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Automated robotic liquid handling/laser-based nephelometry system for high throughput measurement of kinetic aqueous solubility

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Abstract

The ability to rapidly and consistently measure aqueous solubility in a preclinical environment is critical to the successful identification of promising discovery compounds. The advantage of an early solubility screen is timely attrition of compounds likely to fail due to poor absorption or low bioavailability before more costly screens are performed. However, due to the large number of compounds and limited sample amounts, thermodynamic solubility measurements are not feasible at this stage. A kinetic solubility measurement is an alternative to thermodynamic measurements at the discovery stage that provides a rank listing of solubility values with minimal sample requirements. A kinetic solubility measurement is attractive from an automation vantage because it features rapid data acquisition and is amenable to multi-well formats. We describe the use of a robotic liquid/plate handler coupled to nephelometry detection for the measurement of kinetic solubility. We highlight the liquid handling validation, serial dilution parameters, and a comparison to the previous method. Experiments to further enhance throughput, or increase confidence in the automation steps, are described and the effects of these experiments are presented. In our integrated nephelometry method, we observe rapid liquid handling with an error of less than 10%, after a series of validation studies, and a sample throughput up to 1800 compounds per week. We compare the nephelometry method with our semi-thermodynamic flow-injection analysis (FIA) method, and find a 75% bin agreement between the methods.

Keywords: Nephelometry; Kinetic solubility; Robotics; High-throughput

1. Introduction

Physicochemical profiling at the early discovery stage has become a matter of considerable interest in the pharmaceutical industry, as poor bioavailability is a leading factor in compound attrition. The ability to rapidly measure absorption properties (solubility, log P, pK_a) concurrent to activity and transport screens will provide a data-based molecular property assessment so that promising compounds will quickly pass into exploratory development and, conversely, undesirable compounds will quickly fail. Moreover, reduction of discovery stage attrition has the potential for significant cost savings, provided that discovery screens can assure comparable data quality in lieu of more costly absorption, distribution, metabolism and excretion or the "ADME" determinations. Formulation and synthesis optimization, ranking compounds based on favorable properties, and risk assessment, are established benefits of an early physical property screen [1]. An integrated process for measuring solubility, chemical stability, log P, and pK_a in a preclinical environment provides a comprehensive report of physical properties [2].

Aqueous solubility is used to gauge dissolution, absorption and bioavailability of a compound. The ability to acquire solubility determinations at a comparable rate to screening data would identify and eliminate poorly soluble compounds showing good efficacy, thus enabling development of compounds with both good efficacy and good solubility. Despite all the touted benefits of an early-stage physical property screen, there are severe limitations on sample amount, time,

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and resources. Traditional thermodynamic solubility measurements (allowing a solid to equilibrate with a liquid medium, followed by sample quantification) are not feasible at the early discovery stage because of the large sample requirement, low throughput, and labor-intensive sample preparation. An alternative to equilibrium solubility is kinetic solubility in which compounds are pre-dissolved in dimethyl sulfoxide (DMSO) and the solubility is measured as the concentration at which the sample precipitates from aqueous medium. A kinetic solubility measurement is not intended to serve as a substitute for a thermodynamic solubility value because crystal lattice effects are negated when the compound is dissolved in DMSO. Thus, any effects on solubility due to changes in polymorph form cannot be investigated. However, at an early-discovery stage, the use of a kinetic solubility measurement is advantageous because there is a minimal sample requirement, it is more amenable to automated methods, and often does not require sample preparation. Moreover, kinetic solubility is appropriate in the discovery stage not only from a compound optimization perspective but also from a screening development vantage, since most screens are run using compounds in a 0.5–5% (v/v) DMSO medium [1].

Several kinetic solubility methods have been described in the literature, using a range of chromatographic and light scattering techniques. Flow-injection analysis (FIA) is a semi-thermodynamic method in which pre-solvated compounds equilibrate with pH 6.5 phosphate buffer overnight prior to analysis [2]. A flow cytometry method that is capable of measuring solubility between 22 and 556 μg/mL has been described in public forums [3]. Lipinski et al. have published a method to measure solubility by adding 10 μg/μL DMSO stock solution dropwise to pH 7 phosphate buffer in a cuvette and using turbidity to detect precipitation [4]. Yet, the practice of pharmaceutical companies to store their compounds in collections of 96- or 384-well plates has stimulated the desire to identify a technology approach capable of deriving solubility directly from this arrayed format.

In a 96-well plate format, pH-solubility profiles were demonstrated using robotic liquid handling to add aqueous medium, an orbital shaker to mix samples for 3–6 h followed by filtration and a direct UV assay [5]. Direct plate reading by ultraviolet–visible spectrophotometry (UV-vis) or nephelometry adds a desirable feature to kinetic solubility measurements because multiple wells can be assayed simultaneously [6]. A nephelometric method to determine solubility for compounds directly diluted in the microtiter plate format was shown to yield experimental results comparable to existing industry methods, but was outlined as a standalone workstation process [7]. Using a liquid handler to perform direct dilution of predissolved samples and nephelometric detection, Quarterman et al. demonstrated the utility of rank solubility analysis in a single plate [8]. While the ability to determine a valid solubility measurement in the microplate format is a critical advancement, the added criteria of fast, fully automated plate generation and subsequent data acquisition have arisen. Recent throughput increases in activity and transport laboratories that are screening early-stage compounds in parallel has increased the demand for solubility measurements at rates comparable to these other screens. In our own experience, traditional analytical bench methods have proven to be insufficiently mechanized to keep up with these other higher-throughput operations. New chemical materials are synthesized and submitted to discovery laboratories at an unprecedented rate, necessitating analytical support for these compounds at a comparable rate to hits generated from high throughput screening (HTS) platforms.

One foundation in current robotic screening technology is the implementation of liquid handling systems that utilize 96- or 384-well formats and robotic transportation to increase throughput. In initial studies, 96- and 384-well plates were compared for use. The 384-well plates were unacceptably sensitive to airborne dust and minute air bubbles and yielded an unacceptable percentage of false positives. The larger working volume in the 96-well plates and resulting decreased surface tension minimized air bubbles; dust effects were also significantly lessened. Thus a fully integrated system in the 96-well plate format, capable of rapid assay plate production, took advantage of the compatibility of nephelometry with automated liquid handling. The ability to manage multiple 96-well plates without user intervention to obtain solubility values was developed with the goal of rapid liquid handling while maintaining accurate, reproducible results. Requirements for such a system were: robotic transportation, a multichannel liquid dispenser, nephelometer microplate reader, microplate storage, barcode reader, and associated data management software necessary to allow for these user-free runs.

While the assay's buffer pH and ionic composition was previously chosen and validated, this method is easily transferable to different aqueous buffers. The only requirement is to build a unique liquid class, as defined in the Tecan Genesis software, which will account for changes in viscosity and surface tension. Due to the change from a vial-based assay in our FIA method to a robotic plate-based assay, the sample preparation process and % DMSO had to be reexamined. Based upon literature research, a need to accurately quantitate precipitation, and prior experience, serial dilutions were chosen to create a range of concentrations from 0.1 to 200 μM in the assay plates. Li et al. compared three in vitro precipitation methods: direct dilution, static serial dilution, and dynamic injection [9]. Their results concluded that the static serial dilution method, with a 24-h standing time, was best suited to quantifying precipitation. The solubility enhancement effect of organic cosolvents in modest amounts, such as 1–2% DMSO (v/v), in the solubility medium has been observed to increase the solubility of compounds across the Biopharmaceutics Classification System (BCS) [10]. We theorized that a serial dilution would maintain a constant DMSO volume so that the entire concentration gradient would 'experience' the same degree of enhancement. Our previous FIA method had

used a maximum of 2% DMSO and we wanted to maintain consistency between the two methods. Therefore, the DMSO in the assay plates was kept constant at 2% (v/v) and phosphate buffer (pH 6.5; 0.01 M) was used.

To ensure the data quality while transitioning to this fully automated system, we validated that the protocol liquid transfers were accurate and precise. We also ran a predefined set of compounds through an existing FIA method, operating at acceptable accuracy, concurrent to the nephelometry method to affirm that the nephelometric system generated data that was equivalent to known values. In this paper, we present a platform of commercially available system components, identify an assay protocol adapted for such components, describe protocols to derive the liquid handling parameters necessary to optimize and maintain these assay conditions, and outline a series of data comparison tests designed to commission the system with acceptable confidence in data quality. The resulting system provides for a ninefold increase in throughput over the flow-injection method.

2. Experimental

2.1. Apparatus

Integration of a robotic liquid handling, plate barcode reader, and microplate storage were accomplished on the Tecan Genesis platform (Tecan, Maennedorf, Switzerland), as shown in Fig. 1. The liquid handling system was the Te-MO 96 channel pipettor, fitted with a disposable tip pipetting head capable of loading 100 or 200 μL disposable tips (Tecan). For the solubility assay and Te-MO validation steps, Tecan 200 μL disposable tips for GenMate/Te-MO were used. The 96-well microplates were housed in a high-speed carousel (Tecan) and transportation of plates from the carousel and liquid handler or nephelometer was achieved using Tecan's Robotic Manipulator Arm (ROMA). Reagents were replenished in refillable reagent troughs (Te-MO trough rack liners), and wash solution was supplied for tip wash steps using the Tecan wash and refill system. Sample assay plates

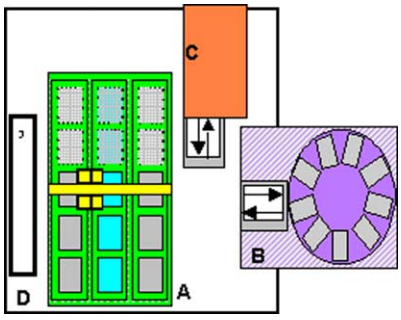

Fig. 1. Schematic diagram of automated solubility system. Components include: (A) Te-MO liquid handling unit, (B) Carousel Plate Storage, (C) Nephelometer, and (D) ROMA arm. The entire unit is covered by plexiglass to minimize contamination by dust.

were read using the BMG Nephelometer (BMG Labtechnologies, Offenburg, Germany), equipped with a 635 nm laser. Data generated by the nephelometer was exported, analyzed, and entered into Oracle database tables using in-house software. This software passes barcodes from the Tecan Genesis software to automatically register each plate and perform solubility calculations. The data handling package allows for removal of outliers, and recalculation of solubility values. A CCS Packard microplate fluorometer (Perkin Elmer, Boston, MA) was used for liquid handling validation studies.

2.2. Materials and chemicals

Five different types of plates: Greiner 655801 and Greiner PS (Greiner-Bio One, Germany); Costar 9017, Costar 3635 and Costar 3615 (Corning, Corning NY, USA) were evaluated to ascertain average background readings and well-to-well variation. Dimethyl sulfoxide (DMSO) and potassium phosphate monobasic were obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). Serial dilutions of samples dissolved in DMSO were performed in Costar 96-well, round-bottom polypropylene plates, called dilution plates hereafter (Corning, Corning NY, USA). 0.1 M phosphate buffer of 200 μL, pH 6.5 were dispensed into Greiner UV-Star clear flat-bottom 96-well plates (Greiner-Bio One, Germany) hereby called assay plates. Volumetric validation assay plates used for the fluorometric test were Costar #3691 black polystyrene 96-well flat-bottom (Corning, Corning NY, USA). Phosphate buffer (pH 6.5; 0.01 M) was prepared using potassium phosphate monobasic adjusted to pH 6.5 with 1 N KOH (Baker, Philipsburg, NJ, USA), and filtered with a 0.2 μm sulfone filter (Corning, Corning NY, USA). The 10 mM sodium borate buffer used for volume validation was diluted from a 40 g/L stock solution of sodium borate (VWR Scientific, West Chester, PA). The 0.1 and 0.4 mM 5,6-carboxyfluoroscein test solution was prepared by adding 5,6-carboxyfluoroscein (Molecular Probes, Oregon, USA) to DMSO. Proprietary compounds were supplied in-house and used as-received.

2.3. Solubility assay protocol

Replicate sets of assay plates were prepared to offset spurious data due to scratched or dirty wells, a diagram of the dilution and assay plates preparation is in Fig. 2. Using the Te-MO liquid handler, 200 μL of phosphate buffer were dispensed into 40 assay plates (two replicates of 20). These assay plates were stored in the carousel while 25 μL of DMSO were dispensed into 19 dilution plates. The master plate, containing 96 samples dissolved in 10 mM DMSO, was serially diluted 2:1 with DMSO on the Te-MO deck. The 20th plate is a true zero plate, containing only buffer and 2% (v/v) DMSO. All 96 compounds are transferred to each dilution plate, and the dilutions occur between multiple plates instead of within one single plate as described by Quarterman et al. [8]. The

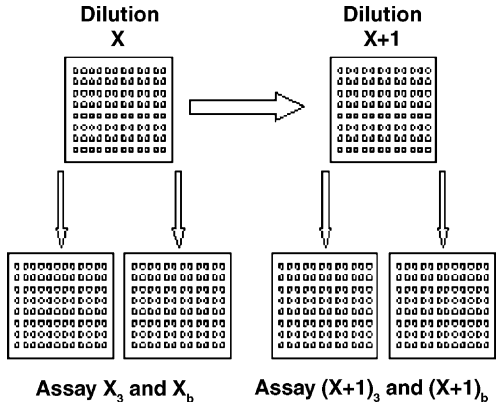

Fig. 2. Flow diagram of the serial dilution process. DMSO (10 mM) samples are received in the Master Plate (MP) and serial diluted in Dilution Plates (DL) filled with DMSO. A 4 μL aliquot from the Dilution Plate is added to a duplicate set of Assay Plates (AP), filled with aqueous buffer. See text for concentration values.

concentration range for the dilution plates was 0.0–10 mM. Samples for assay plates were prepared by adding 4 μL from each dilution plate to two corresponding assay plates, to produce a range in concentration from 0.0 to 200 μM. After sample addition, assay plates were stored again in the covered carousel to avoid airborne contamination. The ROMA was used to transport the assay plates to the Nephelostar for measurement, starting with the zero plate, to ensure equal settling time of precipitation. The solubility point was taken as the concentration at the plate where the nephelometric reading deviated from the background, using a standard deviation algorithm.

2.4. Liquid handler validation

The Tecan Te-MO was assessed for its volumetric accuracy during cross-plate transfer protocols, and its intra-plate precision across wells over multiple transfers. Volume dispensing and aspiration from the Te-MO liquid handler was validated for the appropriate liquids over wide volume ranges, and subsequently optimized to balance rapid liquid handling with accuracy and precision requirements. A liquid transfer validation protocol was developed to minimize cross-plate contamination and verify maintained precision of small-volume DMSO transfers throughout the solubility range. For volumes less than 10 μL, gravimetric testing for accuracy and visual testing of precision was impractical because variations between wells are not readily detected. However, it was necessary to validate liquid handling at these low volumes to ensure an accurate 4 μL transfer of DMSO from the dilution plates into the assay plates.

Statistical comparison between individual mean plate fluorescence $\left(\sum \text{RFU}/96\right)$, in relative fluorescence units (RFU), and target fluorescence from a reference plate containing 204 μL of 1.96 (M 5,6-carboxyfluorescein in 2% DMSO/buffer gave overall plate accuracy (%OPA) to the target volume based upon the following equation:

$$\%\text{OPA} = 100 - \text{ABS} \times \left[\left(\frac{\text{Target RFU} - \text{Mean Plate RFU}}{\text{Target RFU}}\right) \times 100\right]$$

Additionally, well values were compared to mean fluorescence values at a low concentration and a high concentration reference plate (containing 204 μL of 1.575 μM and 2.344 μM 5,6-carboxyfluorescein in 2% DMSO/buffer, respectively), to determine if individual wells maintained acceptable accuracy limits. In-plate precision between wells was calculated by looking at corresponding coefficients of variance (%CV), where S.D. is the standard deviation:

$$\%\text{CV} = \frac{\text{Mean Plate RFU}}{\text{S.D.}} \times 100$$

For instances when precision could be calculated, a coefficient of variance of 10% or less was deemed acceptable.

2.5. Volume addition validation

Volumes of 10 μL or greater were visually inspected for precision and measured gravimetrically for accuracy. To validate the serial dilution transfers of 25 μL, the Te-MO dispensed 25 μL of DMSO into five, pre-weighed in duplicate, polypropylene plates. After addition of DMSO, plates were again weighed in duplicate. The average volume dispensed per well (AVD) was determined by the following equation:

$$\text{AVD}\,(\mu\text{L}) = \frac{\text{Final Weight} - \text{Initial Weight}}{96 \times \text{RD} \times 1000}$$

where RD constitutes the relative density of the solution being added. The overall accuracy (%OA) of the 25 μL addition was computed by:

$$\%\text{OA} = 100 - \text{ABS}\left[\left(\frac{\text{TVPW} - \text{AVD}}{\text{TVPW}}\right) \times 100\right]$$

where TVPW is the target volume per well, in microliters. Gravimetric measurements confirmed volume accuracy across the plate, while visual inspection provided facile determination of obvious outliers. The gravimetric validation was repeated for 50 μL DMSO additions, 200 μL phosphate buffer additions, and 200 μL borate buffer additions. For low-volume additions, accuracy and precision were evaluated by measuring relative fluorescence of a DMSO stock solution of 0.1 mM 5,6-carboxyfluorescein, diluted in 10 mM sodium buffer. Statistical analysis assessed sample plate data to reference plates of equivalent volume and percentage DMSO with known concentrations. The sample and reference plates were read at identical settings in the same fluorescence reader. The Te-MO transferred 4 μL of 0.1 mM 5,6-carboxyfluorescein in DMSO from a pre-filled dilution plate into five separate black polystyrene plates, each containing 200 μL of the borate buffer. These five plates

were linearly shaken for 30 s, and were read by the fluorometer at an excitation wavelength of 485 nm and an emission wavelength of 530 nm, with a 0.5 s per-well read time.

2.6. Combined volume addition and volume transfer validation

A trial of the assay protocol was performed to monitor cross-plate sample carry-over in the serial dilution step and assay plate creation by loading a pre-filled mock compound plate containing 75 μL of 0.4 mM 5,6-carboxyfluorescein in DMSO into the carousel and using the Te-MO create nine subsequent dilution plates. A 4 μL aliquot from each dilution plate was transferred to a corresponding assay plate and read offline on the fluorometer. All plates were checked for intra-plate precision, and mean fluorescent values for 10 plates were analyzed for ascending RFU linearity to confirm the dilutions, based on:

$$R^2 = 1 - \frac{\mathrm{SSE}}{\mathrm{SST}}, \quad \text{where SSE} = \sum (x_i - \bar{x}_i)^2 \quad \text{and}$$

$$\mathrm{SST} = \left(\sum x_i^2\right) - \frac{\left(\sum x_i\right)^2}{N}$$

2.7. Nephelometer settings

For optimal 96-well plate reads on the Nephelostar, BMG recommends a laser beam focus of 2.5 mm, and a plate positioning delay of 0.3 s. Optimal values for the remaining parameters of 96-well Nephelostar plate reads are assay-dependent. The variables of shaking assay plates prior to reading, laser gain, measurement time per well, and multiple cycles per plate read (for obtaining an averaged read value) were evaluated to determine the optimal nephelometer settings for the solubility protocol. Optimal settings were obtained by first using pre-validated equipment to create assay plates offline, single assay plates were read multiple times for varied parameters and subsequent changes to data output were recorded. During tests, parameters were manipulated independently to ensure accurate interpretation of data, and the assay plate was kept inside the Nephelostar to minimize exposure to dust.

2.8. Effect of standing on nephelometry readings

In traditional thermodynamic solubility measurements, minimum standing times of 24 h are used to assure sample-medium equilibrium. For kinetic solubility methods, such wait times are unacceptable due to evaporation of buffer or precipitation of sample out of DMSO. To assess appropriate kinetic solubility standing times, two sets of assay plates containing 124 compounds were created and read after 30 min, 4 h, and 24 h of preparation to determine if compound precipitation or compound re-solvation would occur for the assay conditions. Plates were covered in a controlled environment to prevent buffer evaporation. Assay plates were loaded offline in the nephelometer at the specified time intervals and read using the assay settings previously described. Data was exported, sorted by compound, and analyzed using consistent criteria to derive the appropriate solubility "bin" at each read time. The "bins" were: solubility was reported as >50 μM, it is labeled soluble; 10–50 μM, partially soluble; <10 μM, not soluble. The calculated solubility values were statistically compared to determine any significant time-resolved changes when sample were left standing.

2.9. Validation and comparison of experimental data

Nephelometric solubility was shown to give acceptable results for reference standards, but in order to use it as a high-throughput method, it was necessary to compare solubility values as measured by the flow-injection analysis (FIA) method with the new nephelometric method for pharmaceutical compounds. Moreover, it was critical to have an understanding if (and how) compounds in each therapeutic project would be affected by the change to a kinetic solubility method. Consistent data between the two methods not only increased confidence in the kinetic method, but also provided direct feedback on experimental results to enable a transition from the existing process, as solubility data is important to direct synthesis efforts. In our first comparison, 594 research compounds were first analyzed by FIA. The same stock solution was later used, 2–4 weeks after the FIA experiment, to prepare a plate for nephelometric analysis. A follow-up study tested another 367 compounds between the two methods, where samples for each method were prepared concurrently and each method ran simultaneously. Comparisons of the data were done with a bin method described above. The methods were a match if both results belonged to the same bin, and were considered not a match if one method labeled the compound soluble and the other method labeled it insoluble.

2.10. Nephelometer data output

For the nephelometry method to be successful, the data handling and analysis mechanism must be able to track and integrate large volumes of data. The approach to data handling was to consider it as vital to the overall success of the method. Dilution and assay plates were barcoded, an in-house program passed the barcodes and concentration values to a database and linked them with the nephelometric data. A locally written interface allowed for rapid manipulation of the data, outliers could be easily seen in graphical user interfaces (GUI), as demonstrated in Fig. 3. The data analysis software also gave a graphical representation of the plate, each well was color coded to reflect high, medium, and low solubility results. The program used a standard deviation algorithm to determine the solubility point. Both replications appeared on the same graph, and an outlier could be removed with a mouse click. A database collected all the data, including information

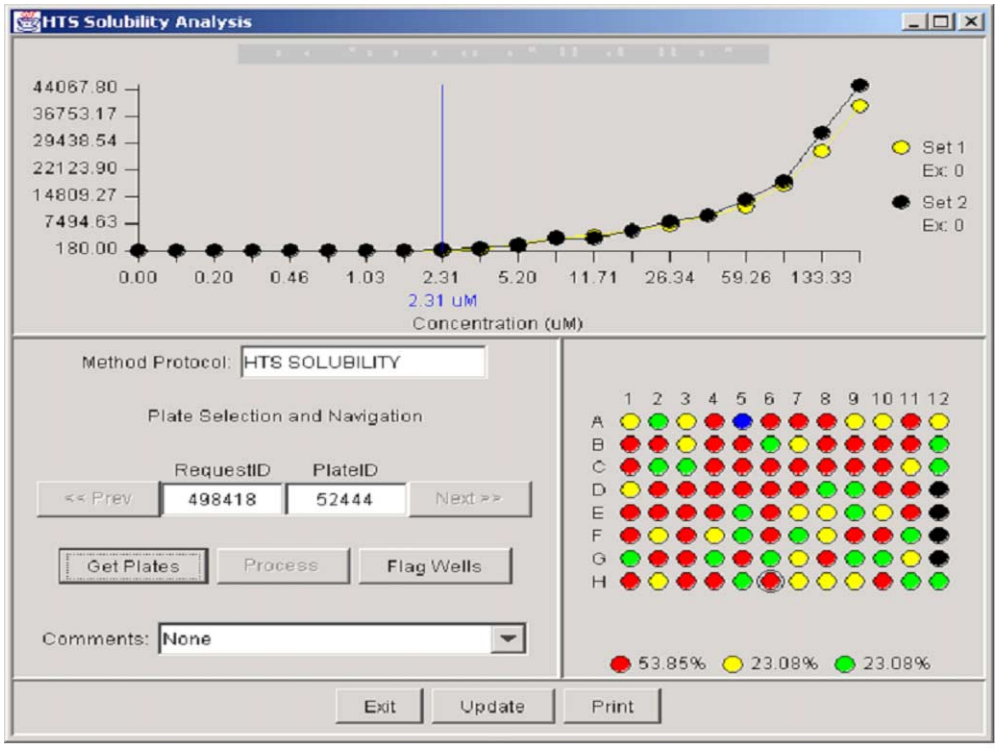

Fig. 3. GUI of in-house solubility analysis program. Green wells indicate a reported solubility >50 μM, yellow wells report solubility 10–50 μM, red wells report solubility <10 μM.

on outliers, and posted the results, which were immediately available for viewing.

3. Results and discussion

3.1. Plate quality

Nephelometer readings are affected by scratches and dust, often yielding an incorrectly low solubility value. To avoid nephelometer readings from scratches or dust, it is integral that the assay plates have an unmarred and dust-free bottom. While an individual bad well may be removed as an outlier, statistical integrity requires that the overall plate average and the median values are close together, and that the standard deviation between wells is as low as possible. Two Greiner and three Costar plates were evaluated for overall quality and compliance with the automated method based on manufacturer-provided information of well clarity and performance in traditional absorbance tests. The plates chosen for the evaluation were tested in triplicate. Testing indicated a wide range in plate quality for nephelometry, shown in Table 1. The median readings were lower than the average for all the plates, implying that a small number of high readings were raising the average. The Greiner 655801(UV-Star) plates were used for the remainder of the experiments, as they yielded the lowest background and smallest standard deviations allowing us to better distinguish increases in nephelometer scatter due to true sample precipitation.

3.2. Liquid handler validation

To ensure assay data integrity, two requirements must be satisfied: accurate sample concentrations in both the dilution and assay plates, and a constant 2% (v/v) DMSO/buffer volume ratio in the assay plates. Large volume additions and transfers were considered acceptable if the average volume dispensed per well, as measured gravimetrically, was within 10% of the target volume and visual verification indicated negligible variation between individual wells. The Te-MO default settings for the head motor were initially used at each test volume and modifications were made if necessary. These motor settings were incorporated into production protocols once five sequential plates met criteria. 25 μL DMSO addi-

Table 1
Comparison of nephelometer baseline values for various plates

Plate type	Average reading	Median reading	High reading	Low reading	Standard deviation
Greiner 655801	194	182	361	94	54
Greiner PS	447	416	1275	305	156
Costar 9017	211	198	699	128	64
Costar 3635	570	564	1796	440	102
Costar 3615	414	391	2369	309	154

tions measured gravimetrically had an average volume per well accuracy ranging from 92.13% to 94.50%. The 50 μL DMSO additions ranged from 98.79% to 99.78% accuracy and 200 μL phosphate additions maintained 99.28–99.78% accuracy. No visible in-plate variation was noted on any of the test plates, thus volume transfer precision for all aforementioned transfers was acceptable. The 200 μL, 10 mM borate buffer additions yielded gravimetric accuracies ranging from 99.22% to 99.48% with good precision, which was considered suitable to use this addition protocol to verify the 4 μL transfer of 5,6-carboxyfluorescein in DMSO. The initial 4 μL transfers gave overall plate accuracy ranging from 84.92% to 89.41%, and CV ranging from 1.66% to 1.87%. The low accuracy was deemed unacceptable, and was addressed by optimizing Te-MO motor settings affecting the rate of liquid aspiration and dispensing. We initially observed that the volume delivered was higher than the target volume; the Te-MO transfer motor's calibration offset was decreased by 0.17 to lower the delivered volume. This approach was taken primarily to compensate for the higher viscosity of DMSO and capillary effects that may have become significant at this low volume. Changing the offset parameter directly lowered the number of motor steps incremented per requested μL. Other changes that were incorporated included slightly slowing the aspiration speed to 5 μL/s and adjusting the dispense speed to 10 μL/s to maintain intra-plate precision for the lowered volume. These settings were revalidated and all five plates were within acceptable criteria yielding overall plate accuracy ranging from 90.20% to 95.99% and in-plate CV ranging from 2.12% to 2.78%. No well had accuracy outside of ±20% of 4 μL. All validated settings were entered into the assay protocol production script.

3.3. Combined volume addition and volume transfer validation

The 10 plate assay protocol and resulting dilution linearity analysis was initially run without changing or washing tips. A signal versus concentration linearity determination over the series of 10 test plates yielded an acceptable R^2 value of 0.99, but showed individual intra-plate coefficient of variances ranging from 3.51% to 14.73%. This high coefficient of variation indicated that an unacceptable level of inter-plate contamination had occurred during the assay plate creation. A likely cause was that significant residue in the Te-MO tips from 4 μL additions of lower concentration dilution plates affected the target concentration in higher concentration plates. Due to the throughput and cost implications of changing tips for each transfer or between dilution plates to assay plate, a flow-through wash trough (Tecan) was added to the Te-MO deck and a wash step between transfers was incorporated. Deionized water was identified for the wash solvent, and was circulated at approximately 3L/h by an electric pump. Initial wash settings of 75 μL for three cycles using slow aspiration and dispense speeds were unsuccessful in removing the carry-over; the settings were optimized to a wash volume of

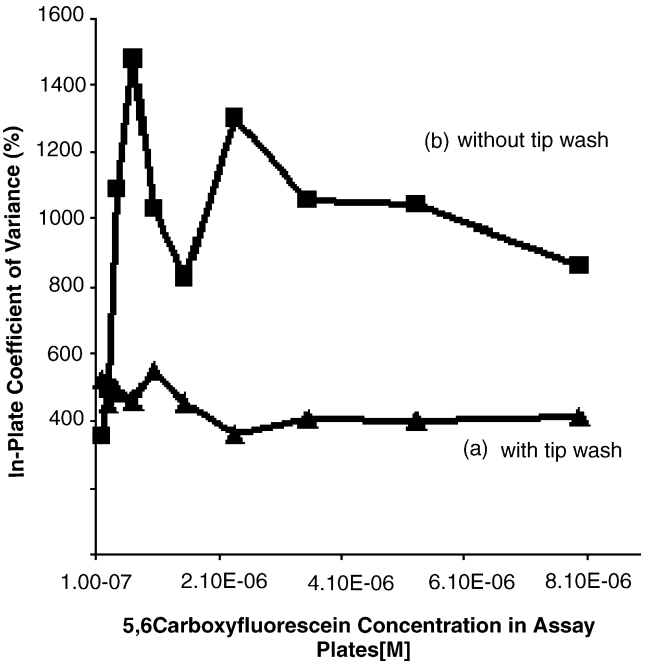

Fig. 4. Assay plate volume transfer precision using the 10-step mock serial dilution plates as the source. With wash step between dilution (a); no wash step between dilution (b).

100 μL between transfers at three cycles per wash with rapid aspiration and dispense speed (greater than 100 μL/s). Employing this optimized tip wash between each serial dilution decreased the variance between wells throughout the solubility range. Fluorescence signals were linear (R^2 = 0.9914) in the concentration range 0.1–8.10 μM. Fig. 4 illustrates the effect of tip wash between serial dilutions. There is a noticeable decrease in intra-plate coefficient of variation when a tip wash was incorporated between serial dilutions (2a) compared to (2b) when no wash was performed between serial dilutions. When the tip wash step and refined wash parameters were integrated into the assay protocol the coefficient of variance ranged from 3.59% to 5.48%, with a consistent R^2 value of 0.99. The DMSO solution, coupled with the intense turbulence of the wash cycle, has been demonstrated to sufficiently eliminate carry-over for pharmaceutical compounds.

3.4. Nephelometer settings

Tests showed no significant advantage of a prolonged pre-read shake to detect sample precipitation; a short pre-read shake was incorporated into the nephelometer settings. Laser gain settings were determined to balance signal sensitivity with instrument output. Low laser gains (5–15) slightly improved the ability of the Nephelostar to distinguish changes in relative precipitation at higher concentrations, but also led to a corresponding diminished sensitivity at the lower concentrations. High gains of 30 or greater led to a slight improvement in low concentration sensitivity, which was deemed desirable. Yet, the improvement was moderate and the potential

Table 2
The effect of multiple cycles per plate read on background and precipitated sample values

Number of cycles	1	2	3
Average read background	191	190	191
Average read precipitated well	23070	22961	23104

of a shortened detector lifetime due to the high gain was undesirable. Mid-range gains (15–30) accurately covered the desired concentration range with the best prospects for sustained performance, and a gain of 22 was selected. Increasing measurement time per well proved comparable to increasing the gain on a linear scale, and was deemed unnecessary due to the gain optimization. Incorporating multiple read cycles per plate to obtain an average read value would improve confidence in data but would increase the total run time. Moreover, increasing the number of read cycles yielded a negligible change to the average read value, for both blank and precipitated samples, as shown in Table 2. The complete final read settings for the solubility assay protocol are outlined in Table 3.

3.5. Effect of standing on nephelometric readings

If kinetic solubility samples are left standing for time intervals approaching the typical stir time for a thermodynamic method, 24 h, the kinetic solubility values begin to approach thermodynamic-like values. Moreover, our previous FIA method left covered samples unstirred overnight and, in order to maintain consistency between methods, we wanted to determine if an overnight standing time was necessary for the robotic method. To study the effect of sample standing on solubility values, 124 proprietary compounds were analyzed at 30 min, 4 h, and 24 h. A representative sample of this data set appears in Table 4. We observed when samples were sorted into bins and the bins compared across the three intervals, only 2.3% of the compounds show differences at the time intervals. The data bin reported was consistent because the plates were covered to prevent buffer evaporation. While implementing the practice of covering plates is appealing, it is not feasible for an automated assay because it would require additional resources, user-intervention and would increase the run time by 1–2 h. Previous research by the authors showed evaporation in uncovered plates, after overnight exposure, was 12%, as determined by unpublished work by the authors. To avoid the problem of evaporation, plates were left uncovered but read within 1 h of preparation. Some samples were observed to have a continually decreasing solubility, indicating that the compound slowly precipitated from the aqueous medium. A broadly applicable solubility protocol with a short standing time may overestimate the solubility for these compounds, but the small percent of compounds that changed bins throughout our kinetic read study suggested that even if the solubility value does change, its bin would likely not change.

Table 3
Nephelostar settings used throughout validation studies and solubility measurements

Number of cycles	1
Measurement time/well (s)	0.1
Positioning delay (s)	0.3
Gain	22
Laser beam focus	2.50
Shake time (s)	10
Orbital shake width (mm)	3

Table 4
Effect of standing on solubility values for representative samples

Molecular weight (g/mol)	Solubility values (μM)		
	30 min	4 h	24 h
701.9	38	57	57
622.6	3.1	3	3.1
400.3	38.0	38	38
480.0	200.0	200	200
363.4	87.0	200	200
295.3	57.0	38	25
497.0	11	11	10.9
300.4	132	132	132
523.5	17	17	16.5

3.6. Validation and comparison of experimental data

The initial cross-method validation using 594 samples did not yield the expected high number of corresponding bins. For this study, only 58% of the samples were in the same bin, 19% were in adjoining bins, and 23% of the sample did not match. Despite the different standing times between the methods, 24 h for the FIA method versus 1 h for the nephelometry method, we determined that these standing times were not the cause of mismatched bins. The delay of 2–4 weeks between preparation of the samples run on the FIA method to preparation of samples, using the same FIA stock solution, run on the nephelometry method was the primary factor in the mismatched bins. The time delay of 2–4 weeks between FIA analysis and nephelometry analysis had caused compounds to precipitate from DMSO. As a result, the nephelometry method generally yielded higher solubility values than the FIA method. Another cross-method comparison was run, using 367 compounds, with both stock solutions prepared simultaneously and the methods running concurrently. Using fresh samples increased the bin matches to 75%, adjacent bins to 21% and decreased the percent of bins that did not match to 4%. We observed that the match percent was project dependent, as shown in Fig. 5, indicating that the nephelometry method would be suitable for compounds across therapeutic areas. A comparison of results from the FIA method with literature equilibrium values indicated that the FIA method would provide solubility values ±20% of the published value [11]. The FIA method was used as a standard to which values generated from the automated method were compared. Fig. 6 provides an overview of how the nephelometric method compares to the FIA method, over a variety of molecular weights, for proprietary compounds. Table 5 contains results from a

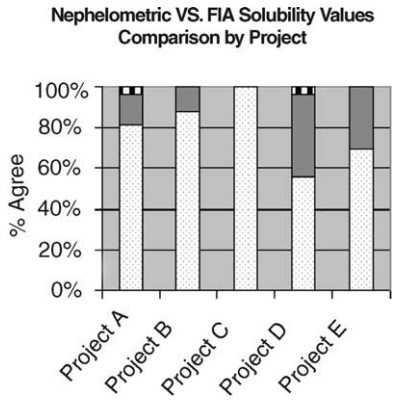

Fig. 5. Second cross-method validation study, using fresh samples and the FIA and nephelometric methods run concurrently. Agreement between methods is between 60 and 95%, dependent on therapeutic area.

Molecular Weight (g/mol)	FIA method	Nephelometric Method
500.7	<3	1.7
461.6	>60	61.6
439.9	28.7	58.7
240.4	>60	48.1
459.6	14.1	27.2
598.1	<3	1.4
464.6	<3	12.2
429.0	<3	85.8
365.2	<3	9.6
499.0	21.6	8.8
412.3	>60	82.5
402.5	9.0	10.6
631.6	3.1	1.4
254.3	37.9	37.9
366.4	38	25
413.6	38	38

Fig. 6. A more detailed comparison of solubility values for different molecular weight compounds, across therapeutic areas. Solubility values are provided in μg/mL.

comparison of solubilities values for commercially available compounds generated by the FIA and nephelometry methods. In general, the use of nephelometry was shown to provide solubility values consistent with equilibrium solubility values [7]. The authors' unpublished comparison of in-house solubility methods (nephelometry versus equilibrium solubility at pH 6.5) for matched lots of proprietary compounds further support Bevan's conclusion. The nephelometric method is especially compatible for compounds that exhibit slow-rate DMSO degradation or slow precipitation out of DMSO, provided that the solubility assay was run within a day of solvated compound preparation. The nephelometry method provides solubility bin values that are comparable to the FIA method, however, an increase in sample throughput and reduction in instrument resources are significant advantages of the nephelometry method over the FIA method. A sample throughput comparison of the FIA and nephelometry methods indicates that the nephelometry method would provide a ninefold increase. The FIA method was capable of analyzing 200 compounds per 6 days, using two instruments, while the nephelometry method could analyze 1800 compounds for the same time frame.

4. Conclusions

An integrated robotic liquid handling and nephelometer system produces solubility values that are comparable to literature values and values determined from a flow-injection analysis method. The increase in throughput, using the defined protocol, is capable of creating the 40 assay plates in 120 min and completing an entire assay from protocol set-up to data analysis in 6–7 h. The evolution of a vial-based assay to an automated plate-based assay required detailed validation of the liquid handling procedure and reevaluation of how to create the concentration range. Validation of the liquid handler for small and large volumes enhanced confidence in the method. Standards run with each plate and quarterly liquid handling validations ensure accurate solubility values. There were no apparent concerns regarding the stability of reagents or the effect of standing on solubility values. We examined the effect of sample standing on solubility values, while maintaining the constant DMSO quantity. Solubility values of most compounds had not changed bins between the 30 min and 4 h testing intervals, we concluded that the 1-h standing time inherent in the solubility protocol had negligible effect on data integrity. Kinetic reads over 24 h showed that there was not a significant change (<5% change) in solubility values when plates were covered. Tests were run to

Table 5
Comparison of solubility values generated by the nephelometry system with solubility values reported in literature

Compound name	Nephelometric solubility (μM)	FIA solubility	Literature solubility (μM)	Bin agreement
Naphthalene	25.6	16	31	Matched
1-Nitronapthalene	22.7	22	26	Matched
Triamcinolone	78.9	>60	82	Matched
Progesterone	3.7	14	12	Adjacent
Ketoprofen	200	N/A	396.7	Matched
Prazosin	26.3	N/A	7.6	Adjacent
Griseofulvin	133.3	N/A	14.74	Adjacent
Digoxin	59.3	N/A	30.7	Adjacent
Propranolol	200	N/A	104.8	Matched

Solubility values are reported as μg/mL unless noted.

determine the ability of the data analysis software to handle these multiple plates; no data handling issues were encountered. Combining a short standing time of 1 h, with the maximum carousel capacity of nine housing units, yields an optimal throughput of 300 compounds per day. Once the production assay was established, changing the aqueous buffer's pH or ionic content would simply require a new liquid class and re-validation of the 200 μL volume handling. A complementary use for this automated system is to run fewer samples at multiple pH's, thus providing a multi-point pH-solubility profile.

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