application note

MultiScreen® Solubility Filter Plate

Quantitative method to determine drug aqueous solubility: optimization and correlation to standard methods

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Abstract

Correlation to standard methodology, reproducibility, solvent (DMSO) effect, and drug recovery (non-specific binding, NSB) were determined for a high throughput aqueous solubility assay. Using a new, 96-well filter plate (MultiScreen Solubility filter plate, Millipore, Billerica, MA), and a protocol compatible with full automation, the aqueous solubility of thirty-two commercial compounds was quantitatively measured. Sample throughput allows for 4 plates a day with 32 samples per plate. Correlation with shake-flask values is reported. Day-to-day, plate-to-plate, and within plate assay variability was determined for a number of compounds whose solubility spans the assay's analytical range (10–500 µM). Effects of incubation time and DMSO on aqueous solubility, as well as NSB, have also been evaluated.

Background

Determining compound solubility in water has become an essential early measurement in the drug discovery process. Poor water-solubility can cause problems in many different *in vitro* testing techniques, leading to unreliable results and/or reproducibility problems. Consequently, candidate compounds can fail early on in their development due to unfavorable physicochemical profiles. An even larger problem results when insoluble precipitates cause false positives in bioassays, potentially wasting valuable resources. Such issues can add significant cost and time to drug development activities.

The standard way to determine the solubility of a compound is to use the shake-flask solubility method.[1] This method is inherently low-throughput, labor intensive, and necessitates the addition of drug in powder form–a requirement which can be incompatible with how compounds are generally maintained (e.g., in DMSO[3]). The shake-flask method involves adding an excess quantity of solid material to a volume of buffer at a fixed pH. This saturated solution is agitated (shake-flask) until equilibrium is reached, generally in 24 to 48 hours. Following separation by filtration or centrifugation, the compound in solution is analyzed and quantified

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by UV/Vis spectro-scopy or HPLC. The MultiScreen Solubility filter plate has been designed and optimized for the determination of aqueous solubility in a high-throughput and automation-compatible workflow. The plate has been developed with the following attributes:

- A 96-well format allows for solubility analysis of multiple drugs in a single plate
- Plate design compatible with all standard laboratory robotics and analytical equipment
- Low sample volume; 10 µL at 10 mM
- Direct quantitation of compound in solution
- Functional over a wide pH and excipient range
- High drug recovery for reliable determination of soluble compound concentration
- Good particle retention to remove insoluble compound
- Compatibility with aqueous organic solutions (e.g., ≤5% DMSO in pH 3–12 buffers)
- Reproducible and repeatable results

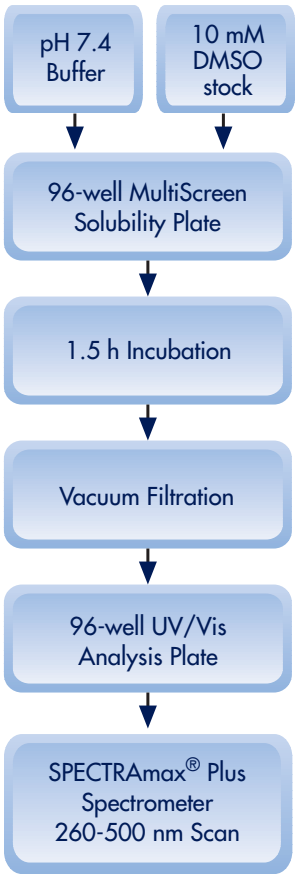
Introduction

The MultiScreen Solubility filter plate with modified track-etched poly-carbonate, 0.4 µm membrane is a single-use, 96-well product assembly that includes a filter plate and a cover. The device is intended for processing aqueous solubility samples in the 100–300 µL volume range. The vacuum filtration design is compatible with standard, microtiter plate vacuum manifolds. The plate is also designed to fit with a standard, 96-well microtiter receiver plate for use in filtrate collection. The MultiScreen Solubility filter plate was developed and is QC tested for consistent filtration flow-time (using standard vacuum), low aqueous extractable compounds, high sample filtrate recovery, and its ability to incubate samples as required to perform the solubility assay. The low-binding membrane has been specifically chosen for high recovery of dissolved organic compounds in aqueous media.

The aqueous solubility assay (see Figure 1) allows for the determination of a drug or compound's aqueous solubility by mixing, incubating and filtering a solution in the MultiScreen Solubility filter plate. After the filtrate is transferred into a 96-well collection plate using vacuum filtration, it is analyzed by UV/Vis spectroscopy to determine solubility. Additionally, LC/MS/MS or HPLC can be used to determine compound solubility, especially for compounds with low UV/Vis absorbance (greater than 270 nm) and/or compounds with lower purity (<90%). For quantification of aqueous solubility, it is recommended that a standard calibration curve be completed and analyzed for each compound prior to determining aqueous solubility.

Test solutions are first prepared by adding an aliquot of concentrated drug or compound (typically 10 µL of 10 mM drug in DMSO) to 190 µL of buffer at a defined pH to achieve a final concentration of 500 µM in 5% DMSO. The buffer solutions are mixed in a covered 96-well MultiScreen Solubility filter plate for 1.5 hours at room temperature. The solutions are then vacuum filtered into a 96-well, polypropylene, V-bottomed collection plate to remove any insoluble precip-itates. Upon complete filtration, 160 µL/well are transferred from the collection plate to a 96-well UV analysis plate and diluted with 40 µL/well of acetonitrile. The UV/Vis analysis plate is scanned from 260–500 nm with a UV/VIS micro-plate spectrometer to determine the absorbance profile of the test compound.

Figure 1: Aqueous Solubility Assay



Materials

Reagents

- 10 mM stock compound in DMSO (10 µL per analysis replicate; i.e., per well)
- Universal buffer reagents: ethanolamine, potassium dihydrogen phosphate, potassium acetate
- 0.15 M potassium chloride solution
- 80:20 Universal Aqueous buffer:acetonitrile solution
- 1.0 N hydrochloric acid
- Acetonitrile (ACN) #A998SK-1 (Fisher Scientific—Atlanta, GA)

Materials

- Biohit Proline™ single and multi-channel pipettors (Biohit-Helsinki, Finland)
- Stericup™ filter unit, 0.22 µm, cat. SCGP U05 RE, or equivalent (Millipore–Billerica, MA)
- 96-well polypropylene, V-bottomed collection plate, cat. 4506-51201, or equivalent (Greiner Bio-One, Inc.–Longwood, FL)
- Wheaton Redi-Pak 1oz. wide-mouth amber glass bottle, cat. 02-911-7 (Fisher Scientific–Atlanta, GA)
- Zirconia grinding media, cat. 08-412-15C (Fisher Scientific–Atlanta, GA)
- Millex®-LCR filter units, cat. SLCR013NL (Millipore–Billerica, MA)
- 10 mL Luer-Lok™ tip syringe, cat. 309604 (Becton Dickinson–Franklin Lakes, NJ)
- Greiner deep-well Master-blocks, 96-well 2.2 mL, cat. 4507-80280 (Bellco Glass, Inc.–Vineland, NJ)
- Greiner UV-Star™ analysis plates, 96-well, flat-bottomed, cat. 4506-55801 (Bellco Glass, Inc.–Vineland, NJ)

Equipment

- Lab-Line® Titer-Plate shaker
- Vacuum/Pressure pump, cat. XX55 000 00, or equivalent (Millipore–Billerica, MA)
- MultiScreen vacuum manifold, cat. MAVM 096 OR, or equivalent (Millipore–Billerica, MA)
- SPECTRAmax® Plus UV/Vis micro-plate reader or equivalent (Molecular Devices-Sunnyvale, CA)
- SOFTmax® Software (Molecular Devices-Sunnyvale, CA)
- Bottle rolling station
- Oversized can

Quantitative Protocol for Determination of Aqueous Solubility

(See Millipore Protocol Note PC2445EN00 for full quantitative protocol)

a. Prepare Universal Aqueous buffer solution to pH 7.4 (see Millipore Protocol Note PC2445EN00 entitled, "Determination of aqueous compound solubility using a 96-well filter plate to remove precipitated solids prior to UV/Vis spectroscopic analysis), filter with Stericup filter unit to remove any particulates, and store at 4 °C for up to one month prior to use.

b. Using a multi-channel pipettor, dispense 190 µL/well of pH 7.4 buffer from step a into a MultiScreen Solubility filter plate.

c. Dispense 10 µL/well of stock compound (normally at 10 mM in DMSO, from a 96-well polypropylene, V-bottomed plate), directly into the buffer in the MultiScreen Solubility filter plate with a multi-channel pipettor.

d. Cover with lid, and then mix with gentle shaking (100-300 rpm) at room temperature for 1.5 hours.

e. Filter the contents of the MultiScreen Solubility filter plate into a clean polypropylene, 96-well V-bottomed collection plate on the MultiScreen vacuum manifold with grid at 10–12 in. Hg. (N.B., Filtration by vacuum requires that there is liquid in all 96 wells.)

f. For analysis with UV/Vis spectroscopy, transfer 160 µL/well from the collection plate to a UV/Vis compatible, 96-well plate and add 40 µL of acetonitrile to each well. Data collection is obtained using a UV/Vis microplate reader.

g. Alternative methods to quantify dissolved compound versus standard curves such as HPLC-UV/Vis or LC/MS/MS are available. See Millipore Protocol Note PC2445EN00 for further details.

Shake-Flask Protocol for Determination of Aqueous Solubility[1]

a. To a wide-mouth glass bottle, add 20 mL of pH 7.4 buffer and a sufficient quantity of a compound to over saturate the aqueous volume (≥10 mM).

b. Add 2 grinding media to each of the glass bottles, close and seal the lid. Place securely into an oversized can such as a small paint can, ensuring that the bottle does not move within the can while it is rolling.

c. Place the paint can with the shake-flask solution on the rolling station, allowing sufficient time for the solution to reach equilibrium, approximately 48 hours.

d. Remove the shake-flask solution from the paint can.

e. Aspirate 2 mL of the solution into a syringe, and attach a Millex-LCR filter unit to the syringe.

f. To allow for any binding to the membrane, discard the initial 1 mL volume.

g. Collect the second 1 mL of solution from the syringe to a distinct well of the deep-well block.

h. Remove 160 µL, in triplicate, from the deep-well block to three wells of the UV analysis plate.

i. Add 160 µL of the pH 7.4 buffer per well to one column (a–h) of the UV analysis plate as background blanks.

j. Add 40 µL of acetonitrile to each sample well in the UV analysis plate, and mix by pipetting 5X.

k. Read the spectrum of the plate from 260 nm to 500 nm in 10 nm increments in the SPECTRAmax Plus plate reader.

l. Some compounds are freely soluble and their OD (optical density) values are large and out of the range of reliable quantitation. Therefore, these shake-flask solutions must be diluted as follows:

   1) Repeat steps e through g.

   2) Dilute by an appropriate dilution factor (compound specific) to reduce the OD to a value in the range of 0.5 to 1.5. Perform dilution in a separate well of the deep-well block by adding filtered solution to the proper amount of pH 7.4 buffer to achieve the desired dilution factor.

   3) Mix the diluted solution by pipetting 5X.

   4) Repeat steps h through k using the newly diluted solutions in place of the original filtered solutions.

Data Collection

Data were collected using a Molecular Devices SpectraMax Plus microplate spectrometer. For accurate and reliable results, UV/Vis absorbance spectra for a specific drug or compound in 5% DMSO were obtained in duplicate at five concentrations (500, 200, 50, 12.5 and 3.13 µM). Standard curves based on the 5 calibration concentrations were used to determine aqueous solubility.

Data Analysis

In order to determine the solubility concentration of a sample, a standard curve for each compound must first be prepared from known concentrations. Figure 2 illustrates superimposed calibration scans for glybenclamide at concentrations of 500, 200, 50, 12.5, and 3.125 µM. Using a wavelength >260 nm at or near the maximum absorbance (300 nm for glybenclamide), standard curves were generated as exemplified in Figure 3. The slope of the line (0.0011 in this example) was then used to calculate drug concentration from the absorbance for each aqueous filtrate sample (see Equation 1). The aqueous solubility spectrum and calibration spectra for each compound were superimposed to confirm compound identity and integrity. The absorbance of the aqueous solubility filtrate sample is read from the wavelength selected to construct the standard curve.

Figure 2: Glybenclamide Spectral Scan in 5% DMSO

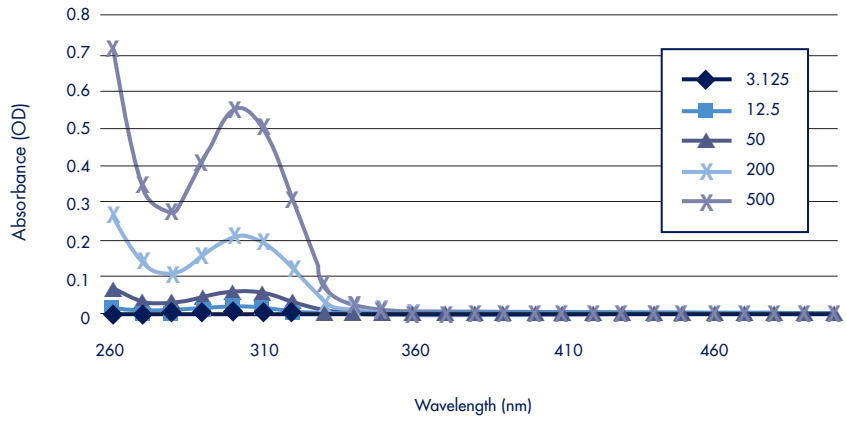
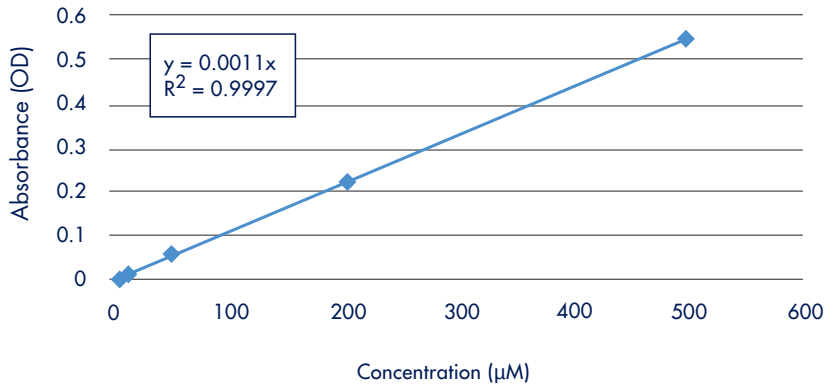


Figure 3: Glybenclamide at 300 nm



Equation 1– Aqueous Solubility Calculation

The final drug concentration in the filtrate is determined by dividing the absorbance by the slope of the line from the calibration curve and multiplying by a factor of 1.25 to account for dilution with acetonitrile prior to obtaining the absorbance spectrum.

$$\text{Aqueous Solubility} = \left(\frac{A_{max}\,Filtrate}{slope}\right) \times 1.25$$

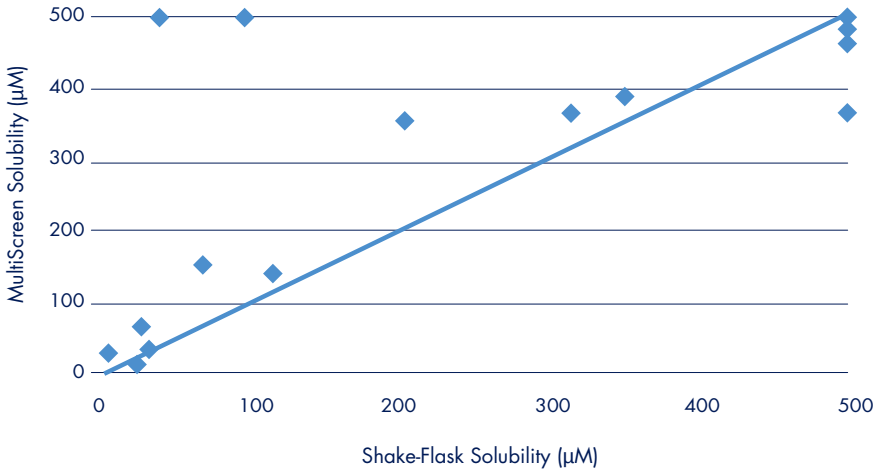
Results

Correlation

The solubility data obtained using the MultiScreen Solubility filter plate and the shake-flask method is displayed in Figure 4. Aqeuous solubility using both methods was determined at pH 7.4 for 32 commercial drugs of varying aqueous solubility. Five point standard curves of each drug in 5% DMSO were generated for each aqueous solubility determination. Shake-flask aqueous solubility values were determined as outlined in the **Protocol**.

As can be seen in Figure 4, the aqueous solubility measured using the MultiScreen Solubility filter plate correlates with the shake-flask method. The diagonal line of Figure 4 represents equal concentrations for both methods. In general, the Multi-Screen Solubility filter plate assay over-estimates shake-flask solubility data. The positive bias, which is somewhat desirable in a screening method, is at least partially attributed to the presence of DMSO in the filter plate assay. Also shown in Figure 4 are two drugs (glybenclamide and nifedipine) which do not correlate well to the shake-flask method. For both compounds, a solubility in excess of 350 µM was determined using the MultiScreen Solubility filter plate while the shake-flask method produced values of less than 100 µM. For these two compounds, the presence of 5% DMSO significantly elevates their solubility. Decreasing the amount of DMSO in the assay can produce solubility values comparable to shake-flask values as displayed in **DMSO Effect on Aqueous Solubility** below.

Figure 4: MultiScreen Solubility Method vs. Shake-Flask



Reproducibility

Well-to-well, plate-to-plate, and day-to-day comparisons were conducted to analyze the reproducibility of the MultiScreen Solubility filter plate assay. Two compounds, low solubility 4,5-diphenylimidazole (4,5–DPI) and moderately soluble ketoconazole (KETO), were assayed in each plate.

Using the standard protocol, two plates were run each day over three different days with each drug consuming 45 wells/plate (6 blanks/plate). The data are presented in Figures 5 and 6.

The well-to-well coefficient of variability (CV) for 4,5-DPI was 4.8% while the %CV for KETO was 4.3%.

The average solubility measured for each drug from plate-to-plate on the same day was within 4% for 4,5-DPI and within 2.5% for KETO. The solubility for each of the compounds measured over different days was 68±7 and 141±5 for 4,5-DPI and KETO respectively.

Figure 5: Reproducibility Analysis for 4,5–DPI

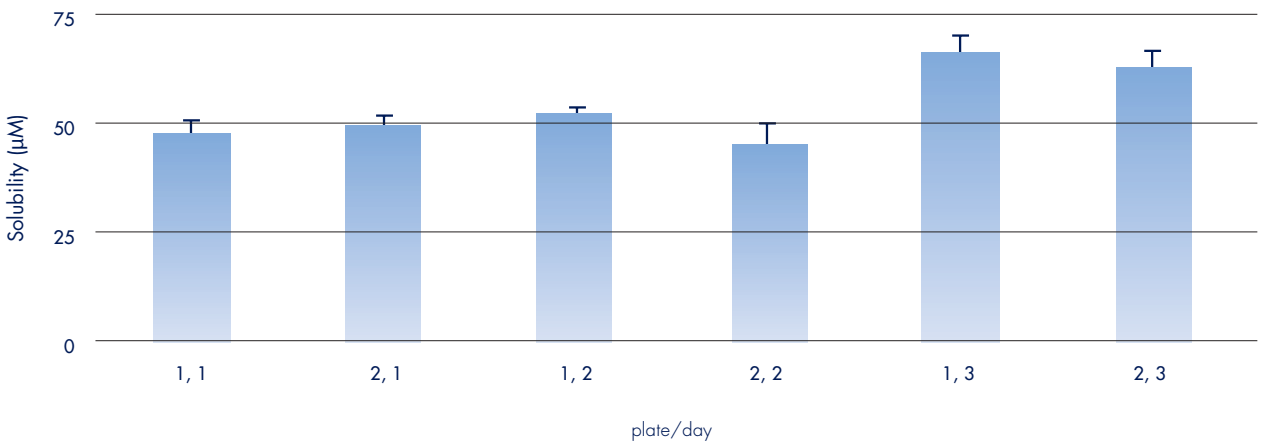
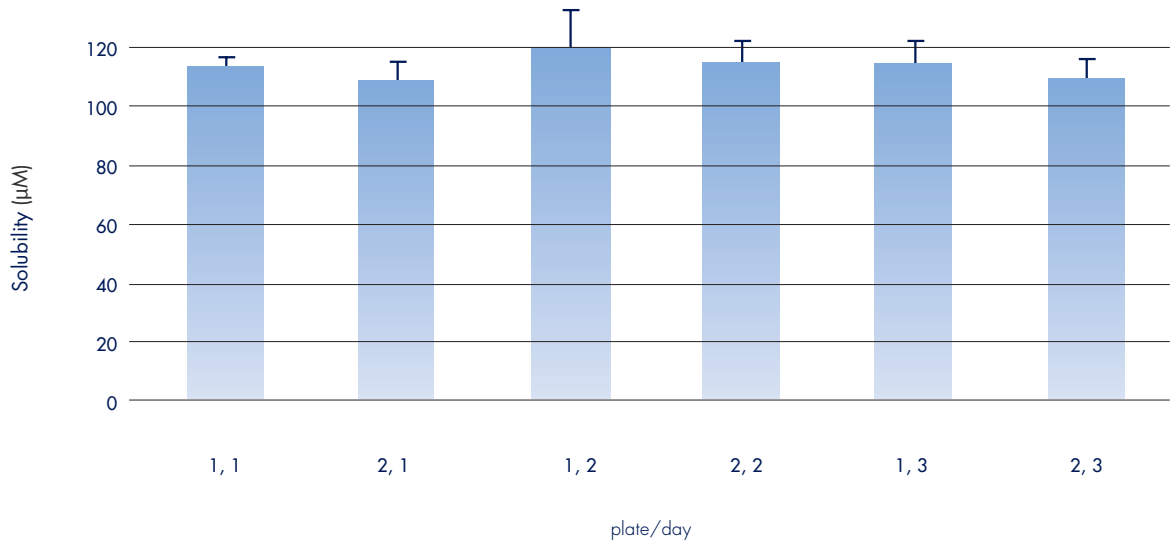


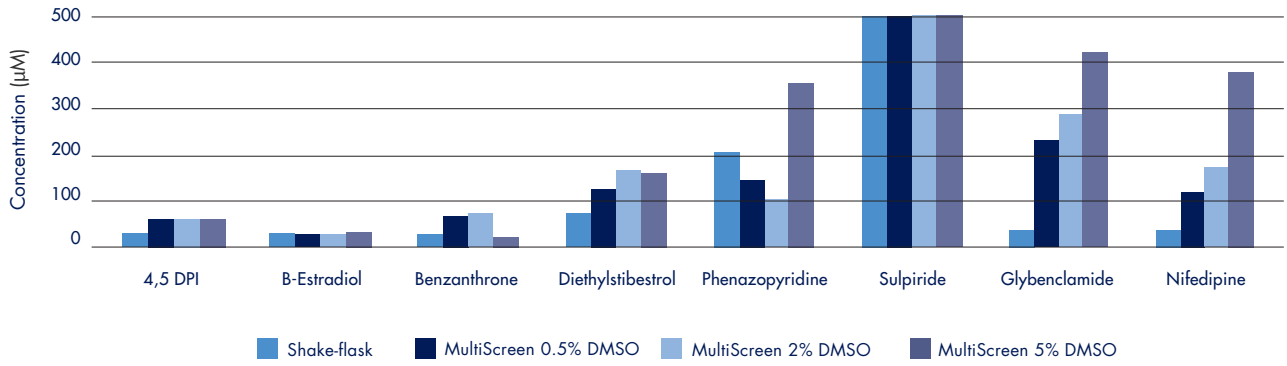
Figure 6: Reproducibility Analysis for Ketoconazole



DMSO Effect on Aqueous Solubility

The protocol, as described, calls for the addition of 10 µL of a DMSO stock solution (normally at 10 mM) to 190 µL of an aqueous buffer, thus resulting in a 5% v/v concentration of DMSO. The use of an organic co-solvent in amounts as low as 5% can increase the aqueous solubility. The effect of DMSO on the apparent aqueous solubility of a number of compounds was determined using the MultiScreen Solubility filter plate assay. The results for eight selected drugs are presented in Figure 7. To produce test solutions with decreased amounts of DMSO, (0.5%, and 2%) the drug concentration of the stock solution was increased 10-fold and 2.5-fold respectively. DPI, ß-estradiol, benzanthrone, all relatively low solubility compounds, and sulpiride, a relatively high solubility compound, are essentially unaffected by the concentration of DMSO. The aqueous solubilities of drugs can be variably affected by the amount of DMSO. Phenazopyridine and diethylstilbestrol are slightly affected, while glyben-clamide and nifedipine are reported in 10-fold excess. If desired, the potential effect of DMSO on solubility can be reduced by lowering the amount of DMSO used. This can be accomplished by making a more concentrated stock solution (e.g., 50 mM) or by lowering the upper limit of the assay (e.g., to 100 µM).

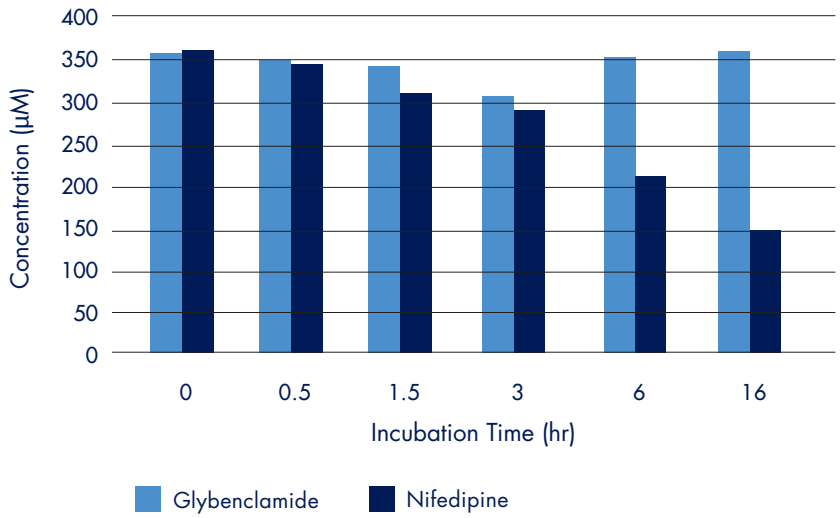
Figure 7: Shake Flask and MultiScreen Solubility at Varying DMSO



Incubation Time

The incubation time for the MultiScreen Solubility filter plate assay has been optimized to 1.5 hours, significantly shorter than that of the shake-flask method which calls for a minimum of 24 hours. Investigations into the required incubation time showed 1.5 hours to be sufficient for the majority of compounds as exemplified with glybenclamide (Figure 8). The short incubation time does not permit the equilibration of super-saturated compounds which can slowly crystallize in chosen solvent systems. This effect is also illustrated with nifedipine in Figure 8, in which the solubility of nifedipine decreases from approximately 350 µM initially to 150 µM after 16 hours.

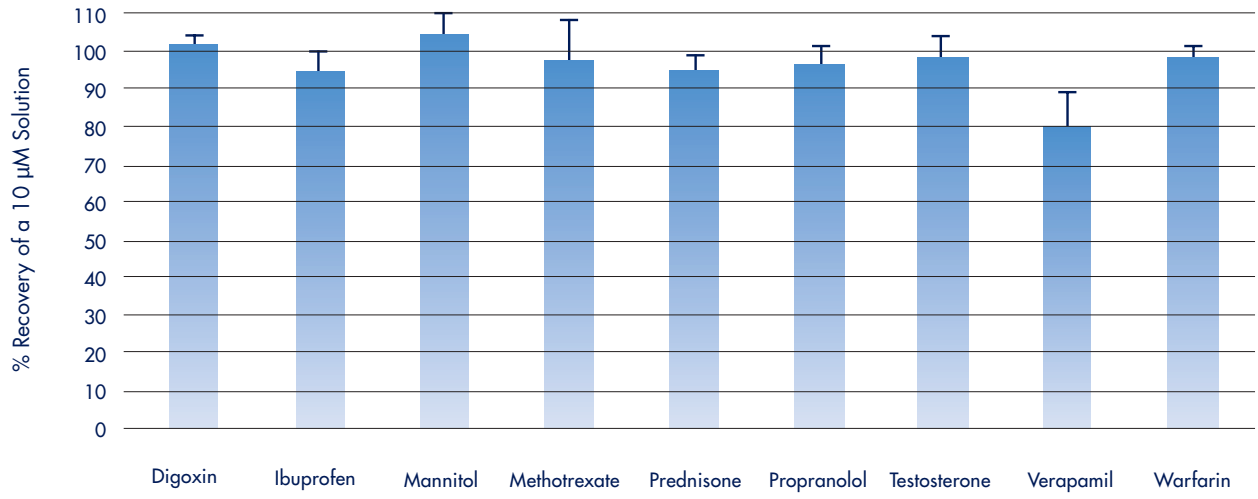
Figure 8: Solubility Time Dependence



Small Molecule Non-Specific Binding (drug recovery)

In order to determine the level of non-specific binding, nine drugs were analyzed for drug recovery in a Multiscreen Solubility filter plate. A 200 µL sample of each compound at 10 µM in 5% DMSO/buffer pH 7.4 was added to individual wells of a Multiscreen Solubility filter plate. The plate was incubated with shaking (300 rpm) at room temperature for 1.5 hr and then vacuum filtered (10-12 in. Hg). The concentration of compound in the filtrate was measured and compared to the starting concentration. All compounds were recovered at a minimum of 80%, with the majority of compounds 90 to 100% recovered. Data showing the drug recovery for the Multiscreen Solubility filter plate is summarized in Figure 9.

Figure 9: MultiScreen Solubility Drug Recovery



Conclusion

The MultiScreen Solubility filter plate provides an automation compatible, high throughput means to estimate the aqueous solubility of hundreds of compound per day. Compatible with compounds dry or maintained in DMSO and requiring a very small sample volume per analysis, the high throughput capabilities of the device provide for analyses of as many as 30 drug candidates per plate. As many as four or more plates per day may be run depending on the level of automation. With 80 to 100 % drug recovery and highly reproducible results, the measured concentrations are reliable, precise and easily integrated into existing chemical profiling and early ADME workflows.

References

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Related Application and Protocol Notes

PC2445EN00: Determination of aqueous compound solubility using a 96-well filter plate to remove precipitated solids prior to UV/Vis spectroscopic analysis

AN1731EN00: Performance and correlation of a 96-well high throughput screening method to determine aqueous drug solubility

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Lit. No. AN1730EN00 Rev. - 9/03 Printed in U.S.A. 03-252

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