

High Throughput Sonication: Evaluation for Compound Solubilization

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Abstract:

Dissolution of organic compounds in DMSO in HTS plate or tube format is a difficult problem as users move to higher compression plate formats. Precipitation of compounds from DMSO screening stocks is a recognized problem in the HTS materials management process. The adverse effect of freeze thaw cycles on DMSO stock solutions stored in plate format as a result of cherry picking operations has led to the gradual replacement of plate-based storage with tube-based storage so as to minimize the number of freeze thaw cycles. Compound solubility in DMSO is markedly decreased by uptake of small quantities of water. We attribute this effect to the non ideal properties of DMSO water mixtures such that cavity formation in solvent, a necessary step in dissolution, is more difficult in wet DMSO than in dry DMSO or in pure water. We report here that efficient compound dissolution is possible even in 384 well format by the use of in-well plate-based sonication. Surprisingly, compounds precipitated from DMSO stocks either by water uptake or repeated freeze/thaw cycles can be re-dissolved by low energy sonication. Finally, we demonstrate that precipitation of compound from DMSO stock solutions is synergistically enhanced by water uptake into DMSO compound stock solutions as well as by increasing the number of freeze thaw cycles.

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Introduction

Sonication has been used in basic science research for over 40 years for such things as degassing of solutions [1], lysing of mammalian [2], fungal [3], or bacterial cells [4], shearing of DNA [5-6], and sonochemistry [7]. Sonication has not been used in the high throughput arenas of pharmaceutical chemistry and screening due primarily to the fact that previously, sonicators could not accommodate the number of samples that needed to be prepared at one time.

Anecdotally, a diverse compound collection may have up to 20% of the compounds precipitated or degraded to at least some extent. The proportion of insoluble compounds could potentially be higher within a closely related chemical library or structural class. As a consequence, when a high throughput screening (HTS) campaign is performed against a compound collection, a large number of samples are either not in solution to the extent expected or are not in solution at all, resulting in an inordinate number of “false negative” results. In a worst-case scenario, the loss of this data can lead to misinterpretation of the screening results and consequently, directing chemistry down the wrong path or missing potential lead compounds. More recently, systematic investigations have been undertaken to better understand the extent of this problem [8-10].

Compound solubility is governed by two basic laws: kinetics and thermodynamics. Sonication can address one of these, kinetics, but is not likely to affect the other, thermodynamics. If a compound is soluble at a given concentration, then sonication can be used to accelerate that compound's solubility. That is, if the compound would “normally” take hours or days to dissolve, sonication can be used to drive that compound into solution in a matter of seconds. However, sonication, in most cases, can do nothing about the thermodynamic portion of the equation. That is, if a compound is only soluble to a concentration of 10 mM in its lowest energy crystalline form, then sonication cannot be used to drive it into solution at a higher concentration. It will, however, insure that the compound is driven into solution to the maximum extent allowed by thermodynamics. The caveat to this statement is that because sonication inputs energy into the sample, it may, in a local environment convert a thermodynamically more stable less soluble crystal form into a higher energy more soluble form, perhaps even to an

amorphous form. This is one possible reason why sonication is effective in redissolving precipitated materials. Conversely, high energy sonication has been applied to induce crystallization of supersaturated conditions [11-12].

In this paper, the degree of compound precipitation is measured as a function of multiple freeze thaw cycles and of water contamination present in a sample. 16 commercial compounds chosen specifically to be representative of the chemistry found in typical drugs along with a small Pfizer compound subset were tested. Compound precipitation was induced through a combination of water contamination to the DMSO (dimethyl sulfoxide) dissolving the samples and by multiple freeze thaw cycles. The ability of a multi-well high throughput sonication system was then tested to determine its ability to drive these precipitated compounds back into solution. Optimal sonication conditions were determined along with heating profiles during sonication, possible contamination of samples with metal ions from the sonication device, and effects of sonication on compound stability were determined.

In general, sonication was capable of driving many of the precipitated compounds back into solution and had no effect on compound stability. In addition, compounds that initially went into solution with difficulty (>5 hours vortexing) were driven into solution rapidly with sonication (less than 20 seconds).

Material and Methods

SonicMan™, high throughput sonication system and disposable 96 and 384 lids were from MatriCal, Inc. (Spokane, WA). The sonicator generates sound energy at 20 kHz from 0-1150 watts. Pulse “On Time” and “Off Time” are configurable in 0.1 second intervals up to 20 seconds. Transmitted energy is estimated to be 12 watts/pin for a 96-well plate and 3 watts/pin for a 384-well plate at full power, which is proportioned evenly across all pins.

Commercial compounds were purchased from Sigma Chemical Company (St. Louis, MO) or ICN (MP Biomed, Irvine, CA). These compounds Baclofen, 4-Acetamidophenol, Chloroquine diphosphate, Salbutamol, (+)-Dehydroisoandrosterone, Haloperidol, Albendazole, Loperamide Hydrochloride, Ketoconazole, EDTA, Terazosin, Mebendazole, and Tamoxifen Citrate Salt, were chosen on a basis to represent a cross

section of physical properties that might be found in a typical drug-like compound collection. Other compounds were from the Pfizer compound collection. All other reagents were from Sigma Chemical Company, St. Louis, MO.

Compound Precipitation

Compounds were dissolved in neat DMSO from a bottle that had not previously been opened and thus was not contaminated with water. Compounds at final concentrations of 30 mM, 10 mM, and 4 mM were dissolved in neat DMSO, 90% DMSO/10% water, 80% DMSO/20% water, 70% DMSO/30% water, or 100% water (DMSO/Water on a vol/vol basis). Samples containing water were first dissolved in neat DMSO and water was added to the final concentration. The samples were then sealed and stored at $-20\text{ }^{\circ}\text{C}$ and allowed to stand at that temperature for a minimum of 2 hours. Compounds were then removed from the freezer and allowed to thaw in a water bath at room temperature ($23\text{ }^{\circ}\text{C}$) for 2-4 hours. This freeze thaw cycle was repeated up to 30 times.

At 0, 10, 20, and 30 freeze thaw cycles, samples of each compound were removed and analyzed for the presence of precipitant by light scatter at 400 nm, 500 nm, and 600 nm. In several cases, the dissolved compounds were colored and by taking optical density (OD) readings at three wavelengths, a wavelength where the compound did not absorb could be found. All subsequent precipitation data was compared at that wavelength. OD is only a qualitative measure of precipitation and while an increase in OD at the given wavelengths indicates more precipitation, OD at long wavelength is not linearly related to amount of precipitate.

Sample Heating

Sample temperature was measured as a function of sonication. The application of sound energy to a sample can cause significant heating to the sample. To measure sample temperature, 30 or 80 μL DMSO or water was placed in a 384 well plate or 250 or 500 μL of DMSO or water was placed in a 96 (1.5 mL) tube format. Samples were sonicated with one of three sonication protocols: 1) 1 second on, 1 second off; 2) 5 seconds on, 1 second off; or 3) 10 seconds on, 1 second off. After 10 seconds of total

sonication, the plate was removed from the Sonicator and a lid containing a series of 16 rapid response thermal couples was placed on the plate. The time between the completion of sonication and the first temperature measurement was less than 5 seconds. Temperature measurements were taken in edge rows, edge columns, and the middle of plate to determine whether edge effects were present. Temperature was measured continuously from time zero (placement of the lid onto the sonicated plate) until the sonicated samples reached room temperature.

Sonication Uniformity

Ideally, every well on a 96, 384, or 1536 well plate would receive the same amount of sonic energy. In order to determine the uniformity of sonication across these plate types, a 20 μ L sample of 10 nM fluorescein in 30% sucrose, 10 mM MOPS buffer at pH=5 was layered below a 60 μ L sample of 50 mM CAPSO buffer at pH=9.5 in all wells of a 384 well plate. 30% sucrose is a very viscous solution and no mixing occurs upon buffer addition. Fluorescein has a very low fluorescence at pH=5 and the fluorescence increases dramatically with increasing pH. The samples were sonicated with the three different pulse types indicated above and the plate read on a Bio-Tek Synergy HT Multi-Detection fluorometer.

Trace Metal Ion Analysis

The disposable 96, 384, and 1536 lids are comprised of a series of metal pins protruding through a universal lid that can be fitted over the plate. The metal pins protrude through the universal lid and into the solution in the wells of the plate. When a plate containing a lid is placed into the sonicator, a sonic horn with dimensions of 116mm X 77mm couples with the “pins” and transfers sonic energy from the horn through the pins and into the solution in the well. The pins are comprised of a brass stock (60-63% Copper, 35.5% Zinc, 2.5-3.7% Lead) with a 5 micron nickel coating covered by a 10 micron gold coating. To test for the presence of metal ions in solution post sonication, a series of 60 μ L DMSO samples were sonicated with 8 X 10 second pulses with 1 second pauses. These are very extreme sonication conditions and would not typically be used in a “normal” laboratory setting. Post sonication, the samples were sent to ACS Labs,

Houston, TX for trace metal ion analysis to detect the presence of copper or tin in solution.

Compound Stability

Although sonication has been used extensively in most chemistry labs to help solubilize compounds, it was necessary to determine if the sonicator behaved differently from single probe sonicators and caused any degradation to the compounds. The 16 commercial compounds were dissolved in neat DMSO to a concentration of 30 mM and subjected to 8 X 10 sonic pulses. Samples were then centrifuged to remove any particulate matter and an aliquot of the supernatant sent for HPLC analysis. These samples along with control samples that had not been sonicated were sent to Alturas Analytics, Moscow, ID for LC/MS analysis to determine if any of the compounds were modified, or degraded by the sonication. HPLC analysis was performed on a HSC18 Supelco 2.1X50mm column, 0.3 mL/min., 0.1% formic acid/2 mM Formate/MeOH, Gradient 5-95% Organic in 12 minutes, 10 μ L injection.

Results and Discussion

The sonicator instrument was designed as a high throughput sonication system capable of sonicating samples in 96 standard, 96 deepwell, and 96 tube format, 384 well plates in standard and deepwell format and in 1536 well plates. The system is comprised of two main components: a sonication device capable of producing from 1 to 1150 watts and a “disposable” sonication lid (Figure 1A). Initial experiments suggested that production of a sonic horn with 384 independent probes small enough to enter a 384 well plate would not be feasible due to both cost and part failure. A horn, so designed, is very expensive and with the amount of energy transmitted through individual pins, the pins had a very high failure rate leading to loss of the entire horn. Second, the cost of such a horn made it prohibitively expensive if and when it became contaminated or if a probe was accidentally bent or broken. Finally, a custom horn would have needed to be built for each possible plate type and application.

The sonicator with its disposable lid system by passes these problems through the use of a single horn with dimensions of 115mm X 77mm, thus covering the entire surface of any microwell plate. Disposable lids can be prepared in essentially any conceivable format and each of these lids can be directly coupled to the sonic horn. This provides the system with broad versatility since a single instrument can be used to sonicate 96 well plates both standard, deep-well and in tube format, 384 well plates, both standard and deepwell, along with 1536 well plates. The lids are disposable parts so that should they become contaminated or fail due to prolonged sonication, they can be easily and relatively cheaply replaced. A “pin lid” has a useful life of approximately 200-300 seconds of sonication at 100% power, (potentially much longer life spans with lower power settings). With typical sonication times of from 20-40 seconds per sample, each lid will sonicate from 5 to 15 plates before it fails. A lid failure is obvious in that the “head” of the pin shears from the pin body and is left free floating in the top of the lid.

The sonicator generates approximately 70 decibels at 3 feet from the instrument. OSHA requires hearing protection for any work environment where the decibel level is greater than 85 decibels at 3 feet from the source of the noise. Consequently, no hearing protection is needed when using the sonicator and it typically generates less noise than many readers, liquid handlers, and robotics found in an HTS lab. This acoustic dampening is made possible by the installation of a specialized multi-composite foam rubber (MatriCal, Inc) that is used to coat the entire inner surface of the device. The device also does not generate any harmful EM effects nor any magnetic field effects and is fully CE and UL compliant.

Figure 1C is a diagram of a typical lid construction. The lid is an injection molded part coated on both sides with silicone rubber layers and “pins” inserted through it. The 40 durometer silicone rubber on the upper surface provides a compliant layer such that when the sonic horn is applied to the surface of the lid, any pins that are slightly higher will be depressed until all pins are in uniform contact with the sonic horn. This insures that each pin in any format will be fully coupled to the sonic horn. The soft 20 durometer silicone rubber layer on the bottom of the lid is used to seal each well from other wells surrounding it such that there is no cross contamination between wells during

sonication. The silicone rubber layer is resistant to all solvents typically used in HTS, other biological applications, and most chemical applications.

The pins are constructed from brass with a nickel coating that in turn is electroplated with a 10um thick gold coat. Initial experiments with plain brass pins indicated that certain chemicals, especially those with very low pH or containing reactive sulfur groups could react with the brass pins. This is not the case with the electroplated gold coated pins.

Cross Contamination Experiment

As with any type of sonication, the possibility exists that microdroplets are formed during sonication and that these droplets could migrate from one well of the microwell plate to another. In order to test whether well to well cross talk was occurring, 96 and 384 well plates were “checker boarded” with either a 10uM Fluorescein solution or with a 10 mM solution of bromothymol blue dye. The plates were then sonicated with 10 second pulses and the plates read (either fluorescence or absorbance) after each pulse. While conditions could be found where cross talk could be induced, such as with long pulses at high energy in wells containing in excess of 100 μ L in a 120 μ L total volume well (384 well format), under typical sonication conditions, no cross talk was observed. Even after the sonic lids had been subjected to 250 seconds of total sonication, at about the point where the lid begins to fail due to metal fatigue, there was no evidence that the silicone rubber gaskets failed causing any cross talk between wells.

Sample Heating

During sonication, a significant amount of energy is transferred from the sonic horn and into solution. For a process such as driving compounds into solution or for resolubilizing compounds, the goal is to keep the sample temperature below 60 °C. This temperature was chosen since in normal lab synthesis procedures chemists commonly expose final compounds to 60 °C as in procedures such as concentration by rotary evaporation, solvent removal in drying pistols, etc. Short term exposure of compounds to 60 °C should not have any effect on compound stability. For applications such as cell lysis, it is necessary to keep the well temperature below 37 °C and for shearing DNA, the

well temperature needs to be kept below the melting point of double stranded DNA (typically less than 90 °C).

Three sonication protocols were chosen to determine the degree of heating associated with various sonic pulse lengths. Protocols of a 1 second pulse followed by a 1 second pause, a 5 second pulse followed by a 1 second pause, and a 10 second pulse followed by a 1 second pause were performed. 96 Matrix tube plates containing 0.5 mL of either water or DMSO and 384 well polypropylene plates containing 60 µL of either water or DMSO were used. The appropriate lid was placed on each plate and the plate sonicated with one of the sonication protocols listed above. After 10 seconds of total sonication time (10 X 1 second pulse, 2 X 5 second pulse, or 1 X 10 second pulse) the plates were removed from the sonicator, the sonication lid removed and a lid containing a series of 15 thermocouples was placed on the plate. The thermocouples were placed in wells A1, D1, H1, A3, D3, H3, A6, D6, H6, A9, D9, H9, A12, D12, and H12 in the 96 well plate and in wells A1, A12, A24, H1, H12, H24, P1, P12, P24, G8, I8, G10, I10, G14, I14 in the 384 well plate. The temperature of the samples within the wells was measured within 5 seconds of the completion of sonication. The plates were allowed to cool to room temperature and the procedure was repeated with 20, 30, 40, 50, 60, 70, and 80 seconds of total sonication time.

Figures 2, 3a, and 3b show the results of this experiment. As can be seen from these experiments, total sonication times of up to 40 seconds could be used for applications such as compound dissolution or redissolution since the sample temperatures did not exceed the desired 60C. However, for cell lysis type experiments, total sonication times of less than 10 seconds would be desirable. Short sonication times lead to less heating than longer sonication times. Figure 3b, however, shows the cooling curve of a sample after sonication. This data indicates that the 1 second “off” time does very little in relation to sample cooling since it was observed that there was negligible cooling in 1 second. The difference in temperature observed between the various pulse protocols is most likely due to the difference in friction between the sonic horn and the pins in the lid. With increased sonication time, friction between the sonic horn and the pins leads to heating of the pins. This heat is then transferred by conduction to the samples within the wells. The temperature variability at any given time point is due to differences in heating

between individual wells on the plate with corner wells heating less than wells in the middle of the plate.

Sonication of a dry well leads to pin heating but has no detrimental effects on either the lid or the sonic horn. The longitudinal pin flexing per se generates heat in addition to the heat generated by the friction between the pin and the sonic horn. When the pin is not immersed in a liquid, the pin tends to heat up significantly more than when immersed. In both cases, both when the pin is immersed or when not immersed, the pins can be warm to the touch.

Trace Metal Ion Analysis

Sonic horns are typically constructed from a stiff metal such as titanium that has a low modulus of elasticity. By using a stiff metal in horn production, the degree of horn heating and fracture is minimized. Commercially available laboratory single probe sonicators have been shown to “throw” small pieces of titanium steel into solution during the sonication process. This is observed as a “pitting” on the face of the sonic probe. It was expected that a similar effect may happen with the pins in the disposable lid especially since they are constructed from relatively soft materials such as brass. While metals are not typically soluble in biological buffer solutions, it was necessary to determine if any metal ions were present after sonication because metal ions such as copper, zinc, or lead could potentially be detrimental to down stream processes.

For these experiments, 60 μ L samples of either DMSO or buffers at pH= 3, 7, or 10 were placed in a 384 well plate and the samples were sonicated using a new disposable lid with either plain brass pins or with gold electroplated brass pins for 80 total seconds (8 X 10 second pulses with 1 second pauses between pulses). Ten samples were combined from each sample type and sent for trace metal ion analysis. For the pure brass pins, the DMSO samples contained levels of copper or zinc that were below detectable limits while the buffered samples contained barely detectable amounts of these ions not exceeding 2.5 ppm (data not shown). For the pins that had been electroplated in gold, the amount of trace metal ion in solution was in all cases below detectable levels of 0.02 ppm or approximately 300 nM. In all cases, the amount of metal ion in solution is far below what could be expected to interfere with any biological reaction.

Sonication Uniformity

With 96, 384, and 1536 well formats, it is imperative that all of the wells within a plate are sonicated to the same relative degree. With an optimal design, the sonic horns can provide a uniform output of +/-5% over the contact surface of the horn but the degree and efficiency of coupling to the disposable lids was tested in order to determine the uniformity of sonication across a 96 or 384 well plate. For these experiments, a 20 μ L sample of 10 mM bromothymol blue dye in 50% sucrose, 10 mM MOPS buffer pH = 5 was layered under 60 μ L of 10 mM CAPSO buffer pH=11 in all wells of a 384 well plate. Bromothymol blue is a color sensitive dye that undergoes a color shift from blue (OD_{max} = 615nm) to yellow (OD_{max}=430nm) with decreasing pH. For the 96 1.4 mL tube plate, 50 μ L bromothymol blue in 50% sucrose, 10mM MOPS pH=5.0 was placed in a 1.4 mL tube and allowed to dry to a viscous film for 24 hours. After the sample was concentrated by drying, 500 μ L of 10 mM CAPSO buffer was added to the tube. The appropriate disposable lid was then placed on the plate and the plate subjected to the sonication protocols describe above. At each 10 second sonication interval, the plate was removed and for the 384 well plate, the absorbance (OD 615nm) read in a Molecular Devices SpectrMax spectrophotometer. The 615nm OD reading is a good indication of the degree of sample mixing. For the 96 Matrix Tube, it was not possible to take OD readings from the tubes as the tubes are not suitable for spectrophotometry and removal of aliquots from the tubes would not yield dependable results since the OD of the sample correlates with the area in the tube from which it was taken (yellow at the bottom and blue at the top). For these experiments, data is displayed in photographic format.

The sonication results from both the 96 well format and the 384 well format are shown in Figures 4 and 5 respectively. Since bromothymol blue absorbance is very pH dependent, the mixing of a very viscous low pH, 50% sucrose solution with a high pH buffer is a good indicator of both the mixing power of the instrument and the uniformity of sonication across the entire lid. As can be seen in Figures 4 and 5, all wells in both the 96 and 384 well plates reach equilibrium within 40 seconds of sonication. In addition, all wells within any given plate reach the same degree of mixing within 10 seconds of each

other. This is the expected result with a 5% sonic horn uniformity and a high degree of coupling between the horn and the disposable lid.

Compound Precipitation

16 commercially available compounds were chosen to represent the type of compounds typically be observed in a pharmaceutical compound library collection and purchased as crystalline forms. It was assumed that multiple freeze thaw cycles would be directly responsible for compound precipitation. For compounds dissolved in neat DMSO, multiple freeze thaw cycles had a modest effect on compound precipitation (Table 1). Of the 16 commercial compounds tested at 30 mM concentration, three of the compounds (Haloperidol, Carbendazim, and Methyl-5-Propylthio-2-Benzimidazole Carbamate) showed precipitation with freeze thaw. However, when the compounds were tested at 10 mM concentration, up to 30 freeze thaw cycles had no effect in causing precipitation on any of the compounds. This implied that freeze thaw cycles are not the only factor contributing to the observed precipitation of pharmaceutical compounds so experiments were expanded to examine other contributing conditions.

To test the effect of water contamination of the DMSO solvent on compound precipitation, samples were prepared in neat DMSO, 90% DMSO/10% water, 80% DMSO/20% water, 70% DMSO/30% water, or 100% water. Compound concentrations of 30 mM, 10 mM, and 4 mM were tested with the above conditions. Samples were prepared in 500 μ L aliquots in a 1.5 mL microfuge tube and subjected to multiple freeze thaw cycles. The tubes were vortexed for 30 seconds to resuspend any particulate matter and 50 μ L samples were removed at T=0, 10, 20, and 30 freeze thaw cycles and analyzed by light scatter to determine the degree of compound precipitation.

As can be seen from Table 1, the addition of even small amounts of water significantly increases the degree of compound precipitation and as the amount of water in the sample increases, the degree of precipitation typically increases. Of the 16 commercial compounds tested, over 50% of the compounds at either 30 mM or 10 mM concentration displayed precipitation upon the addition of water. In addition, there is a strong synergistic effect on precipitation between water addition and freeze thaw cycles. This implied that water addition to DMSO is a major determinant in the precipitation of

compounds. Take for example Haloperidol. In this case, the compound appears to be fully soluble in 90% DMSO/10% water but begins to precipitate in 80% DMSO/20% water. This precipitation is exacerbated by freeze thaw cycles. When the 90% DMSO/10% water sample is subjected to 10 freeze thaw cycles, the compound begins to precipitate and the degree of precipitation is increased with increasing numbers of freeze thaw cycles. The same is true for the 80% DMSO/20% water sample except that the freeze thaw effect is exacerbated.

Of the 9 compounds that precipitate in the presence of water at the 30 mM concentration, 33% show a strong synergy in that freeze thaw induces additional precipitation while 4 of the compounds are probably fully precipitated and any synergy, if there, cannot be deduced. Interestingly, there also appears, at least in two cases to be an inverse synergy in that water addition and freeze thaw cycles help drive the compounds into solution. This could be due to the compounds having an inherently higher solubility in water than in DMSO and the difference is simply the length of time exposed to solvent and is consistent with the predicted aqueous solubilities of these compounds.

This synergistic effect is even more evident in the 10 mM samples. Of the 8 compounds that precipitate in the presence of water, six show the synergistic effects of freeze thaw. The degree of precipitation increased with increasing number of freeze thaw cycles.

The Effect of Water on Compound Solubility in DMSO

The marked effect of water in decreasing organic compound solubility in DMSO can be rationalized by consideration of the non-ideal behavior of solutions of DMSO and water. Ideal behavior in terms of properties is that predicted by consideration of the properties of the individual solvent components. If a compound is very soluble in DMSO and quite insoluble in water then simplistically the ideal behavior scenario would be as follows. If the compound is very soluble in dry DMSO then the solubility in 90% DMSO would be 90% of the dry DMSO value. Non-ideal behavior occurs when the properties of a solvent mixture cannot be predicted from an extrapolation of the properties of the individual solvent components (Figure 6). This is the case with the properties of DMSO water mixtures which show marked non-ideal behavior [13].

An important factor in the solubility of a compound is the energy required to form a solvent cavity within which a compound will fit. Making a larger cavity requires more energy. This is the reason why as a general rule larger compounds show poorer solubility. In dry DMSO, forming a cavity is relatively easy because dry DMSO does not contain any hydrogen bond donor functionality. There is no possibility for the formation of hydrogen bond donor acceptor networks in the solvent because DMSO itself only contains hydrogen bond acceptor functionality. When water is introduced into DMSO the possibility of hydrogen bond networks is present because the hydrogen in water (the hydrogen bond donor) can interact (form a hydrogen bond) with the carbonyl oxygen in DMSO. At a mole ratio of two water molecules to one molecule of DMSO (33% by weight water) the hydrogen bond network is extremely strong and more organized than in water itself. The very strong hydrogen bond accepting ability of the DMSO carbonyl oxygen, the very strong hydrogen bond donor ability of the hydroxyl water hydrogen and a fortuitous combination of water and DMSO bond angles allows the formation of an ice-1 like hydrogen bonded cage structure. This very organized network of hydrogen bonding must be disrupted to form a cavity in the compound solubilization process. The formation of the hydrogen bonded network is responsible for the non ideality of DMSO water mixtures. For example the melting point of dry DMSO drops from 18 degrees centigrade to minus 73 degrees centigrade in 33% by weight water in DMSO. DMSO is more viscous than water. Yet when water is added the viscosity does not decrease (as predicted by ideal behavior). Rather there is a massive increase in viscosity with a peak in viscosity observed at exactly 33% by weight water. This is exactly what would be predicted from the formation of the ice-1 like water DMSO hydrogen bonded cage structure. We believe that the solubility behavior of organic compounds in wet DMSO is exactly analogous to the viscosity behavior of DMSO water mixtures. Even small amounts of water in DMSO greatly decrease organic compound solubility because the formation of ice-1 like hydrogen bonding lattices makes the cavity formation term in solubility very large and difficult to attain.

Compound Stability

Since sonication is a common technique in most chemistry laboratories and is used to help in mixing samples, it was considered unlikely that this high throughput sonication system would have detrimental effects on the compounds. To ensure against sonication induced degradation, the 16 commercial compounds were subjected to a very harsh sonication regimen. In this case, the compounds were sonicated for 8 X 10 seconds with 1 second pauses between the 10 second pulses. This sonication protocol was significantly longer than would typically be necessary to drive a compound into solution (see below) and, from Figure 3, was expected to raise the sample temperature to greater than 70 °C. These compounds were then sent for LC/MS analysis and compared to the same compound that had not been sonicated (the controls). All 16 compounds yielded good LC/MS spectra and the results showed that there were no extra peaks in any of the samples and the areas under the curves were comparable (data not shown). This suggests that there was no degradation of the samples even under rather harsh sonication conditions. The MS analysis indicated that the compounds were not chemically modified as the molecular weights were consistent between the controls and the sonicated samples. While this is a small sample set of 16 compounds, and while it is always a possibility that a compound could be adversely affected by sonication, this data suggests that it would be an unlikely event.

Optimization of Compound Resolubilization

From the temperature study, it was observed that with any of the sonication protocols (1 second on/1second off, 5 seconds on/1 second off, or 10 seconds on, 1 second off) the sample temperature did not exceed the 60C limit until after 50-60 seconds of total sonication. With this as a baseline, it was possible to begin optimizing the other sonication parameters. 50 µL of selected commercial compounds which had been subjected to the various precipitation protocols described above were removed at time zero and analyzed by light scatter. The samples, in a 384 well format, were then subjected to each of the sonication protocols and the degree of precipitation was measured as a function of sonication time.

The horn amplitude is directly related to the amount of power generated by the horn. As the amplitude was increased, the degree of compound dissolution was increased for a given amount of sonication time. However, by increasing the total sonication time, the same relative degree of dissolution could be achieved. With pulse length, either 1 second, 5 seconds, or 10 seconds, it was observed that the longer the pulse length, the higher the degree of compound dissolution (Figure 7A-C). In this case, the 10 second pulse drove compounds back into solution better than the 2 by 5 second pulses which in turn worked somewhat better at driving compounds into solution than the 10 by 1 second pulses. This is fortuitous in that with a 10 second pulse the total plate processing and time to re-dissolution is typically more than twice as fast than re-dissolution with a shorter pulse. Temperature rise is linear with sonication time and nearly independent of pulse length. Consequently, the optimal sonication conditions for driving this particular set of compounds into solution was determined to be 100% Amplitude, 10 second pulses, 4 pulses. Interestingly, these same conditions appeared to be optimal for both the 384 well format and the 96 tube formats tested. While increasing the total number of pulses did, in a very small number of samples, increase the re-solubilization, the increased time and increased heat generated was not justified by the small gain in solubility.

Sonication Effects on Compound Solubility

Liquid compound stock solutions, while usually stored initially in neat DMSO, tend to pick up water quite quickly when exposed to air. The amount of water uptake in neat DMSO is quite rapid when exposed to standard laboratory bench conditions. Compounds that are subjected to relatively long exposure to open air conditions are expected to have a relatively large water content reaching double digit percentages. 46 compounds were chosen from the Pfizer compound collection to represent typical amorphous compounds that have been previously observed to precipitate over time (data not shown). These compounds were dissolved in neat DMSO and then diluted to 10 mM concentration with water to yield a final solution concentration of 70% DMSO/30% water. As had been observed with the commercial compounds, water and freeze thaw cycles had a synergistic effect on compound precipitation.

At time zero, the degree of precipitation of these compounds was determined by light scatter. This yielded a relative measure of how soluble these compounds were in the presence of 30% water. Of the 46 compounds, 15 were fully soluble at a concentration of 10 mM while 28 compounds displayed at least some degree of precipitation, one compound displayed greater solubility and two compounds were indeterminate. All 46 compounds were then subjected to sonication (100% amplitude, 4 by 10 second pulses) and the degree of precipitation remaining after sonication determined by light scatter. Seven of the compounds displayed further precipitation upon sonication suggesting that these compounds had not been fully precipitated and the ability of sonication to create a “seed crystal” allowed precipitation to occur or hastened a process that was already taking place. Fifteen of the compounds were not precipitated so sonication was not expected to have an effect on them. However, of the 21 compounds that were precipitated (28 initial precipitated compounds minus seven that showed further precipitation upon sonication), sonication was able to drive 16 (76%) of these either fully or partially back into solution.

Post sonication, these compounds were subjected to 30 freeze thaw cycles. As with the commercial compounds, it was expected that freeze thaw would have a synergistic effect on compound precipitation. Initially, 15 of the 46 compounds were soluble, however, after 30 freeze thaw cycles, only 3 of the compounds remained soluble and the other 12 showed some degree of precipitation. Three compounds displayed lower OD readings post freeze thaw while freeze thaw had no effect on 16 compounds (these compounds were already precipitated probably to a maximal extent) and 27 compounds (12 which had previously been fully soluble and 15 that showed further precipitation) showed increased precipitation. After 30 freeze thaw cycles, 3 compounds were soluble, 1 was indeterminate, and 42 compounds had precipitated to some extent.

Following the 30 freeze thaw cycles, the compounds were once again subjected to sonication in order to determine the effect of sonication on compound dissolution. Of the 46 compounds, 3 were fully soluble prior to sonication and 4 compounds were so highly precipitated (OD>4.0) that meaningful readings could not be taken. Removing the compounds where sonication effects could not be determined (7 compounds) left 39

compounds where sonication may have an effect. Of these 39 compounds, sonication was able to drive 26 (67%) of these either partially or completely into solution (Table 2)

Conclusion

As is indicated by the data presented here, optimizing conditions to prevent compound precipitation is a complicated process. As can be seen with both the commercial compound and the drug discovery compound sets, water plays a very large part in compound precipitation. However, water is not the only factor that influences precipitation as can be seen from the synergistic effects of water and freeze thaw cycles. Since many pharmaceutical companies maintain their libraries at $-20\text{ }^{\circ}\text{C}$ and since at some point in the compounds history it has been exposed to air thus picking up water, a large number of compounds will be precipitated at common storage concentrations.

Preventing the synergistic effect of water and freeze thaw is not trivial. Repeated freeze thaw cycling of compounds can be eliminated by the use of single aliquot storage systems. However, preventing water uptake is much more complicated. All compound handling processes would have to be performed under a completely dry (0% Relative Humidity) environment. This isn't practical since it would require that entire laboratories with all of the automated equipment therein be kept entirely dry due to the rapid uptake of atmospheric water in DMSO.

Anecdotally, up to 20 % of a pharmaceutical compound library may be affected by precipitation. This would indicate that, in a primary screening setting, a proportion of compounds are not present at all or at such a low concentration that it is effectively not being screened. Obviously, this results not only in a large number of false negative data being generated from the primary screen but also incurs a cost of time, effort, and dollars.

Sonication is currently the only practical method capable of driving precipitated compounds back into solution. While sonication will not drive all compounds fully into solution at a given concentration, that depends on the inherent solubility of that compound, the data above suggests that from 60-80% of compounds will be driven either partially or fully into solution. Evidence is presented here demonstrating the potential of sonication for compound re-dissolution, while careful attention should still be paid to potential compound degradation or sonocrystallization of supersaturated solutions. The

data in this paper is based on light scatter and light scatter is neither a sensitive measure nor is it linear with degree of precipitation. This being the case, suggests that degree of re-solubilization presented here is actually an under estimate of the degree of re-solubilization. In all likelihood, sonication drives nearly every compound into solution to the extent allowed by thermodynamics.

Overall, the data presented above suggests that sonication should play a major role in compound dissolution and re-dissolution, as well as sample mixing and homogenization. It will have its greatest effect when driving compounds into solution upon initial addition of solvent, when re-suspending those compounds post freeze thaw, and post dilution into aqueous buffer. If sonication were employed at some or all of these steps, maximal compound solubility under each separate condition would be assured.

Acknowledgements

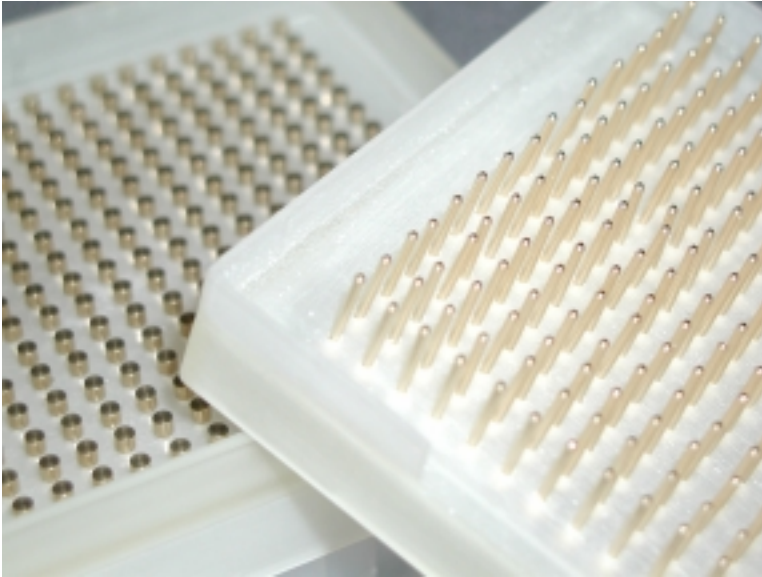
The authors would like to acknowledge Shaughn Robinson for selection of compounds for this evaluation. Dr. Chris Lipinski is an Adjunct Senior Research Fellow to Pfizer Global R&D and a Scientific Advisory member of Matrical, Inc.

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A)



B)

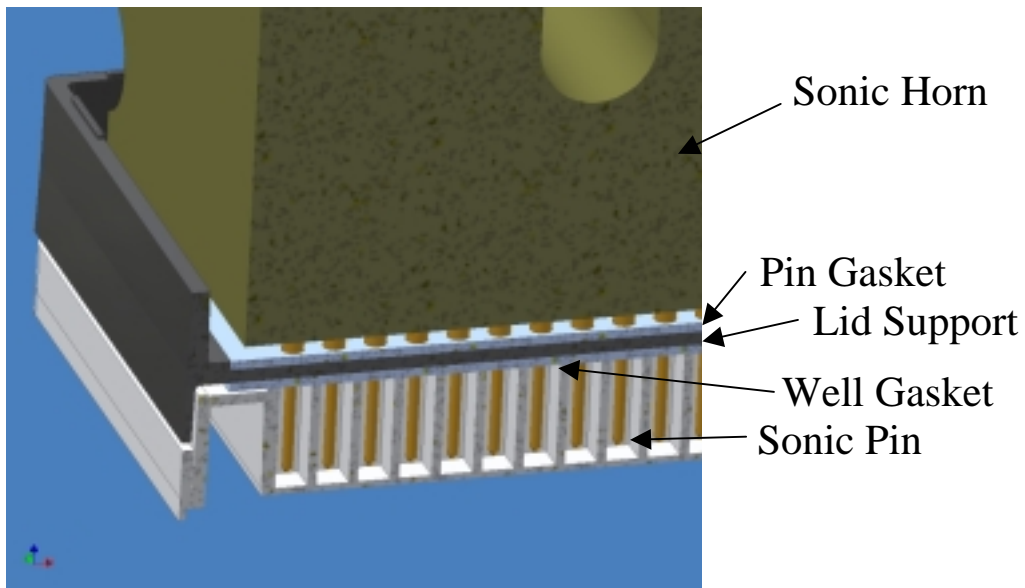


Figure 1. A) Photo of the sonication lid. The lid is comprised of an injection molded part (glass filled polycarbonate) with gold coated brass pins inserted through the lid. B) Diagram of the sonication lid shows the lid with pins inserted, the pin gasket that is used to keep all of the pins at the same height and in contact with the sonic horn, and the well gasket that seals each well during sonication preventing well to well cross talk.

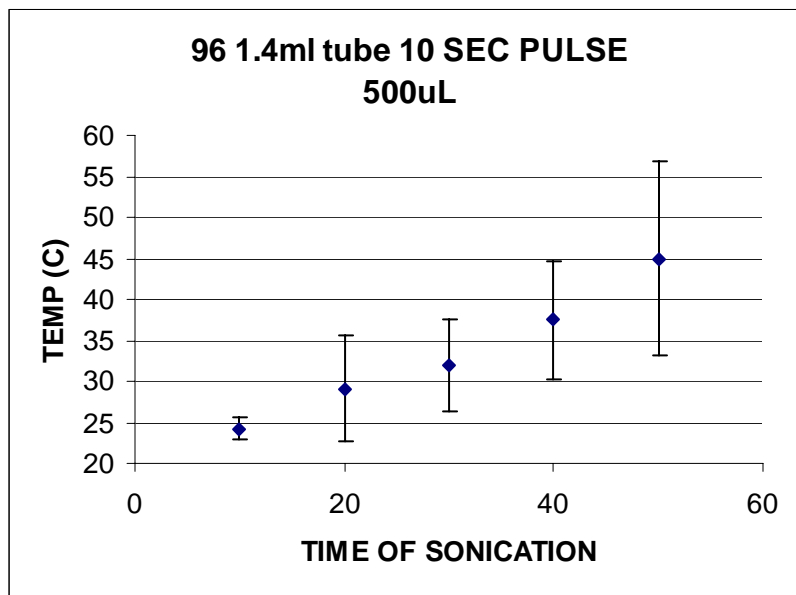
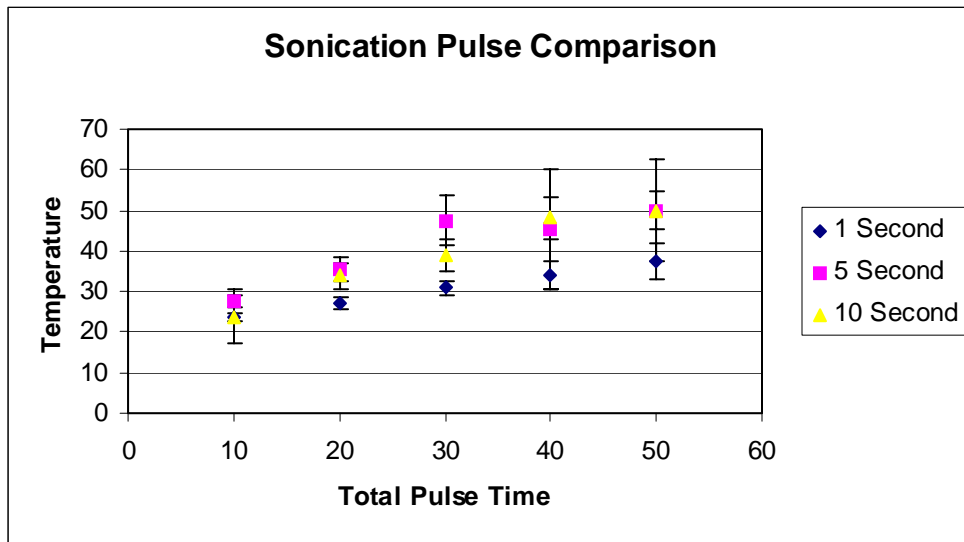


Figure 2. Temperature profile of a 96 well 1.4 mL Tube plate (500 μ L DMSO/well) sonicated with the indicated pulse profile measured at the total indicated sonication time.

3A



3B

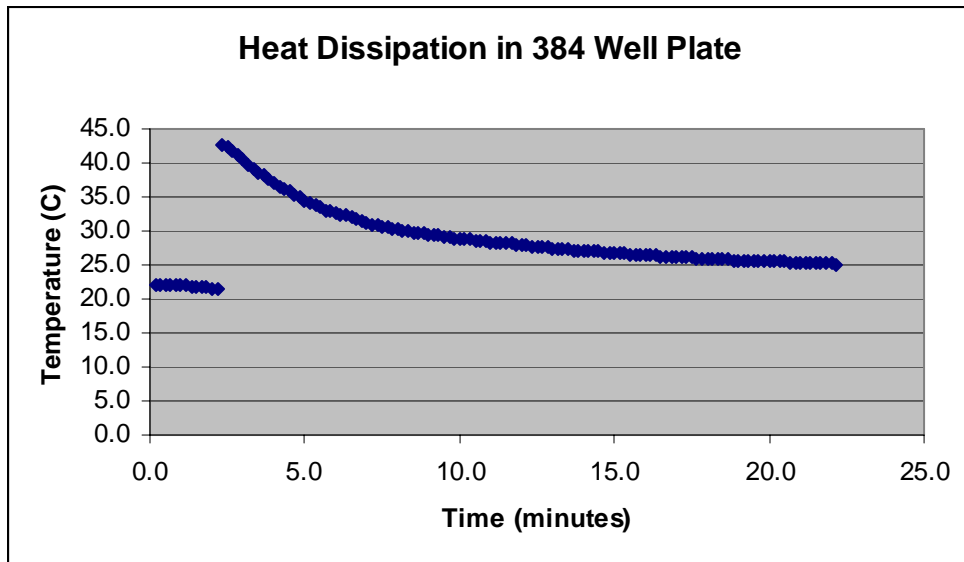


Figure 3: A) Temperature profile of a 384 well plate (50 μ L DMSO/well) sonicated with the indicated pulse profile at total indicated time. B) Typical cooling profile of a well in a 384 well plate post sonication. The plate (70 μ L DMSO/well) was sonicated with a 5 X 10 Second pulse with 1 second pause sonication profile.

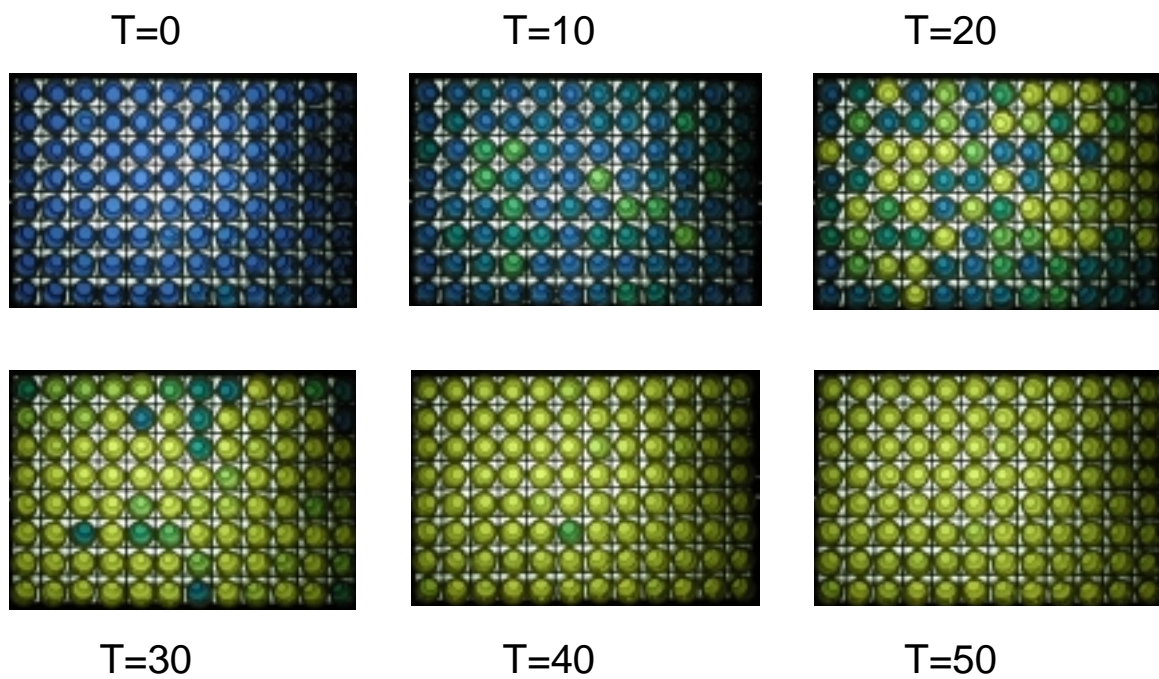


Figure 4: 50 μL (2g/mL sucrose, 0.1M MES, pH=4.0) was placed in the bottom of 96 X 1.4 mL tubes and allowed to dry to a crystalline state. The crystal was then overlaid with 500 μL (0.04 mg/mL Bromothymol blue, 2 mM Carbonate buffer, pH=10.0) and sonicated in the sonicator for the times indicated. At low pH bromothymol blue is a bright blue with an $\text{OD}_{\text{max}} = 615 \text{ nm}$. Conversion of the bromothymol blue to the base form is accompanied by a color change to yellow with an $\text{OD}_{\text{max}} = 430 \text{ nm}$. Sonication is relatively uniform across an entire 96 well tube plate with equilibrium being reached in 50 \pm 5 seconds.

Figure 5

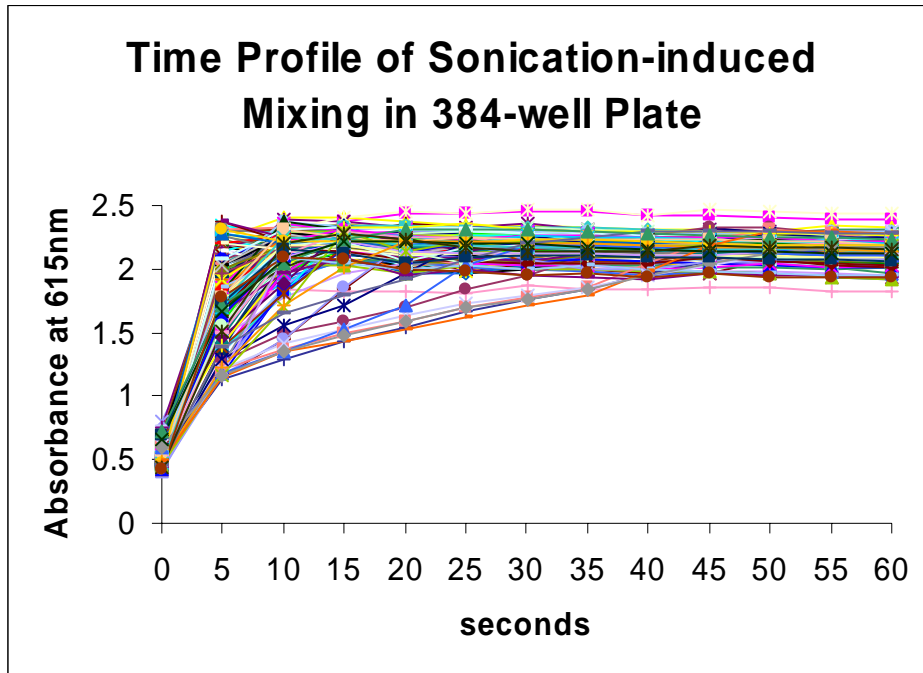


Figure 5: 15 μL of a 50% sucrose solution containing bromothymol blue in 10 mM MOPS buffer pH = 5.0 was pipetted into the bottom of a 384 well glass bottom plate and overlain with 45 μL of 10 mM CAPSO buffer pH=11.0. At time zero, an after each 5 seconds of sonication, the wells were read at OD=615nm to monitor the conversion (sample mixing) of the yellow form of the dye to the blue form. All wells reach equilibrium with 40 seconds of mixing.

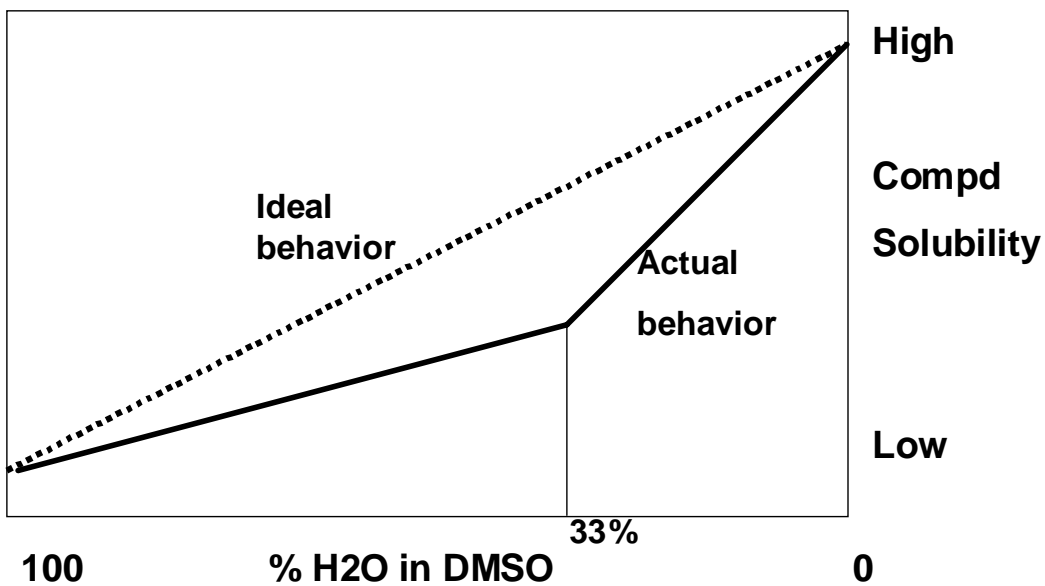
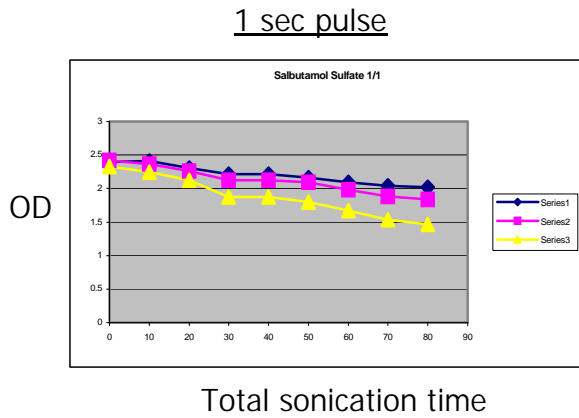
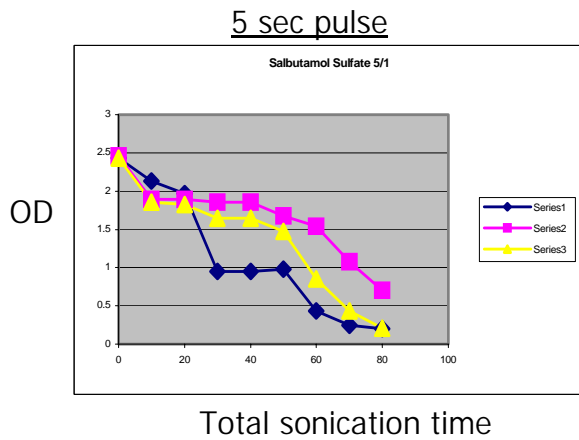


Figure 6: Non-ideal behavior proposed for DMSO:water solvent system

A)



B)



C)

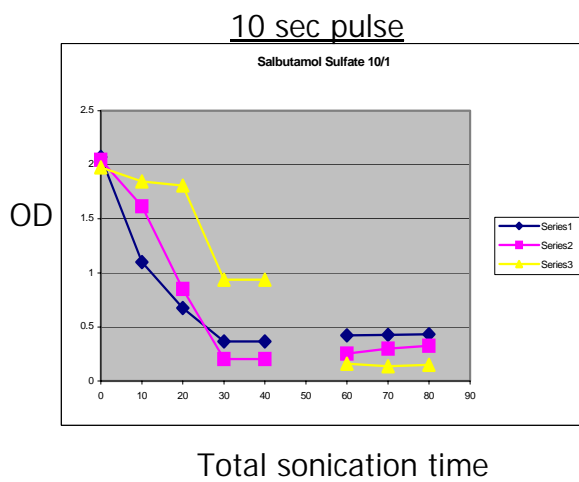


Figure 7A-C: Effect of pulse length on re-dissolution of salbutamol at 30 mM in 10% H₂O/90% DMSO

Table 1: Commercial Compounds

Wavelength(nm)	Analysis					delta F/T	F/T Effect	Post Sonication	delta OD	Sonication Effect
	OD	0 F/T	10 F/T	20 F/T	30F/T					
(10mM Compound)										
HALOPERIDOL	400									
Neat DMSO	400	0.050	0.058	0.055	0.079	0.029	N	0.114	-0.036	N
90%DMSO/10% Water	400	0.050	0.070	0.060	0.084	0.034	N	0.121	-0.038	N
80%DMSO/20% Water	400	0.048	0.297	0.220	0.282	0.234	Y	0.226	0.056	N
70%DMSO/30% Water	400	0.528	0.508	0.401	0.433	-0.095	N	0.737	-0.304	N
100% Water	400	0.078	-	-	0.057	-0.021	N	0.117	-0.060	N
(+/-)-BACLOFEN	400									
Neat DMSO	400	0.643	0.613	0.747	0.567	-0.076	N	0.837	-0.270	N
90%DMSO/10% Water	400	0.693	0.684	0.841	0.640	-0.053	N	0.913	-0.273	N
80%DMSO/20% Water	400	0.610	0.575	0.532	0.451	-0.160	N	0.376	0.075	N
70%DMSO/30% Water	400	0.646	0.542	0.499	0.445	-0.201	N	0.428	0.017	N
100% Water	400	0.029	-	-	0.030	0.001	N	0.041	-0.011	N
CHLOROQUINE DIPHOSF	400									
Neat DMSO	400	0.235	0.207	0.353	0.335	0.101	N	0.454	-0.119	N
90%DMSO/10% Water	400	0.336	0.228	0.566	0.493	0.157	N	0.629	-0.137	N
80%DMSO/20% Water	400	0.330	0.150	0.391	0.211	-0.119	N	0.086	0.125	Y
70%DMSO/30% Water	400	0.157	0.057	0.062	0.083	-0.074	N	0.055	0.028	N
100% Water	400	0.031	-	-	0.031	0.000	N	0.053	-0.022	N
ALBENDAZOLE SULFOXI	400									
Neat DMSO	400	0.087	0.105	0.059	0.099	0.012	N	0.152	-0.053	N
90%DMSO/10% Water	400	0.098	0.102	0.057	0.106	0.007	N	0.180	-0.075	N
80%DMSO/20% Water	400	0.047	0.726	0.532	0.675	0.628	Y	0.737	-0.063	N
70%DMSO/30% Water	400	3.038	2.976	2.529	3.060	0.022	N	2.998	0.061	N
100% Water	400	0.236	-	-	0.162	-0.074	N	0.136	0.026	N
SALBUTAMOL SULFATE	400									
Neat DMSO	400	0.060	0.069	0.064	0.081	0.021	N	0.109	-0.029	N
90%DMSO/10% Water	400	0.081	0.078	0.050	0.070	-0.011	N	0.104	-0.034	N
80%DMSO/20% Water	400	0.044	0.046	0.047	0.038	-0.006	N	0.039	-0.001	N
70%DMSO/30% Water	400	0.045	0.045	0.046	0.045	-0.001	N	0.061	-0.017	N
100% Water	400	0.035	-	-	0.035	0.000	N	0.047	-0.012	N
KETOCONAZOLE	400									
Neat DMSO	400	0.050	0.052	0.066	0.057	0.007	N	0.068	-0.012	N
90%DMSO/10% Water	400	0.054	0.053	0.060	0.057	0.003	N	0.068	-0.011	N
80%DMSO/20% Water	400	-	-	-	-	-	-	-	-	-
70%DMSO/30% Water	400	-	-	-	-	-	-	-	-	-
100% Water	400	1.009	-	-	0.131	-0.878	Y	0.431	-0.300	N
LOPERAMIDE HCl	400									
Neat DMSO	400	0.046	0.045	0.077	0.045	-0.002	N	0.056	-0.012	N
90%DMSO/10% Water	400	0.047	0.051	0.064	0.044	-0.003	N	0.057	-0.014	N
80%DMSO/20% Water	400	0.043	0.042	0.043	0.041	-0.002	N	0.045	-0.004	N
70%DMSO/30% Water	400	0.042	0.041	0.041	0.039	-0.003	N	0.044	-0.005	N
100% Water	400	0.349	-	-	0.452	0.103	N	0.255	0.197	Y
EDTA, DISODIUM SALT	400									
Neat DMSO	400	0.076	0.091	0.063	0.081	0.005	N	0.103	-0.022	N
90%DMSO/10% Water	400	0.209	0.110	0.093	0.303	0.094	N	0.305	-0.003	N
80%DMSO/20% Water	400	0.124	0.113	0.093	0.057	-0.068	N	0.054	0.003	N
70%DMSO/30% Water	400	0.155	0.116	0.087	0.146	-0.009	N	0.132	0.014	N
100% Water	400	0.029	-	-	0.032	0.004	N	0.348	-0.316	N
MEBENDAZOLE	400									
Neat DMSO	400	0.264	0.235	0.209	0.209	-0.056	N	0.245	-0.037	N
90%DMSO/10% Water	400	0.327	0.280	0.233	0.242	-0.086	N	0.305	-0.064	N
80%DMSO/20% Water	400	-	-	-	-	-	-	-	-	-
70%DMSO/30% Water	400	3.559	4.000	2.326	3.528	-0.031	N	3.997	-0.469	N
100% Water	400	1.275	-	-	1.441	0.166	N	4.000	-2.559	Y
TERAZOSIN HCl	400									
Neat DMSO	400	0.050	0.050	0.062	0.051	0.001	N	0.070	-0.020	N
90%DMSO/10% Water	400	-	-	-	-	-	-	-	-	-
80%DMSO/20% Water	400	-	-	-	-	-	-	-	-	-
70%DMSO/30% Water	400	-	-	-	-	-	-	-	-	-
100% Water	400	-	-	-	-	-	-	-	-	-
TAMOXIFEN CITRATE	400									
Neat DMSO	400	0.046	0.050	0.046	-	-0.001	N	0.119	-0.073	N
90%DMSO/10% Water	400	0.065	0.242	0.224	-	-0.159	Y	0.128	0.096	Y
80%DMSO/20% Water	400	0.710	0.430	1.543	-	0.833	Y	1.304	0.239	Y
70%DMSO/30% Water	400	0.542	0.554	0.574	-	0.032	N	0.366	0.207	Y
100% Water	400	0.566	-	-	0.580	0.014	N	0.426	0.154	N
DEHYDROEPIANDROSTE	400									
Neat DMSO	400	0.083	0.091	0.042	0.088	0.004	N	0.102	-0.015	N
90%DMSO/10% Water	400	0.089	0.105	0.042	0.093	0.004	N	0.095	-0.003	N
80%DMSO/20% Water	400	0.043	0.040	0.040	0.044	0.001	N	0.046	-0.003	N
70%DMSO/30% Water	400	2.081	0.039	0.039	0.042	-2.039	Y	0.053	-0.011	N
100% Water	400	0.497	-	-	0.299	-0.198	N	0.538	-0.239	N
ACETAMINOPHEN	400									
Neat DMSO	400	0.061	0.068	0.041	0.073	0.012	N	0.117	-0.045	N
90%DMSO/10% Water	400	0.077	0.075	0.042	0.092	0.015	N	0.082	0.010	N
80%DMSO/20% Water	400	0.042	0.037	0.041	0.041	-0.001	N	0.049	-0.008	N
70%DMSO/30% Water	400	0.038	0.037	0.038	0.040	0.002	N	0.050	-0.011	N
100% Water	400	0.029	-	-	0.030	0.001	N	0.037	-0.006	N
CARBENDAZIM	400									
Neat DMSO	400	0.053	-	-	0.047	-0.006	N	0.083	-0.036	N
90%DMSO/10% Water	400	0.055	-	-	0.119	0.064	Y	0.117	0.002	N
80%DMSO/20% Water	400	1.003	-	-	0.201	-0.801	Y	0.340	-0.139	N
70%DMSO/30% Water	400	1.008	-	-	0.441	-0.567	Y	0.482	-0.041	N
100% Water	400	1.976	-	-	1.535	-0.441	N	1.276	0.259	Y
FEBENDAZOLE	400									
Neat DMSO	400	0.054	-	-	0.051	-0.003	N	0.112	-0.061	N
90%DMSO/10% Water	400	0.049	-	-	0.614	0.565	Y	0.101	0.513	Y
80%DMSO/20% Water	400	1.856	-	-	1.435	-0.419	N	1.863	-0.427	N
70%DMSO/30% Water	400	3.129	-	-	2.992	-0.138	N	3.539	-0.547	N
100% Water	400	1.976	-	-	0.204	-1.772	Y	0.224	-0.020	N
METHY-5-PROPYLTHIO-2-BEZIMIDAZOLE CARBAMATE	400									
Neat DMSO	400	0.046	-	-	0.058	0.012	N	0.095	-0.037	N
90%DMSO/10% Water	400	0.051	-	-	1.316	1.264	Y	1.565	-0.249	N
80%DMSO/20% Water	400	0.115	-	-	1.367	1.251	Y	0.308	1.058	Y
70%DMSO/30% Water	400	0.516	-	-	1.096	0.581	Y	1.513	-0.417	Y
100% Water	400	0.657	-	-	0.157	-0.500	Y	0.557	-0.399	N

Table 2: Pfizer compounds

Compound #	Analysis			0 F/T			0 F/T			30F/T			Sonication		
	Wavelength(nm)	400	500	600	OD	0 F/T	Sonication	delta OD	Effect	30F/T	delta F/T	Effect	Sonication	delta OD	Effect
1		0.15	0.05	0.05	500	3.05	3.182	-0.13	N	3.24	0.06	N	3.3939	-0.1537	N
2		2.16	0.08	0.04	500	1.83	2.9855	-1.16	Y	2.31	-0.68	N	4	-1.6914	Y
3		4.00	0.06	0.04	500	0.17	0.0982	0.07	Y	0.20	0.11	N	0.0636	0.141	Y
4		0.11	0.06	0.05	400	1.21	0.4637	0.75	Y	1.00	0.54	Y	0.5486	0.4516	Y
5		1.66	0.80	0.64	600	0.76	0.7958	-0.03	N	0.89	0.10	N	0.9609	-0.0667	N
6		1.31	0.57	0.27	500	2.47	0.8829	1.59	Y	1.66	0.77	Y	0.9811	0.6758	Y
7		0.05	0.04	0.04	400	2.41	2.6392	-0.23	N	2.32	-0.32	N	2.3646	-0.0423	N
8		0.09	0.05	0.05	400	3.30	0.2462	3.05	Y	4.00	3.75	Y	3.3427	0.6573	Y
9		0.05	0.04	0.04	400	3.19	3.1716	0.01	N	2.84	-0.33	N	2.9449	-0.1079	N
10		0.05	0.04	0.04	400	0.72	0.1322	0.59	Y	0.32	0.19	Y	0.1318	0.1915	Y
11		3.10	0.05	0.04	500	0.40	0.3078	0.10	N	0.64	0.34	Y	0.3154	0.3287	Y
12		0.08	0.05	0.05	400	1.37	1.0598	0.31	N	1.86	0.80	Y	2.0229	-0.1593	N
13		0.12	0.05	0.04	500	0.09	0.0643	0.03	N	0.31	0.24	Y	0.0556	0.252	Y
14		0.06	0.04	0.03	400	0.06	0.4304	-0.37	Y	0.2625	-0.17	Y	0.1218	0.1407	Y
15		0.06	0.04	0.03	400	2.76	0.4669	2.30	Y	2.1645	1.70	Y	0.291	1.8735	Y
16		0.07	0.05	0.04	400	3.63	0.142	3.49	Y	2.5457	2.40	Y	0.0772	2.4685	Y
17		0.07	0.04	0.04	400	0.72	0.188	0.53	Y	0.8386	0.65	Y	0.2025	0.6361	Y
18		0.06	0.04	0.04	400	0.11	0.1481	-0.04	N	0.2941	0.15	Y	0.1122	0.1819	Y
19		1.11	1.01	0.98	400	1.06	0.9193	0.14	N	0.9884	0.07	N	1.0404	-0.052	N
20		0.08	0.05	0.04	400	0.10	0.1667	-0.07	N	0.5075	0.34	Y	0.1056	0.4019	Y
21		0.09	0.05	0.04	400	0.40	0.1512	0.25	Y	0.7577	0.61	Y	0.2044	0.5533	Y
22		0.04	0.04	0.03	400	0.80	0.0952	0.71	Y	0.9601	0.86	Y	0.0906	0.8695	Y
23		0.12	0.06	0.04	400	1.51	0.1674	1.35	Y	1.1199	0.95	Y	0.4276	0.6923	Y
24		0.09	0.04	0.04	400	3.28	1.9014	1.37	Y	2.4199	0.52	N	2.2123	0.2076	N
25		ND	ND	ND	400	3.39	2.9191	0.47	Y	2.9239	0.00	N	2.6237	0.3002	N
26		0.10	0.04	0.03	400	1.49	4	-2.51	Y	0.8315	-3.17	Y	0.8665	-0.035	N
27		ND	ND	ND	400	0.97	1.5245	-0.56	Y	0.2421	-1.28	Y	0.0706	0.1715	Y
28		3.01	0.83	0.36	600	0.34	0.3691	-0.03	N	0.87	0.50	Y	0.5383	0.3299	Y
29		3.60	2.77	2.52	500	1.83	0.0786	1.75	Y	1.70	1.63	Y	0.106	1.5978	Y
30		1.65	1.41	1.33	400	1.00	0.1952	0.80	Y	1.64	1.45	Y	0.2034	1.4372	Y
31		2.42	0.05	0.04	500	2.38	4	-1.62	Y	2.98	-1.02	Y	4	-1.0218	Y
32		4.00	0.09	0.06	500	0.16	0.223	-0.06	N	0.91	0.69	Y	0.1359	0.7786	Y
33		0.29	0.09	0.06	400	0.13	0.1502	-0.02	N	0.44	0.29	Y	0.1267	0.3127	Y
34		0.09	0.04	0.04	400	0.47	0.4585	0.01	N	0.37	-0.09	N	0.2679	0.1005	N
35		0.10	0.05	0.05	400	1.61	2.1919	-0.58	N	1.64	-0.56	N	1.9981	-0.3627	N
36		1.08	0.06	0.05	500	2.76	3.3382	-0.58	Y	3.28	-0.06	N	3.5499	-0.2691	N
37		4.00	1.44	0.16	600	4.00	3.4935	0.51	Y	4.00	0.51	Y	3.9387	0.0613	N
38		4.00	0.20	0.09	500	4.00	4	0.00	N	4.00	0.00	N	4	0	N
39		4.00	4.00	4.00	400	4.00	4	0.00	N	4.00	0.00	N	4	0	N
40		3.77	0.23	0.07	600	0.15	0.1763	-0.03	N	0.76	0.59	Y	0.5448	0.219	Y
41		3.48	0.20	0.09	500	0.60	3.1908	-2.59	Y	0.37	-2.82	Y	0.3894	-0.0186	N
42		3.58	2.25	2.17	500	0.04	0.06	-0.02	N	1.25	1.19	Y	0.0422	1.2032	Y
43		0.46	0.16	0.04	500	0.20	0.3848	-0.19	N	0.31	-0.08	N	0.282	0.0255	N
44		0.50	0.37	0.30	400	0.10	0.1441	-0.04	N	0.07	-0.08	N	0.0757	-0.0086	N
45		0.08	0.04	0.04	400	1.66	0.189	1.47	Y	0.92	0.73	Y	0.154	0.7638	Y
46		0.04	0.03	0.03	400	0.04	0.1849	-0.15	N	0.04	-0.15	N	0.0447	-0.0061	N