A Combination Turbidity and Supernatant Microplate Assay to Rank-Order the Supersaturation Limits of Early Drug Candidates

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ABSTRACT: A unique opportunity exists at the drug discovery stage to overcome inherently poor solubility by selecting drug candidates with superior supersaturation propensity. Existing supersaturation assays compare either precipitation-resistant or precipitation-inhibiting excipients, or higher-energy polymorphic forms, but not multiple compounds or multiple concentrations. Furthermore, these assays lack sufficient throughput and compound conservation necessary for implementation in the discovery environment. A microplate-based combination turbidity and supernatant concentration assay was therefore developed to determine the extent to which different compounds remain in solution as a function of applied concentration in biorelevant media over a specific period of time. Dimethyl sulfoxide stock solutions at multiple concentrations of four poorly soluble, weak base compounds (Dipyridamole, Ketoconazole, Albendazole, and Cinnarizine) were diluted with pH 6.5 buffer as well as FaSSIF. All samples were monitored for precipitation by turbidity at 600 nm over 1 h and the final supernatant concentrations were measured. The maximum supersaturation ratio was calculated from the supersaturation limit and the equilibrium solubility in each media. Compounds were rank-ordered by supersaturation ratio: Ketoconazole > Dipyridamole > Cinnarizine ~ Albendazole. These *in vitro* results correlated well with oral AUC ratios from published *in vivo* pH effect studies, thereby confirming the validity of this approach. © 2014 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 103:3022–3032, 2014

Keywords: supersaturation; precipitation; turbidity; supernatant; microplate; solubility; UV/Vis spectroscopy; HPLC

INTRODUCTION

Low aqueous solubility presents a significant challenge to drug discovery and development, hindering *in vitro* screening assays as well as *in vivo* oral absorption studies.[1,2] Supersaturation, a state in which concentration exceeds equilibrium solubility for the most thermodynamically stable solid form, provides an opportunity to overcome inherently poor solubility and increase permeation flux across an *in vitro* cellular or *in vivo* intestinal barrier.[3] The ability to select drug candidates with inherently superior supersaturation propensity during the drug discovery stage can reduce downstream formulation efforts and development timelines.

To be effective, supersaturation must be generated as well as maintained—a process aptly described by a spring and parachute analogy.[3] The generation stage requires shifting the compound from a high- to low-solubilizing environment. This can be achieved through either a pH shift,[4] solvent shift,[5] or solid form shift via a salt, cocrystal, or amorphate.[6] The metastable supersaturated state is maintained only if precipitation is delayed, prolonged, or incomplete. In general, increasing applied concentration increases the magnitude of supersaturation, but does so over shorter periods of time.[6] Ultimately, an upper concentration limit exists for supersaturation.[7] Although this limit has been correlated with amorphous solubility in water[8] as well as in high or low pH buffers,[9] it has not been determined whether this relationship also exists in biorelevant media intended to mimic the pH and composition of intestinal fluid.

Turbidimetric analyses are well suited to monitoring for the presence of precipitate over time, inferring complete supersaturation from the absence of light scattering particles. The degree of turbidity, however, does not directly correlate with the extent of precipitation, as the turbidity signal decreases with increasing particle size but increases with increasing particle concentration.[10] Thus, this technique determines the existence, onset, and duration of precipitation, but not the extent.

Alternatively, supernatant analyses directly measure the concentration remaining in solution, thereby inferring the amount of precipitation. This can be an efficient technique for single-time point analysis. However, removing the precipitated solid for multiple compounds over multiple time points slows throughput and requires larger sample amounts, which limits the utility of this approach in the discovery environment.

Although neither turbidity nor supernatant analyses alone provide a complete assessment, utilized together, these orthogonal techniques become complimentary. Furthermore, both analytical techniques are microplate compatible. This is especially valuable in the discovery environment for efficient sample handling and material conservation, given that time and material supply limitations are often acute.

Supersaturation assays reported in the literature compare polymorphic form[6,7,11] or precipitation-resistant formulation[5,12–14] options to enhance supersaturation for a single compound and concentration. These assays do not, however, allow for supersaturation and precipitation comparisons between different compounds over multiple applied concentrations. A new assay is therefore needed for such intercompound supersaturation comparisons.

Confirming the validity of this new approach requires an *in vivo* supersaturation versus nonsupersaturation comparison,

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which results from the inherent nature of the compound rather than polymorphic forms or formulations. Fortunately, the gastrointestinal pH shift represents an example of nondelivery-specific *in vivo* supersaturation, at least for ionizable compounds. A weakly basic compound dissolves to a greater extent in acidic versus neutral pH gastric media and can supersaturate upon transition into the intestinal lumen, thereby providing enhanced oral absorption. This phenomenon is known as the pH effect[15] and represents a valuable *in vivo* comparison for confirming the relevance of *in vitro* supersaturation assessments.

This work describes a unique, straightforward, robust, and material sparing supersaturation assay, developed to determine whether a compound precipitates and how much remains in solution as a function of applied concentration over a specific period of time. The assay operational parameters were optimized to ensure samples were initially homogeneous, turbidity signal was sufficiently sensitive to changes in solid concentration, and that changes in turbidity signal were because of precipitation rather than particle settling.

Four poorly soluble, weak base model drugs (Dipyridamole, Ketoconazole, Albendazole, and Cinnarizine) were diluted via dimethyl sulfoxide (DMSO) solvent shift in a microplate to multiple applied concentrations with two commonly used simulated intestinal fluids:[16] neutral pH buffer or FaSSIF. The DMSO solvent shift method was chosen because early drug candidates are often stored in DMSO solutions. The solvent shift method also ensures all compounds are completely dissolved at the start of the experiment so that undissolved solids are not confused with precipitated solids. Although the rate and extent of supersaturation at each applied concentration is fixed and cannot be independently adjusted, a range of supersaturation rates and extents are probed by testing multiple applied concentrations. Turbidity was monitored over 1 h to detect for precipitation, followed by supernatant concentration analysis of filtrate to determine the amount of compound remaining in solution. One hour was chosen as a reasonable time frame to maintain supersaturation to achieve oral absorption in the upper intestine.[17] The compounds were rank-ordered by degree of supersaturation relative to thermodynamic solubility and compared with results from published *in vivo* pH effect studies.

EXPERIMENTAL

Materials and Media Preparation

Dipyridamole, Ketoconazole, Albendazole, and Cinnarizine were purchased from Sigma–Aldrich, St. Louis MO. Spectroscopic grade DMSO was purchased from EMD and acetonitrile, methanol, and water were HPLC grade and obtained from JT Baker (Billerica, MA) or Sigma–Aldrich. Polypropylene 96-well microplates were purchased from Corning (Center Valley, PA). Flat-bottom 500 μL capacity Pyrex glass well inserts and round base support plates were purchased from Analytical Sales and Service. These glass insert wells provided good optical clarity, solvent resistance, and cost-effective disposability to prevent cross-contamination. Phosphate buffer solutions were prepared with sodium monobasic phosphate and sodium dibasic phosphate from Sigma–Aldrich. The pH was measured with a Beckman pH meter and adjusted with 1 M NaOH or 1 M HCl solutions from Sigma–Aldrich. SIF powder was purchased from ePhares and used to prepare FaSSIF according to the specified guidelines.[18] Polystyrene sphere particle standards were obtained from Duke Scientific. McCormick brand blue food dye solution was purchased from a local grocery store.

Analytical Procedures

Master DMSO stock solutions (~10 mg/mL for Dipyridamole and Ketoconazole, 4 mg/mL for Cinnarizine, and 2 mg/mL for Albendazole) were serially diluted with DMSO (1.33-fold for Dipyridamole and Ketoconazole, 1.5-fold for Cinnarizine, and twofold for Albendazole), and 20 μL aliquots were transferred to pyrex glass insert microplates purchased from Analytical Sales and Service. Samples were diluted 20-fold with 380 μL of either buffer or FaSSIF (concentration range of ~5–500 μg/mL for Dipyridamole and Ketoconazole, 0.2–200 μg/mL for Cinnarizine, and 0.1–100 μg/mL for Albendazole), mildly vortexed at the lowest setting sufficient to achieve orbital mixing for 20 s and then monitored for turbidity with a Tecan Infinite M200 instrument. Turbidity was monitored at 600 nm as none of the model compounds absorbed light at this wavelength. Experiments were conducted for 1 h at ambient temperature with intermittent shaking between readings (e.g., 20 s of orbital mixing at 60 rpm). Precipitate was assessed for birefringence with a Zeiss Axiolab polarized light microscope. After 1 h, 250 μL of each sample were filtered through a 0.2-μm PVDF(polyvinylidene difluoride) plate filter purchased from Corning into a polypropylene microplate containing 250 μL of acetonitrile. Unfiltered 100 μL aliquots were diluted into 100 μL of acetonitrile in a polypropylene microplate. Sample plates were sealed with aluminum foil and mixed on a benchtop vortexer for 10 min prior to HPLC analysis.

HPLC analyses were performed with a Waters 2795 system, 2990 PDA detector, Waters Xbridge C18 5 μm, 4.6 × 50 mm^2 column using Empower 2 software. Both the column and sample compartments were maintained at 25°C. Injection volumes ranged from 10 to 100 μL depending upon the sample concentration. Mobile phase A was 0.025% sulfuric acid and mobile phase B (MP-B) was methanol. Gradient elutions at 1.5 mL/min began with 35% MP-B, increased linearly to 98% MP-B over 3 min, remained at 98% MP-B for 1 min, and returned to starting conditions to re-equilibrate. Appropriate detection wavelengths were selected for quantitation of each compound.

Equilibrium solubilities were measured after stirring 10 mg of solid compound in 10 mL of either buffer or FaSSIF media containing 5% DMSO for 24 h at ambient temperature. Samples were monitored by UV/Visible spectroscopy with a pION μDiss Profiler dissolution system using fiber optic probes to ensure equilibrium solubility had been achieved. Supernatant was filtered through a 0.2-μm Anotop filter after presaturating both the 1-mL polypropylene syringe and the filter with 5 × 1 mL aliquots prior to filtrate sample collection. Samples were diluted 1:1 with acetonitrile and analyzed by HPLC. Excess solid was assessed for birefringence with a Zeiss Axiolab polarized light microscope.

Blue food dye solution was diluted 1:50 into PEG400. PEG400 was used as the stock solvent to mimic a worst-case high-viscosity scenario and clearly differentiate the dispersal techniques. For each test, 20 μL aliquots were placed in glass insert microplate wells and diluted with 380 μL of pH 6.5 buffer. Absorbance was monitored at 630 nm via the Tecan Infinite M200 instrument with or without shaking for 20 s between readings. For external mixing, the plate was secured to and