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Automated approach to couple solubility with final pH and crystallinity for pharmaceutical discovery compounds

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Abstract

The design and validation of a novel high-throughput system for thermodynamic solubility determination requiring only 5 mg of sample is described. The system uses a sintered nickel filter assembly to recover excess solids from saturated solutions for rapid crystallinity assessment via powder X-ray diffraction (PXRD). Moreover, the system measures the pH of filtrates to provide a final pH value with the solubility measurement. The limit of detection for the UV–vis plate reader used on this system is ∼0.001 mg/ml, while the practical upper limit is ∼3 mg/mL. The solubility measurements of 60 proprietary Pfizer compounds were used to validate the nickel filter assembly against a more conventional polyvinylidenedifluoride (PVDF) filter. Additionally, a comparison was made between a subset of 10 compounds run on the automated system and a more traditional shake-flask method employing HPLC analysis. In both cases, a favorable comparison was obtained.

Keywords: Automation; pH; Pharmaceutical discovery; Solubility; X-ray diffractometry

1. Introduction

The determination of aqueous solubility values plays a critical role in drug discovery as solubility and dissolution can impact the fraction of drug absorbed from the gut, as well as oral bioavailability [1,2]. Initial screens focus on kinetic solubility measurements for compound libraries since they offer the advantage of short equilibration times and use of dimethylsulfoxide (DMSO) solubilized samples [3–6]. These assays are well suited for initial binning [6] of compounds and categorizing different chemical series. Computational approaches are an option for small subsets of new chemical entities (NCEs), however applied across diverse discovery sets, these methods still fall short of desired accuracy required for decision making in drug discovery programs [7]. As compounds progress towards candidate selection, more accurate determinations are needed. The desire for automated thermodynamic solubility determination without the method development necessary for HPLC techniques has lead to assays using UV–vis plate readers [8]. Compared to the kinetic screens, these systems typically sacrifice sample throughput and require more compound. Thermodynamic solubility determining methods, however, produce more accurate solubility values necessary for formulation development and ADMET (absorption, distribution, metabolism, excretion and toxicity) profiling.

The accurate determination of a solution pH corresponding to a measured aqueous solubility value is critical for ionizable compounds, as a difference of one pH unit can cause a 10-fold difference in solubility. Additionally, the crystalline or amorphous nature of the undissolved solid in the saturated solution can have a significant impact on the final measured solubility value. To overcome fluctuations in pH, solubility screens are commonly run using a buffered solution at a physiologically relevant pH of interest [9], allowing for NCE comparison under uniform conditions. Solubility values are often reported under the assumption that the final pH is maintained by the buffer solution. While this can be the case for poorly soluble compounds, it is not uncommon for the solution pH to be significantly different from that of the starting buffer, especially for freely soluble compounds. Accurately measuring the saturated solution pH avoids the necessity to maintain buffer capacity, and with a measured

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or predicted pK_a of an NCE one can generate a pH-solubility profile using the Henderson–Hasselbalch equation [10].

In a drug discovery setting, minimal effort is exerted into crystallizing compounds, and as a direct result the solid form is rarely well understood. Recent results suggest that this could be at least partially responsible for discrepancies between kinetic and thermodynamic solubility values [11]. An amorphous form has a higher energy than its crystalline counterpart leading to a higher apparent solubility [12]. However, amorphous solid is typically less physically and chemically stable than its crystalline counterpart, making a crystalline phase preferred for development. Since the higher apparent solubility of amorphous solids can be misleading, many laboratories do not attempt thermodynamic solubility measurements until crystalline material becomes available. However, phase transformations to more stable crystal forms are known to occur when solid compound is stirred in solvent over a period of time [13,14], which means amorphous solids and metastable crystal forms may transform to more stable crystalline forms during equilibration. It is therefore advantageous, whether in the discovery or development setting, to obtain powder X-ray diffraction (PXRD) data for the solid phase remaining in the saturated solution at the time of filtration.

Providing pH and crystallinity information along with solubility results allows discovery teams to make better informed decisions about the relative strengths or liabilities of the biopharmaceutical properties of their NCEs. This report describes the integration of commercially available equipment to provide solubility with corresponding pH measurement and crystallinity check, at a rate of ∼100 NCEs per week. The system requires only 5 mg of each NCE and is capable of measuring solubility values in a range of ∼0.001–3 mg/mL. Sixty proprietary Pfizer compounds in solid form as well as solutions of known concentration were used to validate the accuracy and precision of the system.

2. Experimental

2.1. Instrumentation

The solubility workstation utilizes a Tecan Freedom Evo™ (Tecan, Maennedorf, Switzerland) as the main robotic platform as shown in Fig. 1. The system includes an eight-tip liquid handler system with 1-mL syringes. The deck also includes a pick-and-place arm for manipulation of test tubes as well as the pH probe and a Robotic Manipulator arm for transferring plates. The deck integrates a balance (SAG285 Mettler™ Toledo, Greifensee, Switzerland), shaker (Te-Shake™, Tecan), vacuum manifold (Te-Vac, Tecan), vortexer (Reax Top, Heidolph, Germany), pH meter (InoLab™ Level 2, WTW, Germany) and UV plate reader (Safire™, Tecan). PXRD data is collected on a Bruker-AXS D8 Discover™ PXRD unit (Bruker, Madison, WI). The unit is equipped with a video camera mounted along with a laser used for vertical alignment of each sample as well as a motorized *x*, *y*, *z* stage to position samples in the path of the X-ray along with a custom designed holder for the filtration unit.

2.2. Materials

All solubility analyses were performed using a pH 6.5 50-mM phosphate buffer prepared from reagent grade sodium phosphate monobasic monohydrate and sodium phosphate dibasic purchased from Sigma–Aldrich and JT Baker, respectively. To prepare solution standards for the assay, a 75:25 (v/v) 1,2-dimethoxyethane (glyme):water solution is used. The solution solubilizes the vast majority of compounds in Pfizer's NCE library up to ∼0.5 mg/mL, but unlike other solubilizing solvents such as DMSO, its UV wavelength cutoff is much lower. A 75:25 glyme:water solution has an absorbance of approximately 0.3 absorbance units at 230 nm with a 1 cm path length. The glyme:water solution is compatible with commercially available UV-transparent plates at concentrations reached under assay conditions and is sufficiently non-volatile such that evaporation does not significantly affect results under typical operation (data not shown). Reagent grade glyme and HPLC grade water were used as received from Sigma–Aldrich. Hydrocortisone (ICN Biomedicals Inc.), diltiazem hydrochloride, atenolol and enalapril maleate (Sigma–Aldrich) were prepared as solutions of known concentration. All other samples for solubility analysis were received from Pfizer Compound Management. Deep well plates (Uniplate Part No. 7701-5200) and UV transparent microplates (BD Falcon Part No. 353261) were used in the solubility assay.

2.3. Description of automated solubility protocol

To determine the solubility of a compound, the UV–vis absorbance of a saturated solution of the compound is measured

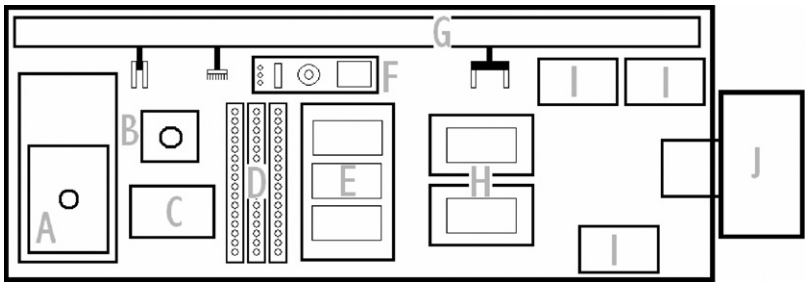

Fig. 1. Schematic diagram of solubility workstation. Components include: (A) balance, (B) vortexer, (C) shaker, (D) test tube holder rack, (E) 96-well plate holder rack, (F) pH measurement station, (G) track with pick-and-place, eight-tip liquid handling, and robotic manipulator arms, (H) vacuum filtration station, (I) plate storage hotels, and (J) UV–vis plate reader.

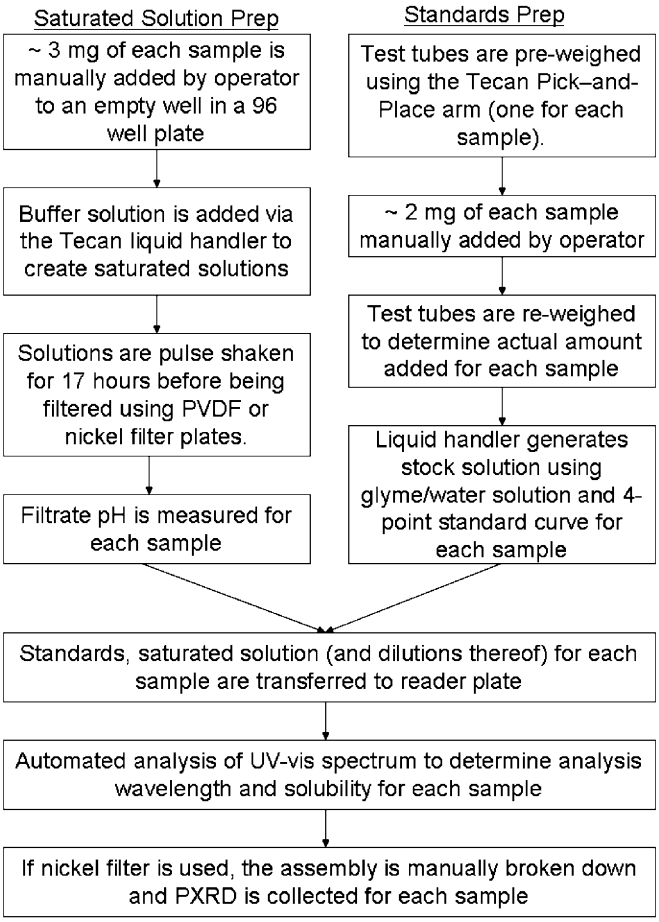

Fig. 2. Automated solubility determination process flowchart.

and the concentration calculated based on a four-point UV–vis standard curve of the compound using the Lambert–Beer Law. Fig. 2 shows a schematic of the solubility protocol designed for discovery compounds.

2.3.1. Saturated Solution Preparation

Approximately, 3 mg of each compound is manually added into wells of a 96-well polypropylene deep well plate. The system adds 1 mL of the phosphate buffer to generate saturated solutions. The samples are then equilibrated using a pulsed shaking routine. The samples are shaken at 1200 rpm for 1 min and then allowed to rest for 9 min. This 10-min cycle is repeated during the 17-h overnight equilibration. After this equilibration step, the samples are transferred to a polyvinylidenedifluoride (PVDF) filtration plate or the nickel filtration assembly (see below) to be filtered using the Te-Vac^{TM} manifold. The pH of the filtrate is recorded before solubility analysis and when using the nickel filtration assembly, the collected solid is analyzed for crystallinity.

2.3.2. Standards preparation

After saturated solutions have been prepared, tare weights are measured for empty culture tubes for each sample. Approximately, 2 mg sample of each NCE is manually added to each tube and the robot reweighs the tubes to calculate sample weight. The samples are dissolved in 5 mL of the glyme/water solution to generate a stock solution of known concentration. These solutions are each vortexed for 20 s to aid the formation of a homogeneous solution. From these stock solutions, aliquots of 1300, 1000, 700, and 200 μL are diluted with glyme/water amounts of 100, 400, 1000, and 1500 μL, respectively. This results in four standard solutions of known concentration.

2.3.3. Solubility analysis

For each sample, 20 μL aliquots from each of the four standard solutions are transferred into a 96-well UV-transparent disposable plate containing 280 μL of the aqueous buffer. This creates four solutions with known concentration in the appropriate range for the UV–vis plate reader. The amount of glyme in these solutions is 5% by volume. Subsequently transferred to the plate for each sample are 280 μL of the saturated solution, a 10- and 100-fold dilution of the staturated solution, along with a blank (the buffer solution containing no compound). To these latter solutions, 20 μL of the glyme/water solution are then added to achieve 5% glyme throughout the plate. The prepared plate is automatically transferred to the UV plate reader that scans all 96 wells from 230 to 350 nm with a 2 nm step size. A standard curve based on the Lambert–Beer law is generated for each wavelength. The NCEs solubility is then calculated from the absorbance of the saturated solution, or its dilutions, at the wavelength where the highest regression coefficient is observed for standard solutions. The wavelength with the highest regression coefficient may not be the most sensitive wavelength for analysis. In these cases, a different wavelength can be manually chosen to evaluate the solubility.

NCEs that fail to dissolve completely in the glyme/water solution produce standard curves that deviate strongly from linearity. These spectra also tend to look choppy as undissolved particles interfere with the absorbance signals. For these compounds, no solubility is reported. In some cases, these solubilities can be determined by generating a lower concentration standard curve (by weighing out less compound) or by using a solvent the compound is more soluble in to generate the standard curve.

2.4. Nickel filter assembly

The nickel filter assembly, utilized for rapid crystallinity check, is shown in Fig. 3. The assembly was custom-machined using commercially available components. The assembly consists of two main components—a filter plate and a guide plate. The filter plate consists of an aluminum support containing sintered nickel with a 0.5 μm pore size in the standard 96-well footprint. The sintered nickel separates out solids and does not diffract in the PXRD analysis range, functioning as a zero background holder for PXRD analysis. Individual flow directors for each well guide the filtrate to the collection plate, avoiding contamination between wells. The guide plate consists of a Teflon® coated block with 96 hollow wells used to provide a 1-mL working volume for the transfer of NCE slurry during the filtration. The two components are compressed together separated via a gasket that provides a tight seal for each well. The assembly is held together using several screws around the perimeter of

Fig. 3. Nickel filtration assembly to check NCEs for crystallinity. An aluminum support with 96 0.5-μm pore sintered-nickel filters is attached to a Teflon® coated block with 96 wells to provide working volume. Screws are used to clamp the two plates together, separated by a tight gasket. A vacuum pulled from below will filter the solid phase from the liquid. After filtration, the assembly is disassembled and the solids collected on the sintered nickel are analyzed by PXRD.

the assembly. After filtration, the operator can break down the assembly to get access to the solid material. Samples are then allowed to dry, lightly compacted, and analyzed by PXRD.

2.5. X-ray protocol

PXRD patterns are collected in reflectance mode while employing a Cu-radiation source with a 0.5-mm collimator operated at 40 kV/40 mA. The NCEs are scanned from 6° to 38° in 2-theta with a 60 s exposure time. Samples are oscillated 0.5 mm in the *x* and *y* directions during the scan.

2.6. pH measurement results

The automated pH measurement routine uses the pick-and-place arm to transport the pH probe (N 5900 A, Schott AG, Germany) to each saturated solution well. The probe reads the pH approximately every 2 s. Once 10 consecutive readings are obtained that have a standard deviation less than 0.5% R.S.D., the solution is considered stable and the average pH value is recorded. To determine the precision of the automated pH measurement, 32 replicate measurements were made using the same buffer solution. Fifty-one aqueous solutions and buffers ranging from pH 1.9 to 10.2 were used to determine the accuracy of pH measurement. The pH of each solution was determined using the automated routine and were also measured separately on an Accumet AR 50 pH meter after calibration with the same pH 4, 7, and 10 buffers.

2.7. Automated solubility results

The automated solubility system was validated in a two step process. The initial step involved using solutions of known concentration in place of saturated solutions. This validated the analytical processes on the system including the preparation and dilution of standards using the liquid handler and the accuracy of the UV plate reader. The results obtained were compared with the known concentrations in order to assess precision and accuracy. The second step of the validation was a comparison of solubility values generated on the system. In addition to the analytical routines validated in the first step, these runs included the automated preparation, filtration, and dilution of the saturated solution.

2.7.1. Comparison of solutions with known NCE concentration

Solutions of known concentration were prepared below known solubility values. Compound was added and diluted to desired range using the phosphate buffer. Samples were stirred overnight before eight replicates were added to the system in place of the normally prepared saturated solutions. The system measured the "solubility" of these solutions to determine how closely the measured concentration matched the concentration they were prepared at. Solutions of hydrocortisone, diltiazem hydrochloride, atenolol, and enalapril maleate prepared at different concentrations ranging from 0.010 to 2.2 mg/mL were run and analyzed on the system.

2.7.2. NCE solubility comparison

To examine how the nickel filter assembly compares to a more standard 0.45 μm PVDF filter plate, a set of 60 discovery NCEs were run on the automated system using both filters. The NCEs were selected from a wide range of therapeutic areas. Included in Table 1 is a brief summary of the properties of the 60 NCEs selected for the study.

A comparison of the automated system with traditional shake-flask data was also used to assess the accuracy and reproducibility of the system. A subset of 10 compounds was chosen for comparison. Eight replicate samples of each compound were run with the nickel filter assembly and PVDF filter plate. These results were then compared with four manually prepared saturated solutions filtered using 0.45 μm polytetrafluoroethylene (PTFE) syringe filters which were analyzed via RP-HPLC. An HP 1100 HPLC equipped with pump, degasser, autosampler, and diode array detector, along with a Zorbax™ C18 (4.6 mm × 50 mm) column were used for the RP-HPLC analysis. The mobile phase composition, injection volume, and detection wavelength were optimized for each NCE.

Table 1
Properties of 60 NCEs tested for solubility comparison

Property	Min	Max	Median
Molecular weight (Da)	219	724	401
c log *P*	−1.19	6.19	2.24
Acids	5	Salts	11
Bases	33		
Zwitterions	3		
Non-ionizable	19	'Rule of 5' compliant compounds	47

3. Results and discussion

3.1. pH measurement results

The automated pH measurement routine showed good reproducibility with 32 replicate values showing a standard deviation of 0.019 and a %R.S.D. of 0.31 indicating that well to well variability should not be a significant source of error. A plot of the manually collected data versus the automated data for 51 buffer solutions had a slope of 1.002 and a correlation coefficient of 0.981 displaying good accuracy for the solubility assay (not shown).

3.2. Comparison of solutions with known NCE concentration

Eight replicates of each solution of known concentration were run through the automated solubility routine. The known concentration was compared with the determined 'solubility' value of these solutions. The data in Fig. 4 show good accuracy and reproducibility for the all tested NCEs. The largest variability was seen at 2.2 mg/mL, the highest concentration tested. Typically, in these cases where the NCE solution is highly concentrated, the saturated solution as well as 10-fold diluted solution yield absorbance readings that fall outside the UV detector's linearity range forcing the system to rely on the 100-fold diluted solution for solubility analysis. Since the 100-fold dilution NCE solution is prepared from a 10-fold diluted solution of the 10-fold dilution, small pipetting errors can be propagated resulting in the larger variability of the absorbance signal and consequently the calculated solubility. Given the limited quantity of compound used for the testing (~5 mg), concentrations in excess of 2 mg/mL will rarely be achieved. Therefore, the reduced accuracy observed at the highest concentrations is not a concern for solubility determinations in a discovery setting.

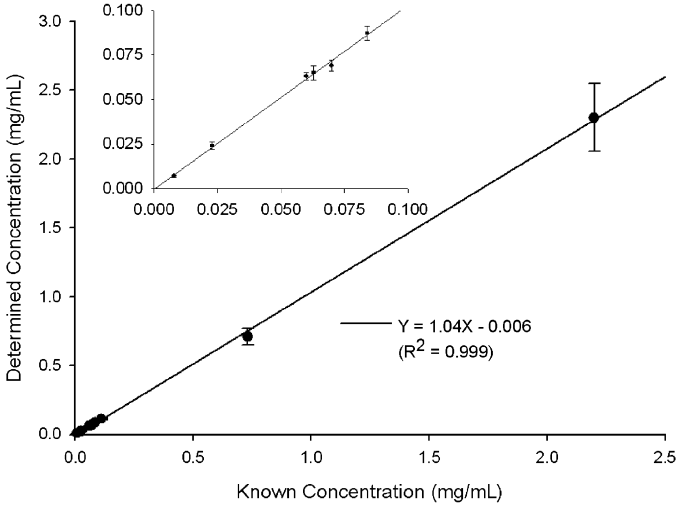

Fig. 4. Comparison of determined solution concentration and known solution concentration of NCE samples run on the automated solubility system. Error bars represent standard deviation from eight runs.

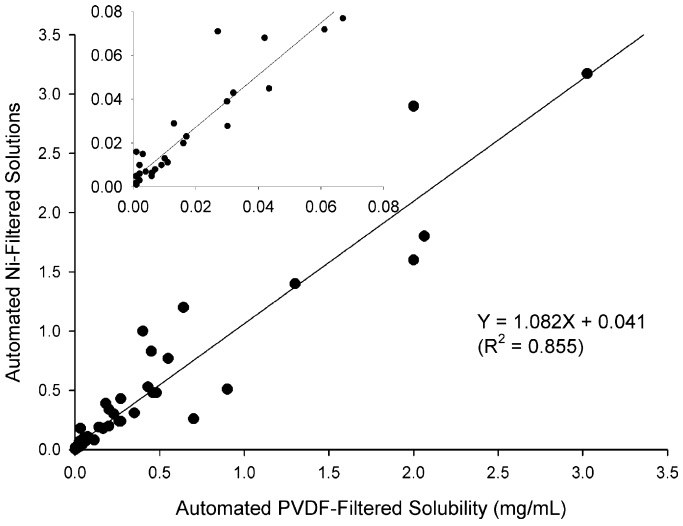

Fig. 5. Comparison of solubility results obtained for 60 Pfizer proprietary NCEs using custom sintered nickel filtration vs. conventional PVDF filtration.

3.3. NCE solubility comparison

The aqueous solubility of a set of 60 Pfizer discovery compounds was determined to examine the filter effects of the PVDF compared to the custom-made nickel filter assembly. The results shown in Fig. 5 indicate a reasonably good agreement between the two filtration systems with a regression coefficient of 0.85. While the 60 chosen NCEs do not represent the entirety of chemistry space of Pfizer's NCE libraries, the results indicate there is no systematic difference between the two filtration methods.

A subset of 10 NCEs was selected for a more detailed comparison. Eight replicates of each NCE were run using the nickel filter assembly and the PVDF filter plate. In addition, four replicates of each NCE were prepared and analyzed by RP-HPLC. A comparison of the nickel filter assembly data and the RP-HPLC data is shown in Fig. 6. There was a strong correlation between the automated runs and the manual RP-HPLC results; however, there was more variability with the replicates of nickel filtered

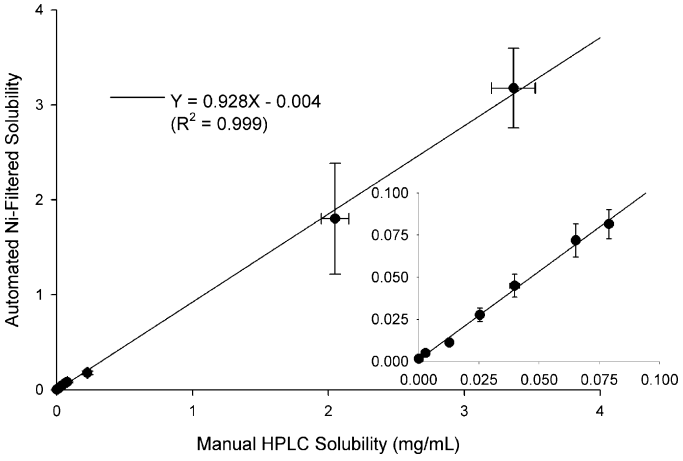

Fig. 6. Comparison of 10 solubility results obtained from sintered nickel filtration samples on the automated system ($n = 8$) and manual RP-HPLC determinations ($n = 4$). Error bars represent standard deviation from replicate samples.

samples. The PVDF filter data for the same set of 10 compounds was similar (slope = 0.925, $r^2 = 0.996$) although the variability was reduced slightly indicating that the error is likely a function of both the automated assay as well as the filtration method. For HT screening the variability was not considered to be of major concern, as it is not expected to affect decision-making by discovery teams. As compound scarcity is ameliorated past the lead-candidate stage or early development stage, conventional, more labor-intensive methods like shake-flask with HPLC analysis are typically employed.

3.4. Solid form conversions

Several NCEs were chosen to illustrate the power and insight that can be gained by including the crystallinity check. Carbamazepine exists in several polymorphic forms [15] and the conversion of the anhydrous form to the dihydrate is known to occur rapidly in water. Anhydrous carbamzepine was obtained and an initial PXRD was run on the sample. This was compared with the sample that was collected after a 17 h overnight equilibration on the solubility system. It is clear from the PXRD scans in Fig. 7 that there was a form change and the post-equilibration sample is the dihydrate form [16].

Forms A and C of chlorpropamide [17] were prepared, characterized (data not shown), and run on the automated solubility system. It appears from the PXRD patterns that the Form C initial sample does have a small, but detectable amount of Form A (peak at 11.8° 2-theta). After 36 h equilibration the samples were filtered and analyzed. The solubilities were both determined to be 2.4 mg/mL. Looking at the initial and final PXRD patterns of the samples in Fig. 8, Form C has converted to Form A, which is known to be the more stable of the two forms. It should be noted that low-angle peaks are sometimes missed by the Bruker AXS D8 Discover™ PXRD unit due to the method of integration combined with preferential orientation. The low-angle peak that appears in the final samples is known to be present in Form A.

A similar experiment was carried out with two forms of furosemide [18]. Both forms were run in the solubility screen

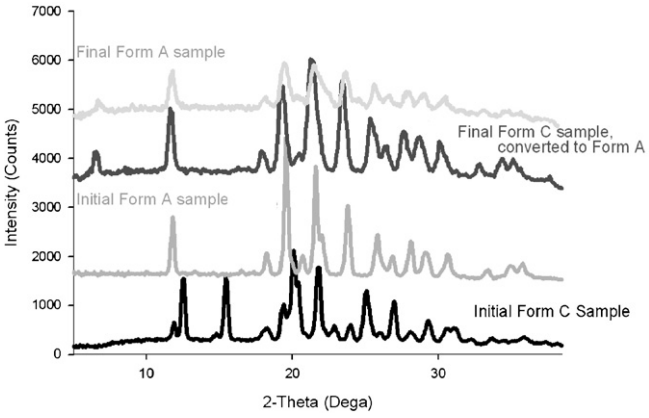

Fig. 8. PXRD scans of initial and final (36-h equilibration) chlorpropamide samples.

and the solubilities were determined to be 1.8 and 2.2 mg/mL. The PXRD of each sample after equilibration was overlaid with initial scans and showed that both samples retained their original form (see Fig. 9).

Another interesting phenomenon seen during compound screening is the conversion of amorphous samples to crystalline samples. Typically no effort is made in early discovery to crystallize the compounds. The test case of Compound A is an example of a compound that was initially an amorphous solid but began to crystallize in the overnight (17 h) slurry (see Fig. 10). During the solubility analysis of the 60 compounds a similar result was observed for the conversion of a salt form to its corresponding free form. A salt form may be freely soluble in water but limited by its free form solubility at the buffered pH of interest. In these cases the free form will crash out of solution and the solubility measured in the screen is actually the solubility of the free form and not the salt. These results can be supported using PXRD to confirm the solid form at equilibrium.

It is worth mentioning that not every sample yields a high quality PXRD signal. Samples that have high solubility will leave little solid for analysis. Also, samples that are strongly hydrophobic and do not wet well have a tendency to coat the

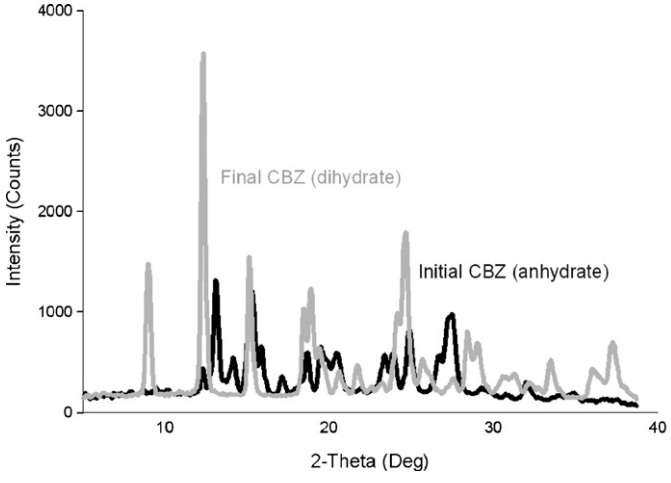

Fig. 7. PXRD scans of initial carbamazepine (anhydrate) sample and carbamazepine (dihydrate) sample recovered post-equilibration (17 h later).

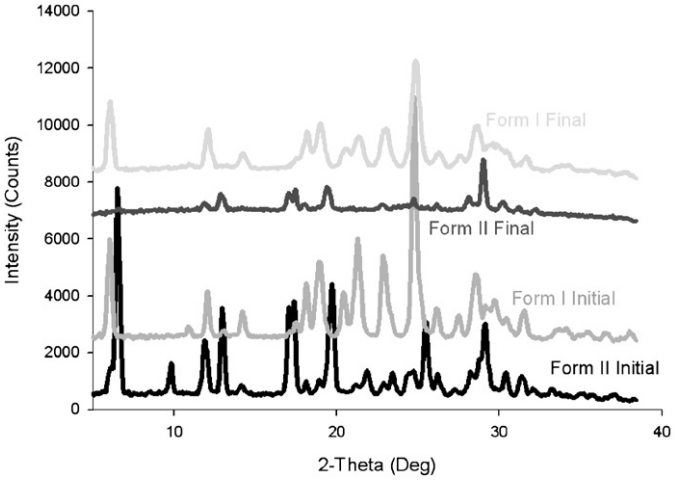

Fig. 9. PXRD scans of initial and final (36-h equilibration) furosemide samples.

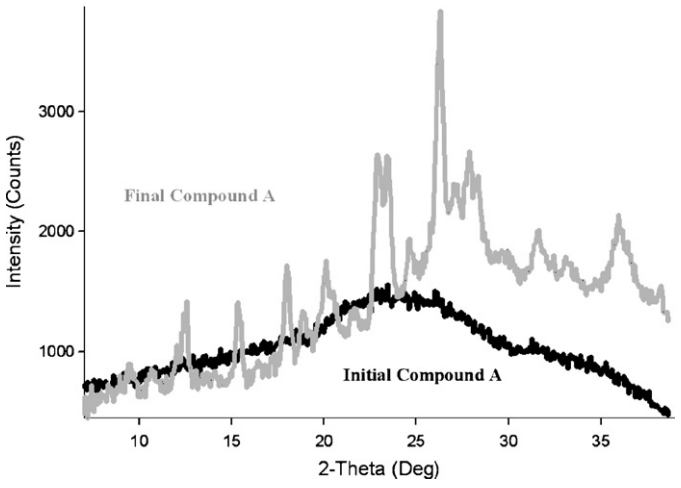

Fig. 10. PXRD scans of initial and final (17-h equilibration) compound A samples.

sides of the well and tip. These samples do not slurry well and consequently do not transfer well for collection of solid.

4. Conclusions

The results of the automated solubility platform described here were, within reasonable limits, consistent with manual determinations using HPLC. The small compound requirements of 5 mg per sample and broad determination range make the platform an ideal tool for early physicochemical profiling. The system is not compatible with compounds that are oils or gels as well as compounds that cannot be solubilized to generate an adequate standard curve. The use of the sintered nickel filtration assembly and integration of the pH measurement offers key advantages over traditional high-throughput solubility determination systems. By tracking solubility and crystallinity information, this system allows the investigator to track form changes across lots, monitor anhydrous to hydrate form changes of samples, or even determine solubility enhancement by metastable forms including the amorphous state. The database of high quality solubility data generated by the system can be used for calculations of intrinsic solubility of crystalline compounds for use as good training sets for computational solubility predictions.

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