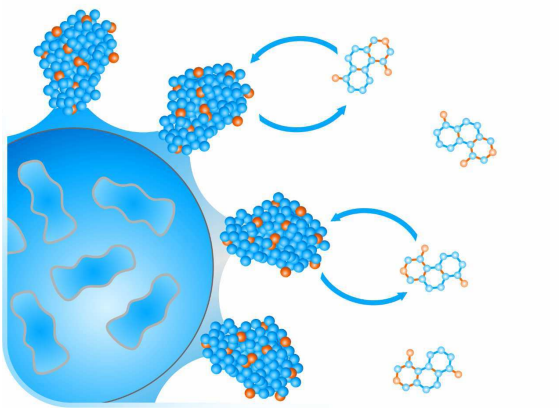

Figure 1: Drug binding to TRANSIL via serum protein

Material and Equipment

CyBi®-Well vario

The CyBi®-Well vario is a simultaneous pipetting instrument with an exchangeable pipetting head using individual pistons for the disposable tips (96 or 384 in parallel). Spinning the top of the tips into a silicon mat results in airtight sealing and ensures uniform tip length for enhanced pipetting performance.

Figure 2: The CyBi®-Well vario with a 384/25 µL head and two stackers

The microplates are moved by a high quality plate carriage and a plate lifter to access the individual wells up to the 1536-well plate format. All this ensures extreme high pipetting precision.
The modular design of the CyBi®-Well vario allows use of the instrument as a stand-alone pipettor e.g. for assay development where it can be equipped with e.g. stackers, barcode readers or a CyBi®-Drop. Of course it can be also integrated into larger screening systems to ensure higher throughput.

The CyBi®-Well vario can use disposable tips ranging from 250 µL to 10 µL. For very low volume transfers 2.5 µL ceramic tips can be used, which allow pipetting down to 25 nanoliter range.

Labware

- TRANSIL® HSA Binding Kit
- Greiner Bio-One, PP-MASTER-BLOCK, 384 Well, Deepwell (781270)
- Greiner Bio-One, UV-STAR, 384 Well, (781801)

Reagents

- PBS Buffer ph7.4, Dulbecco's
- Indomethacin, Ketoprofen, Phenylbutazon, Piroxicam, Warfarin

Kit Description

The TRANSIL® HSA binding kits are shipped frozen to the customer creating flexibility in the time schedule for use. Generally the drug of interest is added to prefilled micro plates at a single concentration.

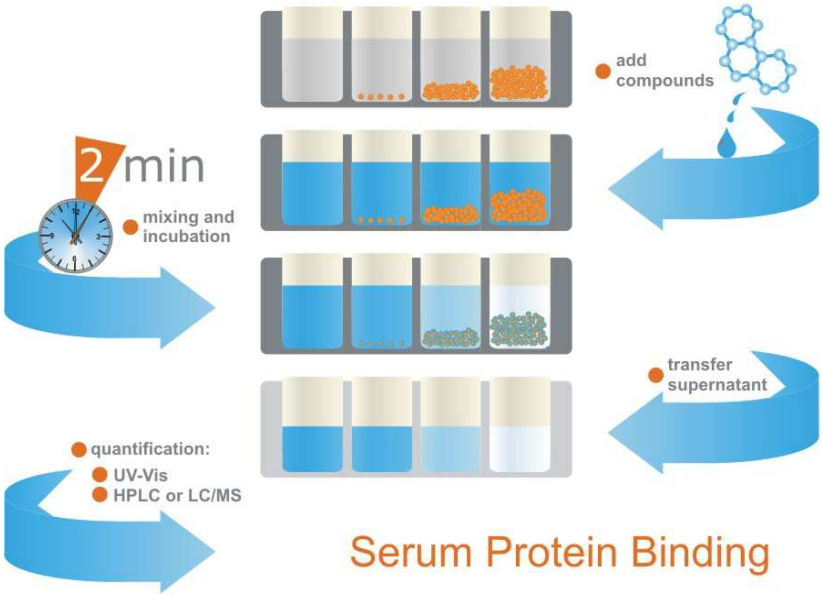

Figure 3: *General description of the TRANSIL Assays*

In order to quantify the concentration of the drug after the assay a reference sample without TRANSIL® is applied.
For a high-throughput determination four wells per compound are used, one reference and three wells filled with different amounts of TRANSIL® titrating the protein against the drug. The plate configuration is customized. In this case four plates were used, one reference plate and three protein plates.
Normally 10 mM stock solutions of the compounds in DMSO are prepared and diluted with buffer up to a final DMSO concentration of less than 5 % and the compound concentrations in the µM range (20 - 70 µM). After addition of the diluted compound to the wells, the wells are mixed by re-suspending the beads and incubated for 2 minutes. After this the TRANSIL® material is separated from the supernatant.
The drug concentration of the reference wells and the sample is analyzed by standard analytical methods like UV absorbance on a plate reader, HPLC or LC/MS. Final analysis of the bound drug yields the dissociation constant K_d and the bound fraction f_b. Figure 3 illustrates the general procedure determining serum protein binding with TRANSIL®.

The assay protocol

The basic steps of the assay were the following:

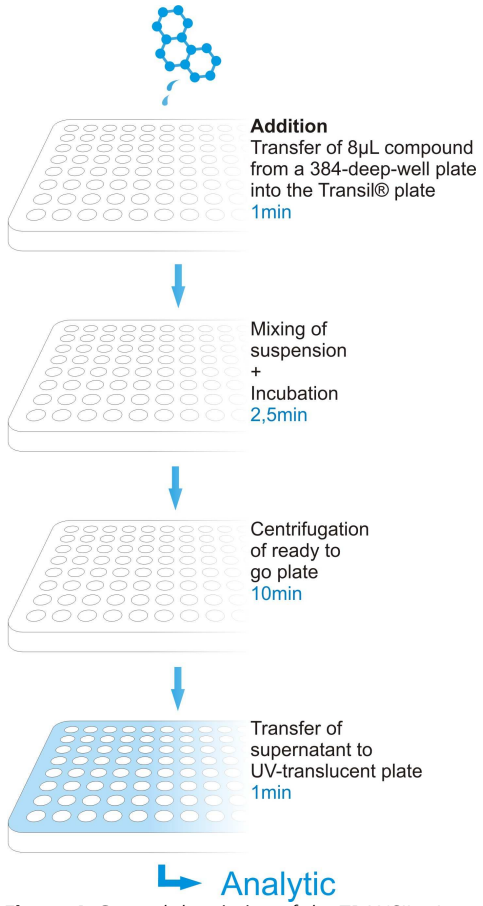

Figure 4: *General description of the TRANSIL Assays*

For all liquid transfers a CyBi®-Well vario with the pipetting head 384/40 with 25uL tips and stackers was used.
The demanding parts of the liquid handling process were to ensure a very effective mixing and the aspiration of the supernatant without aspirating any beads. This was possible by making use of the sophisticated options for liquid handling of the CyBi®-Well vario.
For mixing two different cycles were involved applying different heights of aspiration and dispensing. The first mixing cycle was intended to "break apart" the layers of beads (aspiration and dispensing at 1mm above the bottom of the wells), whereas the dispensing height was set to 7mm ('above ground') for the second cycle in order to ensure an optimal re-suspension. Each of the cycles consisted of 10 aspiration-dispensing steps. During mixing all piston speeds were set to their maximum speeds (ramp-mode: 25 µL/s).
For aspirating of the supernatant the pistons were set to a very low speed (3 µL/s) as well as the stage vertical movement (40 mm/s). The aspiration height was set to be 1mm above the layer of the beads.

Results

The following overall dissociation constants K_d and fraction bound f_b values (representing the fraction of compound bound to HSA at blood concentration) were found:

Compound	K_d	f_b
Indomethacin	1,6E-06	99,7%
Ketoprofen	4,4E-06	99,3%
Phenylbutazon	2,8E-06	99,5%
Piroxicam	1,1E-05	98,2%
Warfarin	1,8E-05	97,1%

The use of the TRANSIL® HSA Binding Kit in combination with a CyBi®-Well vario showed an **excellent correlation with classical approaches** (equilibrium dialysis).

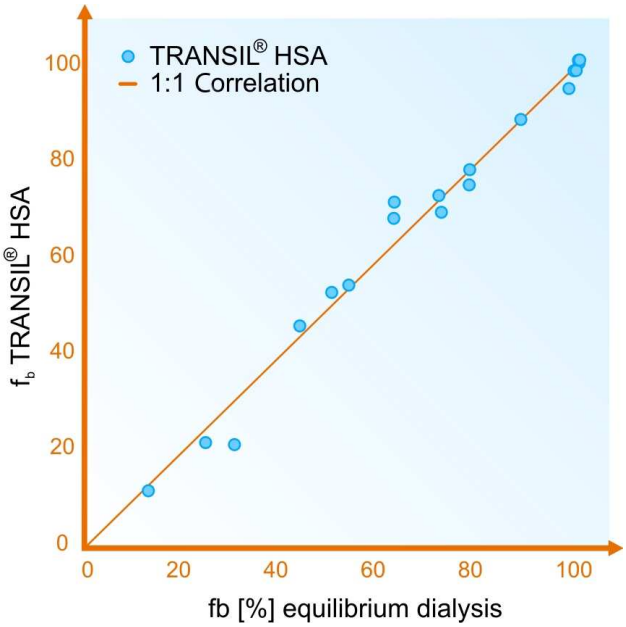

Figure 5: *Validation of the TRANSIL® HSA approach*

The TRANSIL® HSA Binding Kit and the use of the CyBi®-Well vario allowed a very high throughput **of over 700 samples/h**.

The robustness of the described method and its easy handling makes the combination of TRANSIL® kits and CyBi®-Well vario an ideal tool for processing ADME studies in a fast and cost effective way. High quality data are generated when the combination of kit and automation is used.