

MultiScreen® Solubility Filter Plate

protocol note



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

Introduction

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.¹ Alternatively, and even more problematic, insoluble precipitates have been shown to cause false positives in bioassays, potentially wasting valuable resources. Such issues invariably add significant cost and time to drug research projects.

The standard method of determining the solubility of a compound involves adding an excess quantity of solid material to a volume of buffer at a set pH. This saturated solution is agitated until shake-flask equilibrium is reached. Following separation by filtration or centrifugation, the compound in solution is analyzed and quantified by UV spectroscopy or HPLC. This shake-flask solubility method is inherently low throughput and labor intensive.

A more efficient technique for solubility determination is by a filtration-based assay. For automation-compatible, 96-well filtration assays,

Millipore has introduced MultiScreen Solubility filter plates. The 96-well plates are specifically designed and optimized for rapid solubility determination with results that correlate to shake-flask method. The plates have the following attributes:

- 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

General Considerations

Aqueous solutions are mixed and incubated in the MultiScreen Solubility filter plate, which is then operated in a vacuum mode to retrieve the filtrate for analysis by UV spectroscopy to determine the concentration of soluble component. Solubility concentration can also be determined by LC/MS/MS or HPLC, detection methods convenient for compounds

with low purity (<90%) or low extinction coefficients. Using the methods that are described in this protocol note, the aqueous solubility for thirty compounds at a preselected pH value can be determined in two hours.* The solubility assay described here can be performed manually or can be fully automated. For automation protocols, visit www.millipore.com/automation.

*Up to 4 plates and 120 drug compounds can be run in triplicate and analyzed in eight hours.

Materials

Reagents

- 10 mM stock compound in DMSO (10 μ L 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 buffer:acetonitrile solution
- 1.0 N Hydrochloric acid

Equipment

- Lab-Line® Titer-Plate shaker
- MultiScreen Solubility Filter Plate MSS LB PC 10 (Millipore Corp. – Billerica, MA)
- MultiScreen Vacuum Manifold MAVM 096 OR (Millipore Corp. – Billerica, MA)
- Millipore Vacuum/Pressure Pump XX55 000 00 (Millipore Corp. – Billerica, MA)
- UV/Vis microplate reader (Molecular Devices – Sunnyvale, CA)

Materials

- Biohit Proline™ single and multi-channel pipettors 0.2 – 10 μ L, 50 – 1200 μ L (Biohit Oy – Helsinki, Finland)
- Stericup™ Filter Unit SCGP U05 RE (Millipore Corp. – Billerica, MA)
- 96-well disposable UV-Star™ analysis plate No. 4506-55801 (Greiner Bio-One GmbH – Frickenhausen, Germany)
- 96-well polypropylene, V-bottom collection plate No. 4506-51201 (Greiner Bio-One GmbH – Frickenhausen, Germany)
- 96-well polypropylene, 2.4 mL deep-well plate No. 4507-80280 (Greiner Bio-One GmbH – Frickenhausen, Germany)

Protocol

This protocol is for manual pipetting and liquid transfer.

A separate protocol has been written for automated and robotic methods.

Protocols for automated methods can be found online:

www.millipore.com/automation.

I. Universal Aqueous Buffer Preparation

To quantify the aqueous solubility of thirty drug compounds, 50 mL of buffer is required. With 500 mL of buffer it is possible to quantify the aqueous solubility of three hundred drug compounds.

- a. Prepare 500 mL of pH 7.4 universal aqueous buffer as follows:
 - 250 mL Type I water from a Milli-Q® ultrapure water system
 - 1.36 mL (45 mM) ethanolamine
 - 3.08 g (45 mM) potassium dihydrogen phosphate
 - 2.21 g (45 mM) potassium acetate

Thoroughly mix above reagents.

- b. Adjust the pH to 7.4 ± 0.05 with 1.0 N HCl and q.s. to 500 mL with 0.15 M KCl.
- c. Filter the final universal aqueous buffer solution with a Stericup Filter Unit (or equivalent) to remove particulates and reduce bacterial growth. Buffer may be stored at 4 °C in the dark for up to 4 weeks.

Note: Other buffers at other pHs can be substituted.

Table 1: Universal aqueous buffer preparation.

Buffer Reagent	Solution Molarity	Reagent Amount	ACS Reagent	q.s. Volume
Ethanolamine	45 mM	1.36 mL	>99.0%	500 mL
Potassium dihydrogen phosphate	45 mM	3.08 g	>99.0%	
Potassium acetate	45 mM	2.21 g	>99.0%	

II. Standards and Calibration Protocols

Note: To reduce errors in aqueous solubility analysis due to non-quantifiable compound solubility, it is recommended that each compound's standard calibration be completed and analyzed before starting the aqueous solubility assay.

Preparation of Compound Standards for Calibration

Standards are made in an 80:20 buffer:acetonitrile (AcN) solution to ensure overall compound solubility. Additionally, the level of DMSO in all calibrators is maintained at 5% (v/v), ensuring that the final solvent content of all standards and samples remains consistent. For the preparation of standards, the buffer:AcN solution and DMSO are added to the plate and mixed thoroughly before the addition of 10 mM DMSO stock compound.

This protocol details preparation of the 5 standard calibrators: 500 μM , 200 μM , 50 μM , 12.5 μM , 3.13 μM .

- To prepare a standard curve, identify 10 wells (12 wells if including solvent blanks), for example: A1, A2; A3, A4; A5, A6; A7, A8; A9, A10; and A11, A12 for solvent blanks (see Figure 1).
- Add buffer:AcN solution, DMSO and 10 mM DMSO stock to appropriate wells of a 96-well polypropylene, 2.4 mL deep-well plate, as indicated in Table 2.
- Mix wells A1 and A2 (500 μM) with pipettor (10x).
- Mix wells A3 and A4 (200 μM) with pipettor (10x).
- Transfer 100 μL of solution from A3 to A5, and 100 μL from A4 to A6. Mix wells A5 and A6 (50 μM) with pipettor (10x).
- Transfer 100 μL of solution from A5 to A7, and 100 μL from A6 to A8. Mix wells A7 and A8 (12.5 μM).
- Transfer 100 μL of solution from A7 to A9, and 100 μL from A8 to A10. Mix wells A9 and A10 (3.13 μM).

Figure 1: Standard calibrators in 96-well plate (all units are μM).

	1	2	3	4	5	6	7	8	9	10	11	12
A	500	500	200	200	50	50	12.5	12.5	3.13	3.13	0	0
B												
C												

Table 2: Preparation of standard calibrators.

	500 μM	200 μM	50 μM	12.5 μM	3.13 μM	0 (blank)
Well Locations	A1, A2	A3, A4	A5, A6	A7, A8	A9, A10	A11, A12
Buffer:AcN Solution	285 μL	380 μL	285 μL	285 μL	285 μL	285 μL
DMSO	—	12 μL	15 μL	15 μL	15 μL	15 μL
10 mM DMSO Stock	15 μL	8 μL	—	—	—	—
200 μM Standard (from A3 and A4)	—	—	100 μL	—	—	—
50 μM Standard (from A5 and A6)	—	—	—	100 μL	—	—
12.5 μM Standard (from A7 and A8)	—	—	—	—	100 μL	—

Preparation of Eight Individual Standard Curves

- a. For eight standard calibration curves (one plate), prepare approximately 50 mL of 80:20 (v:v) pH 7.4 universal buffer:acetonitrile, by combining 40 mL of pH 7.4 universal buffer (refer to *Section I, Universal Aqueous Buffer Preparation*) with 10 mL of acetonitrile.
- b. Using a multi-channel pipettor, dispense buffer:AcN solution into a 96-well polypropylene, 2.4 mL deep-well plate. Add 285 μL /well to columns 1, 2, and 5–12. Add 380 μL /well to columns 3 and 4.
- c. Using a multi-channel pipettor, dispense DMSO to the same plate. Add 12 μL /well to columns 3 and 4 and 15 μL /well to columns 5–12.
- d. Using a single channel pipettor, dispense 10 mM DMSO stock to wells in columns 1, 2, 3, and 4 of the 2.4 mL deep well (same) plate. For the first compound, add 15 μL /well to A1 and A2 and 8 μL /well to A3 and A4. For the second compound, add 15 μL /well to B1 and B2 and 8 μL /well to B3 and B4. Continue this pattern for rows C, D, E, F, G, and H using a different 10 mM DMSO stock compound for each subsequent row.
Note: If there is a limited quantity of 10 mM DMSO stock compound, use less of the amounts listed but keep the same final concentration.
- e. As in steps e – g in *Preparation of Compound Standards for Calibration*, serial dilute (1:4) 100 μL from the wells in column 3 to the wells in column 5, using a multichannel pipette. Mix 10x. Repeat with 100 μL from column 4 to column 6. After mixing, transfer 100 μL from column 5 to column 7 and 100 μL from column 6 to column 8. After mixing (10x), transfer 100 μL from the wells in column 5 to the wells in column 7 and 100 μL of wells in column 6 to the wells in column 10. Columns 11 and 12 are blanks. See Figure 2 for the concentration of compounds in the wells.
- f. Cover the 2.4 mL deep-well plate with a polystyrene lid or equivalent, and shake it gently (100 – 300 rpm) for 30 minutes at room temperature (22.5 ± 1 °C). If analysis is not done within 30 minutes of shaking, cover the plate with adhesive film to prevent evaporation.

Figure 2: Set-up for 8 standard calibration curves on a 96-well plate (all units are μM).

		1	2	3	4	5	6	7	8	9	10	11	12
Compound 1	A	500	500	200	200	50	50	12.5	12.5	3.13	3.13	0	0
Compound 2	B	500	500	200	200	50	50	12.5	12.5	3.13	3.13	0	0
Compound 3	C	500	500	200	200	50	50	12.5	12.5	3.13	3.13	0	0
Compound 4	D	500	500	200	200	50	50	12.5	12.5	3.13	3.13	0	0
Compound 5	E	500	500	200	200	50	50	12.5	12.5	3.13	3.13	0	0
Compound 6	F	500	500	200	200	50	50	12.5	12.5	3.13	3.13	0	0
Compound 7	G	500	500	200	200	50	50	12.5	12.5	3.13	3.13	0	0
Compound 8	H	500	500	200	200	50	50	12.5	12.5	3.13	3.13	0	0

- g. After shaking the 2.4 mL deep-well plate for 30 minutes, directly transfer 200 μL from each well to a 96-well disposable UV analysis plate for UV spectroscopy. At this point, it is also possible to use other analysis methods such as HPLC or LC/MS/MS to quantify the soluble compound.
- h. Scan the 96-well disposable UV analysis plate at 10 nm increments from 260 – 500 nm and determine the absorbance for each well of the UV analysis plate at each of the 25 different wavelengths of the scan.
- i. Overlay the spectral scans from each concentration of the compound (see Figure 3).
- j. Identify one or more wavelengths > 270 nm, at which the maximum absorbance for the highest concentration is > 0.1 absorbance units (AU). Select the wavelength to use for standard curve using one or more of the following criteria: The optical density (OD) at 200 μM and 500 μM are less than 2.0 AU; the OD for the 50 μM standard is significantly greater than the OD for the 12.5 μM standard; the relationship between concentration and OD for three or more standards is linear.
- k. Construct a standard curve for the compound by plotting the absorbance vs. concentration at the chosen wavelength (see Figure 4).

Figure 3: The overlaid standard spectra for ketoconazole at pH 7.4. For some compounds, it may be necessary to examine the complete absorbance spectrum (260 – 800 nm) prior to selecting the optimal wavelength for analysis.

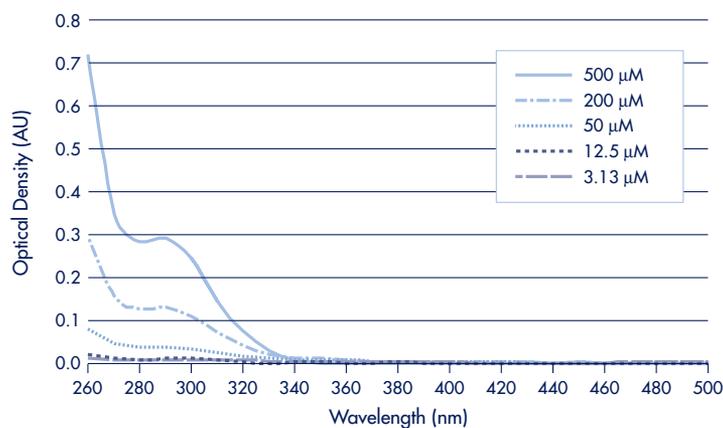
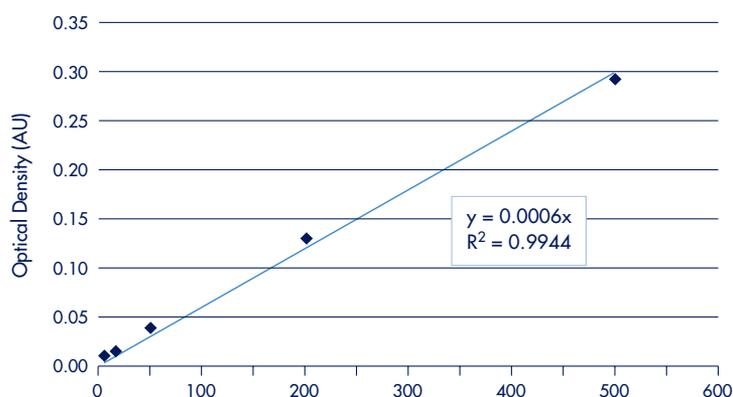


Figure 4: The standard curve for ketoconazole at pH 7.4 (based on absorbance at 290 nm).



III. 96-well Aqueous Solubility Protocols

Note: it is recommended that each compound's standard calibration be completed and analyzed before starting the aqueous solubility assay.

Preparation for 96-well Aqueous Solubility

If the compound standard calibration spectrum from *Section II, Standards and Calibration Protocols*, generates a linear curve with a correlation coefficient (concentration versus absorbance) [r^2] greater than 0.85, the aqueous solubility of that compound can be determined as already outlined (using UV/Vis spectroscopy). If the correlation coefficient [r^2] of any compound's standard curve is less than 0.8, an alternate detection method such as HPLC or LC/MS/MS may be more appropriate than UV/Vis analysis.

Preparation of the MultiScreen Solubility Filter Plate

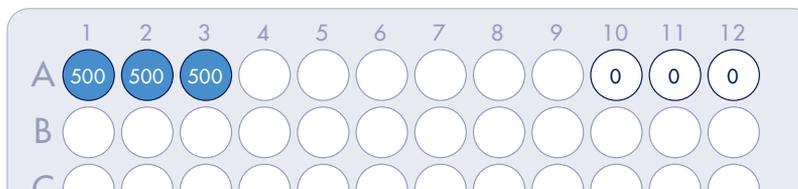
- To prepare a MultiScreen Solubility filter plate, identify 2 or 3 wells for each compound (depending on number of replicates) and 2 or 3 wells for blanks; for example, A1, A2, and A3 for compound 1, and A10, A11, and A12 for 5% DMSO blanks (see Figure 5).
- Add buffer, DMSO (for blanks), and 10 mM DMSO stock to appropriate wells as indicated in Table 3.
- Cover and gently shake (200 – 300 rpm) the MultiScreen Solubility filter plate for 90 minutes at room temperature. After 90 minutes, vacuum filter (10" Hg) the aqueous solution from the MultiScreen Solubility filter plate into a polypropylene V-bottom plate. Transfer 160 μL /well of filtrate from the V-bottom plate to a 96-well disposable, UV-Star analysis plate. Dispense 40 μL /well of acetonitrile to the UV-Star analysis plate, cover and shake gently at room temperature for 5 minutes.
- Scan the UV-Star analysis plate in a microplate spectrometer from 260 nm to 500 nm at 10 nm increments. 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:

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

Table 3: Preparation of MultiScreen Solubility filter plate.

Concentration	500 μM	0 (blank)
Well Locations	A1, A2, A3	A10, A11, A12
Buffer	190 μL	190 μL
DMSO	—	10 μL
10 mM DMSO Stock	10 μL	—

Figure 5: Preparation of MultiScreen Solubility filter plate (all units are μM).



Preparation of Thirty Individual 10 mM DMSO Stock Compounds

- Using a multi-channel pipettor, dispense 190 μL of pH 7.4 universal aqueous buffer (refer to *Section I, Universal Aqueous Buffer Preparation*) into all wells of a MultiScreen Solubility filter plate.
- Dispense 10 μL /well of DMSO into each of six wells for blanks.
- For each compound to be tested, dispense 10 μL /well of the 10 mM DMSO stock solution into each of three different wells (if performing the analysis in triplicate) into wells that have been pre-loaded with (190 μL) of the pH 7.4 universal aqueous buffer (see Figure 6).
- Cover the MultiScreen Solubility filter plate with the polystyrene lid and incubate with gentle shaking (200 – 300 rpm) for 1.5 hours at room temperature (22.5 ± 2.5 $^{\circ}\text{C}$).
- After incubation, place the MultiScreen Solubility filter plate on a vacuum manifold and filter (10 – 12" Hg) each well for 30 – 60 seconds into a polypropylene 96-well V-bottom collection plate.

Figure 6: The layout of an entire MultiScreen Solubility filter plate in which 30 different compounds (in triplicate) and 6 blanks can be analyzed.

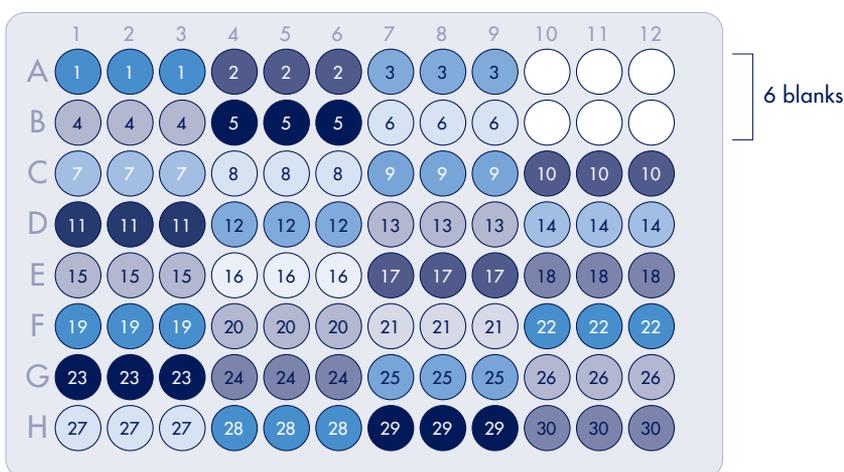
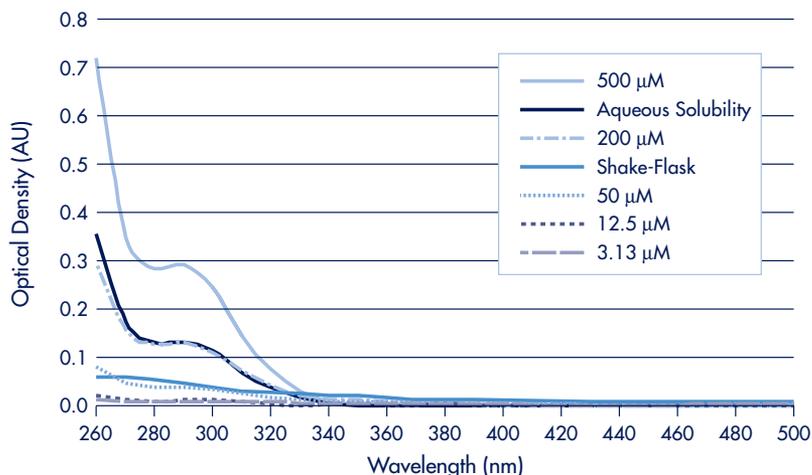


Figure 7: Spectral scans superimposed on calibration scans for ketoconazole at pH 7.4.



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- f.** After filtration, using a multi-channel pipettor, transfer 160 μL of filtrate from each well in the 96-well polypropylene V-bottom collection plate to a corresponding well in a 96-well UV-Star analysis plate.
 - g.** Using a multi-channel pipettor, add 40 μL of acetonitrile to each well of the 96-well UV-Star analysis plate (there will now be a total of 200 μL solution per well comprised of an 80:20 buffer:AcN solution).
 - h.** Cover the 96-well disposable, UV-Star analysis plate with a polystyrene lid and mix by gentle shaking (200 – 300 rpm) for 5 minutes at room temperature (22.5 ± 1 $^{\circ}\text{C}$). If analysis is not done within 30 minutes of shaking, cover the plate with adhesive film to prevent evaporation.
 - i.** After mixing, scan the 96-well disposable UV-Star analysis plate in a microplate spectrometer from 260 nm to 500 nm at 10 nm increments. At this point another analytic method, such as HPLC or LC/MS/MS may be used to quantify the soluble compound in the filtrate.
 - j.** Determine the absorbance for each well of the UV-Star analysis plate at each wavelength of the scan.
 - k.** Superimpose each compound's aqueous solubility spectral scan and calibration scan to determine if the solubility spectra match the calibration spectra. If the absorbance of the aqueous solubility filtrate sample coincides with the absorbance range of the calibration spectra, it is quantifiable. Figure 7 illustrates the superimposed ketoconazole spectral scans (including the scan of the shake-flask method sample).
 - l.** To quantify the amount of soluble compound in the ketoconazole (pH 7.4) aqueous solubility filtrate in Figure 7, divide the OD at 290 nm by the slope of the standard curve and multiply by a factor of 1.25:

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

Results and Discussion

As summarized in Table 4, there is good agreement between the shake-flask and aqueous solubility methods. In most instances, the apparent solubility in the aqueous solubility assay is somewhat higher than the solubility as measured using the shake-flask method. This is almost certainly due to the presence of 5% DMSO in the compound diluent. As reported, the effect of 5% DMSO is estimated to increase apparent aqueous solubility by approximately 40%.

Table 4. Method correlation of shake-flask vs. MultiScreen Solubility filter plates at pH 7.4.

Drug	Solubility (μM)	
	Shake-Flask	MultiScreen
Tamoxifen	3	30
4,5 DPI	25	68
β -Estradiol	31	34
Nifedipine	36	380 (113)*
Diethylstilbestrol	66	156
Clozapine	96	500
Ketoconazole	114	141
Prednisone	500	493
Testosterone	315	365
Phenazopyridine	204	355
2-Naphthoic acid	500	500
Amiloride	500	500
Amitryptiline	500	486
Atenolol	500	500
Caffeine	500	500
Chloramphenicol	500	500
Chlorpromazine	500	464
Diclofenac	500	500
Furosemide	500	500

*Solubility at 0.5% DMSO

Summary and Conclusions

This fully automatable assay can provide aqueous solubility data (in triplicate) for over one hundred-twenty drug compounds per 8-hour day. pKa data on compounds with ionizing groups can also be obtained. There are however some important method limitations. Test compounds and standards must remain soluble over the duration of the assay regardless of the solvent system. Compounds not soluble in 20% aqueous acetonitrile may produce visible precipitate and cloudiness, affecting the UV spectroscopy analysis. If precipitate is present, other analytical methods such as HPLC or LC/MS/MS may be used. Upon visual inspection, other drug compounds may contain color-producing chromophores that interfere with UV spectroscopic absorbance in the spectral range 260 – 500 nm. This chromophore interference may be eliminated by increasing the spectral range to > 500 nm. The fact that the sample is made up in a 5% (v/v) DMSO solution could result in an over-estimation of the compound's solubility in a purely aqueous solution.

Lower amounts of DMSO (0.5%) may be necessary for more accurate quantification (see nifedipine, Table 4). If the compound is not relatively pure (>95%), this method may not be suitable. A complex mixture would require some sort of chromatographic separation prior to analysis. Additionally, some impurities may alter compound solubility or contribute disproportionately to UV spectroscopic absorbance. Finally, it is essential that the test compounds have sufficient UV spectroscopic molar absorptivities (extinction coefficients) to provide the requisite analytical sensitivity.

With these limitations in mind, the assay is still well suited as a high throughput tool for a number of compound screening applications — including the determination of structure-solubility relationships and setting appropriate dosing concentration ranges for subsequent *in vitro* testing programs.

References

1. ASTM: E 1148-02, *Standard Test Method for Measurements of Aqueous Solubility*, Book of Standards Volume 11.05.

Related Application Notes

AN1730EN00: Quantitative method to determine drug aqueous solubility: optimization and correlation to standard methods

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

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