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Integration of a rapid automated solubility classification into early validation of hits obtained by high throughput screening

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Abstract

Besides the structural verification of hits generated by high throughput screening also the determination of physicochemical properties is essential for an efficient lead identification. Especially solubility is fundamental for the correct planning and interpretation of experiments. We describe the set up of a fast automated solubility test within our existing workflow for hit validation to assure compound identity and purity. 384-Well plates with hit validation compound solution are used for analysis employing liquid chromatography and mass spectrometry (LC/MS). The remaining compound solution was used for a fast automated solubility classification employing a nephelometer integrated into a Tecan robotic workstation. Thereby 9000 compounds were classified as poorly- and well-soluble. This rapid and simple test does not require any additional amount of sample or sample processing than before but provides additional information on the hits at an early stage of lead identification.

Validated by a more detailed nephelometric analysis for 500 out of the 9000 compounds in different buffer systems this simple test has shown to produce relevant data.
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1. Introduction

Pharmaceutical profiling has become an important strategy in the lead discovery process with the aim to increase lead candidate quality [1–3]. The ability to make informed decisions upon compound profiling data at an early stage can save project team's effort and time. The pharmaceutical profiling assays have to be feasible for high throughput, rapid turnaround time, and wide coverage of structurally diverse samples.

As a first assay to characterize confirmed hits generated by high throughput screening (HTS) typically an integrity and purity assay is performed employing LC/MS as a crucial step. With cycle times of only 3–5 min and the capability of multiplexing it is possible to achieve throughputs of more than 1000 samples per day with only μg amount of sample [4–7].

In contrast, physicochemical assays predicting adsorption, distribution, metabolism, excretion, and toxicity (ADMET) are often rather time and material consuming. Thus, assays for the determination of lipophilicity, permeability, metabolic profiling, or solubility are performed on a much smaller number of samples. Typically, discovery research protocols involve the initial dissolution of compounds in DMSO for these in vitro or even in vivo tests [8,9]. Thus, access to solubility data based on dimethylsulfoxid (DMSO) stock solution can help to interpret activity test results in screening and subsequently improve structure activity relations. Solubility data also contributes to medicinal chemistry where already in the early stage of lead identification oral adsorption problems could be encountered [10–12].

The more traditional thermodynamical methods such as shake flask are not directly applicable to the screening environment since they do not reflect the individual HTS assay workflow and conditions. Nephelometry is a rather fast approach for solubility determination and often referred to as a kinetical method [13–15]. An advantage of this approach is that DMSO solutions of compounds can be used which are also generally used in biological screening assays.

In this study we analyzed the possibility to set up a fast nephelometric method for classification of solubility in addition

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to LC/MS hit analysis, which is routinely performed for confirmation of compound integrity. We integrated a commercial nephelometer within our automated liquid handling environment employing the same 384-well plates as used for LC/MS hit validation measurements meaning that no additional sample and also sample preparation was needed for this solubility classification. Dependant on two data threshold levels based on the median of clearly dissolved blanks, compounds were ranked into solubility classes. By crosscheck with a more extended solubility test on more than 500 of the 9000 tested compounds we could show that we get useful information with that rapid method.

2. Materials and methods

2.1. Reagents

For LC/MS characterization during analytical hit validation a total of 9000 in-house compounds were supplied by the Boehringer Ingelheim sample repository on 384-well white clear bottom plates (Nunc GmbH, Wiesbaden, Germany) with 2 μl of a 10 mM DMSO stock solution. On each plate two rows were left empty, providing space for solvent blanks required for data evaluation. The compounds were diluted with LC solvent water-acetonitrile (95:5, v/v, containing 0.1% trifluoroacetic acid) right before LC/MS measurement. After LC/MS-measurement the microtiter rack with the remaining solution was analyzed nephelometrically.

Additionally, a subset of these compounds has been selected for extended nephelometric measurement in different buffer systems. Almost 500 selected compounds were supplied by the Boehringer Ingelheim sample repository as 1 mg solid compound in glass vials.

Solvents were of HPLC grade, trifluoroacetic acid was purchased from Sigma–Aldrich Chemie GmbH (Steinheim, Germany). Phosphate buffered saline (PBS) concentrate (10×) was obtained from Fluka (Buchs, CH). PBS (sodium phosphate (0.1 M)/9% sodium chloride, pH 6.9) was diluted 1:10 with water to obtain 10 mM PBS buffer with pH 7.4 before using it for sample preparation. Water was de-ionised and further purified by a membrapure purification system (MembraPure, Bodenheim, Germany).

Commercially available reference compounds have been obtained from Sigma–Aldrich (Steinheim, Germany) with exception of Testosterone (Fluka, Buchs, CH).

2.2. Microtiter racks

Different types of 384-well clear bottom plates were evaluated with regard to well-to-well variation and background readouts: Greiner 781101, Greiner 781095, and Greiner 781096 (Greiner Bio-one, Frickenhausen, Germany); Nunc 265196 and Nunc 242763 (Nunc GmbH, Wiesbaden, Germany); Thermo 95040000, Thermo 311001, and Thermo 312001 (Thermo Electron, Dreieich, Germany). For comparison reasons a 96-well clear plate from Nunc (Nunc 269620) was used.

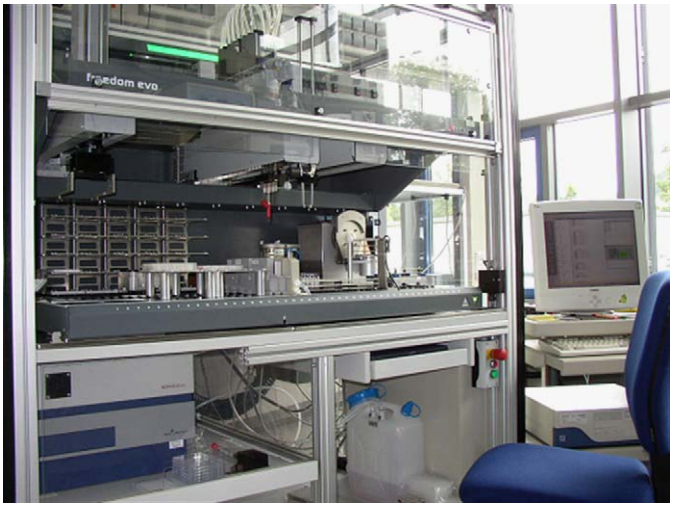

Fig. 1. Tecan workstation for sample preparation and solubility determination with integrated nephelometer.

2.3. Apparatus

For all nephelometric measurements a Nephelostar (BMG Labtech, Offenburg, Germany) was used. It was integrated into a Tecan Freedom EVO workstation (Tecan, Maennedorf, Switzerland) equipped with the following features as shown in Fig. 1: Robotic manipulator (RoMa) arm for transportation of microtiter racks to rack hotels, liquid handling station, or nephelometer; liquid handling arm to prepare dilution series supported by a Variomag Teleshake 1536 Lift (H + P Labortechnik, Oberschleißheim, Germany); pick and place arm together with a de-capping station and shaker for sample preparation of the solids out of the initial glass vials; vial barcode reader for sample administration. Buffers were stored in refillable reagent troughs and freshly prepared for a batch of 96 samples.

2.4. Nephelometer settings

Up to now solubility measurements using nephelometry are reported only on a 96-well plate format [13–15]. Nephelometer settings for 384-well reads as well as the settings for the individual modules of the Tecan workstation (e.g. shaking time, liquid handling classes) had to be optimized and validated. The laser beam focus was optimized to 1.9 mm. Plate positioning delay was set to 0.5 s, two read cycles per plate were used, and measurement time per well was set to 0.5 s resulting in a total measurement time of 12 min for a completely filled 384-well plate.

Results in 96-well plates could be reproduced as described in literature [13]. To check instrument parameters on 384-well plates a dilution series of an internal test compound in PBS was measured on 96-well plate and on 384-well plate with different sample volumes. Periodically instrument set up and check was performed with commercially available reference compounds.

2.5. Fast solubility measurement of LC/MS hit validation solutions

The sample repository supplied 2 μl of a 10 mM DMSO stock solution for each compound. Just before LC/MS measurement this 2 μl stock solution was diluted by a CTC-HTS-PAL-autosampler with dilutor with 80 μl LC solvent. 5 μl of this solution were injected into LC/MS. The remaining solutions were used without further processing for nephelometric solubility measurement. Between sample preparation and injection the microtiter rack was stored in the cooled stacker of the autosampler at 15 °C to avoid significant solvent evaporation. After LC/MS analysis the two blank plate rows were filled with 75 μl of LC solvent, to mimic unprecipitated sample. Plates were placed onto the Tecan robot, shaken with the Teleshake and then placed into the nephelometer.

2.6. Evaluation of nephelometric data from LC/MS plates

Data generated by the nephelometer were exported and analyzed using an in-house Excel-Macro in a "two level" approach. Since wells with precipitated sample show significantly higher signal response compared to the wells filled with solvent blanks, two thresholds were determined empirically by relating visual examination to nephelometric readout to classify the compounds into "clearly dissolved" and "precipitated". Wells with a nephelometric readout below the 9-fold value of the mean of the solvent blanks were defined as "clear", sample wells above a 20-fold threshold were defined as "precipitated". Wells with readouts within these limits were not clearly classifiable (Fig. 2).

2.7. Solubility measurement on solid hit validation compound

A 1 mg of solid compound was supplied by the sample repository. The Tecan robotic system dissolved the compound to obtain a 5 mg/ml DMSO stock solution. Samples were then diluted either with acidic buffer identical to the LC solvent used for sample preparation of the LC/MS samples or with PBS buffer

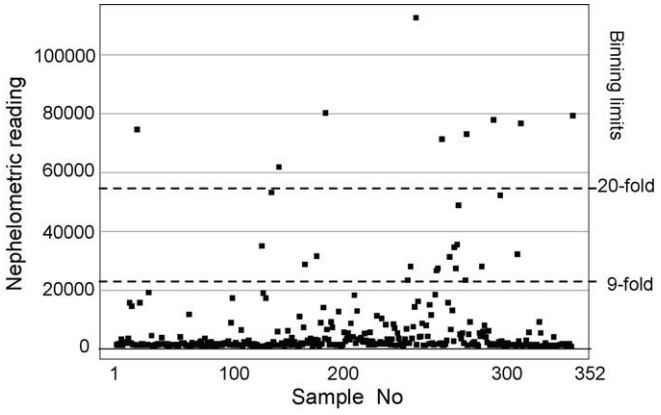

Fig. 2. "Two level" data evaluation of LC/MS compound solutions. Based on nephelometric read-out the samples are binned into clearly dissolved, precipitated, and not classifiable.

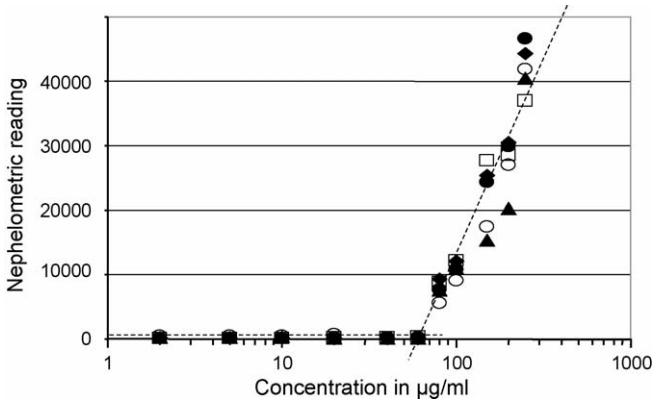

Fig. 3. Solubility determination with segmental regression employing dilution series. The different symbols reflect multiple dilution series to prove reproducibility.

(pH 7.4; 10 mM) to obtain dilution series from 2 to 250 μg/ml in duplicate on 384-well plates. The completed sample plate was automatically supplied to the nephelometer at the bottom of the Tecan robotic system as shown in Fig. 1.

2.8. Evaluation of nephelometric data based on solid compounds

The data obtained for the duplicate dilution series of the solid compounds was exported and analyzed by an in-house application based on XLfit 4 (ID Business Solutions Ltd., Surrey, UK). The software package allows removal of outliers and a recalculation of solubility values. Since the intensity of scattered light is proportional to the numbers of particles in suspension a constant signal is observed in case of a completely dissolved sample similar to the intensity of the wells filled with pure solvent referred to as blanks. As soon as the sample precipitates the signal intensity increases significantly. The intersection of the two lines obtained from segmental regression marks the solubility of the compound (Fig. 3). Solubility data was classified as: poorly soluble (<20 μg/ml), partially soluble (20–80 μg/ml), soluble (>80 μg/ml).

3. Results

3.1. Integration and validation of 384-well plates for automated nephelometric measurements

To assure that 384-well plates can be used for nephelometric solubility determinations we compared the readouts obtained by several 384-well clear bottom plates to evaluate the best performing rack type. Main focus has been put on the median value of the overall average of the nephelometric readout of each well and also on plate quality with regard to scratches. For LC/MS hit validation Nunc square well clear bottom plates (Nunc 242763) showed the highest quality with regard to scratches and well-to-well quality as well as the capability to deal with dilution volume used during sample preparation. Thermo clear plates (Thermo 95040000) were used for analyses with solid compounds. These plates provide round wells with a smaller volume than square

Table 1
Effect of sample volume in 96-well and 384-well microtiter plates on nephelometric signal intensity

Parameters changed (μl)	Nephelometric solubility of in-house compound (μg/ml)	Plate type
96-Well plate, volume 200	41	Nunc 269620
96-Well plate, volume 100	44	Nunc 269620
384-Well plate, volume 100	43	Nunc 242763
384-Well plate, volume 50	44	Thermo 95040000

wells resulting in a longer path length for smaller volumes. Thus, the sample consumption was reduced. Effects of plate quality were reduced since we ran dilution series in all rows as replicates.

Well volume effects signal intensity due to varying path lengths for scattering. Therefore, we assessed the effect of sample volume in 384-well plates and 96-well plates to assure data quality of our test. We prepared dilution series of a solid compound of the Boehringer Ingelheim pool with different end volumes into the different microtiter racks. The measurements have been performed in quadruplicate and showed a deviation of ±5 μg/ml for each measurement mainly resulting from segmental regression parameters. As can be seen from Table 1 data obtained with 96-well plates was reproduced on 384-well plates. A volume of 50 μl showed to be feasible for solubility determinations in 384-well plates.

Table 2 shows the solubility values for commercially available substances obtained nephelometrically by dilution series in PBS buffer as well as the classification based on the "two level" approach compared to literature data. It can be seen that we have been able to provide solubility classification consistent with literature with both methods covering a dynamic range of concentrations typical for early phase pharmaceutical solubility determination.

3.2. Fast nephelometric solubility classification employing LC/MS solutions

The main focus of our investigations was to provide relevant physicochemical data in the early lead discovery phase with a sufficient throughput and efficiently integrated into our hit validation workflow.

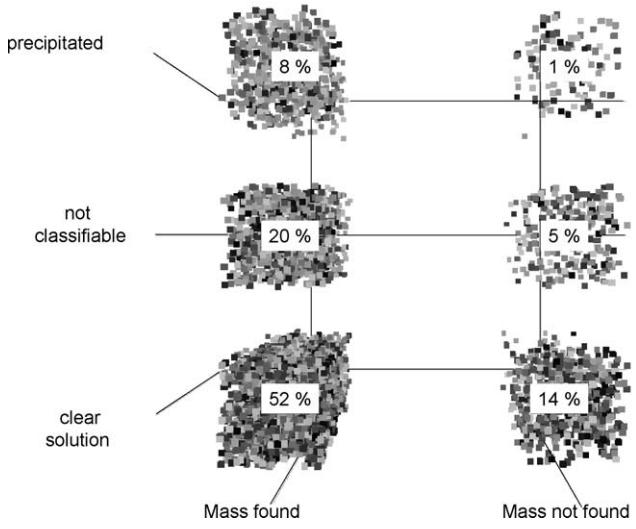

Fig. 4. Results of fast "two level" solubility binning for samples with either "mass found" or "mass not found".

Since only 5 μl of the diluted hit compound solution are used for LC/MS analysis we used the rest of the valuable solution for a fast solubility classification. After LC/MS analysis the microtiter plates were transferred into the nephelometer and data analyzed with a "two level" approach. The median value of the signal intensities of the blank buffer was related to the signal intensity of the hit compound solution in nephelometric analysis. This fast nephelometric solubility test was run on approximately 9000 hit validation compound LC/MS solutions. We plotted LC/MS-result (found/not found) versus solubility result (clear solution/not classifiable/precipitated) as shown graphically in Fig. 4. Data quality was sufficient to sort compounds into solubility classes.

In recent years it has been found in our lab as well as in literature that about 10–20% of all analyzed compounds were not detected by a standard LC/MS method [20]. One key question was whether this is due to solubility problems in LC/MS solvent. Although one could expect compound precipitation to negatively effect LC/MS data we did not find a significant accumulation of precipitated compounds in the "MS not detected" category.

Since the compounds are diluted by the autosampler just prior to injection into the LC/MS system it seems that we do not have to deal with significant solubility problems at that point.

Table 2
Solubility values of reference compounds determined by nephelometry compared to literature

Sample name	Literature data [16–19], aqueous, buffered, pH 7.4 μg/ml	Nephelometric data, based on dilution series in 10 mM PBS, pH 7.4 μg/ml	Nephelometric data, based on "two level" approach classification
Verapamil	140	>200	Clearly dissolved
Corticosterone	>200	>250	Clearly dissolved
Diethylstilbestrol	2–20	<5	Not classifiable
Estradiol	<5	<5	Precipitated
Testosteron	20–30	80–100	Not classifiable
Loratadine	<5	5–15	Precipitated
Bifonazol	<2	<5	Precipitated
Clotrimazol	<5	<5	Precipitated
Triphenylene	<2	<5	Precipitated

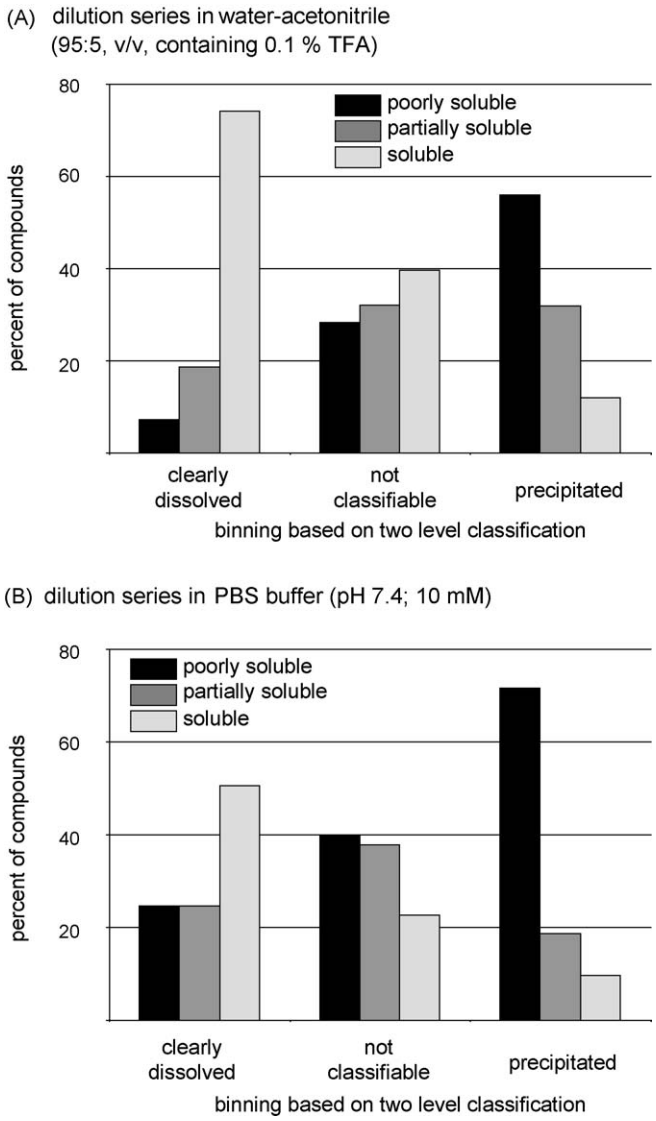

Fig. 5. Cross correlation of the nephelometric results from the fast "two level" and the conventional "dilution series" binning. X-axes show solubility classes based on "two level" solubility binning. The bars indicate the solubility bins obtained by nephelometric data of the dilution series: (A) binning with the solvent used for LC/MS sample preparation and (B) binning in PBS buffer.

Subsequently we verified the solubility results of compounds, for which molecular weight and purity have been confirmed by LC/MS, in order to assure that we detected "real solubility" with this rapid test. Approximately 160 compounds were selected and ordered as solids from each of the three solubility classes that have been built upon the results of the "two level" binning. Starting with solid material stock solutions in DMSO have been prepared. Subsequently dilution series in two buffer systems were prepared and then analyzed nephelometrically. To validate the "two level" data obtained from DMSO liquids for LC/MS the same buffer system as for the MS sample preparation was used for the dilution series (Fig. 5A). Additionally, we used the PBS buffer to mimic conditions more similar to biological assays (Fig. 5B).

Combined results in terms of solubility binning for both buffers are shown in Fig. 5A and B. It can be seen that our fast "two level" analysis data is well correlated to the more explicit data of the dilution series solubility test. Fig. 5A shows that most of the compounds of the fraction MS-detected/clear solution are also binned "clearly soluble" in the acidic buffer of the dilution series solubility test. Also most of the compounds of fraction MS-detected/precipitated are also binned "poorly soluble" in the acidic buffer of the dilution series solubility test. This proves that the data obtained from the LC/MS solutions provides meaningful data. Even in the more physiological PBS buffer (Fig. 5B) results of the fast "two level" method are in good correlation with the results of the dilution series solubility binning.

The "two level" data analysis of the nephelometric results based on the samples solutions used for LC/MS analysis relates to the data obtained by nephelometric analysis of the dilution series in the two different buffer systems. Due to the high throughput capabilities and the uncomplicated workflow integration of the fast "two level" solubility approach became a valuable tool to provide solubility binning on large numbers of hit validation compounds with no additional material needed.

4. Conclusions

The integration of a fast nephelometric solubility test of hit validation compounds plated in 384-well plates provided reliable data for classification of compound solubility. The throughput of this test is linked only to the throughput of the LC/MS hit validation. Data can be obtained with almost no effort and further sample processing and no additional material consumption from the LC/MS sample plates. Dependant on the sample preparation for LC/MS analysis the "two level" method could also provide information on possible compound precipitation in sample wells.

Validation of this test was performed by comparison with nephelometric data from dilution series starting from solid material. Therefore, this rapid "two level" test will further on be performed on a routine basis to give additional solubility information for in silico methods and medicinal chemists. Within the further lead identification process a more comprehensive understanding and interpretation of HTS results can be obtained.

For a more detailed investigation or in case of intriguing data obtained by the fast "two level" solubility test the automated robotic liquid handling platform can add further value dependant on the requests of biology and/or chemistry. Employing dilution series and the possibility to use a variety of buffer solutions including multiple pH values or buffer even containing ingredients that are specific for biological assays the method has proven to deliver results consistent with literature data as well as with in-house shake flask and NMR solubility data.

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