Overcoming the Problems Associated with Long-Term Storage of Compounds in DMSO

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Introduction

The "Diversity Set" is a discovery tool consisting of 1990 compounds selected from the DTP repository to represent a unique array of pharmacophores from the 250,000-item chemical library. It is made available to investigators at NCI-Frederick and elsewhere in 96-well microtiter plate format for testing in a wide variety of screens. These compounds are solubilized in DMSO and frozen in storage for extended periods of time. Some compounds tend to form a precipitate as they are frozen and thawed. This may affect the accuracy and reproducibility of results, both of which are dependent on the compound being stable and in solution. The Natural Products Support Group undertook studies designed to identify the problematic compounds, determine compound solubility after various lengths of storage, and devise a method to partially alleviate the problems associated with long-term storage. Information about the Diversity Set may be found on the Developmental Therapeutics Program web site at: **http://dtp.nci.nih.gov/branches/dscb/diversity%5Fexplanation.html**

Background

Since sample storage in DMSO and distribution as a DMSO solution is normal operating procedure for high-throughput screening, compound solubility and stability are of utmost importance for obtaining accurate bioassay results. This is especially true as amounts and volumes used in testing decrease to the microgram/microliter or nanogram/nanoliter levels. Many "bad things" can happen to DMSO solutions, such as evaporation, precipitation, contamination, and decomposition, just to name a few. Since DMSO is highly hygroscopic (Figure 1), water absorption quickly occurs, which greatly affects the solubility of some compounds in DMSO in freezer storage. As seen in Figure 2, the freezing point of DMSO is radically depressed by the presence of water, requiring much lower temperatures to freeze the sample.

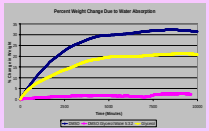

Figure 1

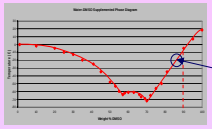

Figure 2*

Compounds in storage at -20°C are not frozen solid if the water content is greater than 10%.

There are many considerations when dealing with compound storage in DMSO, such as:

- How long is safe (Rx, precipitation)?
- At what concentration (increased precipitation at higher concentrations)?
- In what type of container (leachate, borosilicate or soda glass, brown or clear)?
- At what temperature (Rx, precipitation)?
- Using what freeze/thaw cycle?
- Using multiple freeze/thaw cycles?

Also, the behavior of the compound in solution must be considered, such as initial solubility, inherent chemical stability, and the solubility of the compound after drying. The Natural Products Support Group frequently prepares microtiter plates for screening labs from the Diversity Set master plates and plates submitted from outside sources. Precipitation has been observed in roughly 20% of the wells of the 10 mM Diversity Set plates (Figure 3). These plates, along with a 1 mM set, have been in freezer storage for a long time (>2 years). Intuitively, it is fair to assume that the concentration of drug in DMSO in these wells is no longer 10 mM. The question then arises concerning the actual concentration of drug in solution being tested.

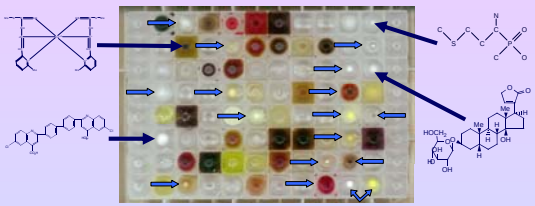

Figure 3

Experiments and Methods

Experiment #1 – Measurement of compound in DMSO solution above a precipitate.

After identifying which wells of the Diversity Set contained precipitate, a database search was performed using an in-house MassLynx/OpenLynx database to determine the feasibility of using the compound as a sample for this study. Prior to this experiment, the plated Diversity Set samples were analyzed by HPLC/MS to determine relative purity. The criteria used to select compounds for this experiment were: a) the correct mass ion had to be detected during the initial analysis; and b) the compound must have a sufficient retention time on a C18 HPLC column.

Reference materials prepared at 10 mM in DMSO were used to construct a standard curve, from 10 mM to 1 mM, and injected on to a Waters HPLC/MS/ Sedex ELSD system. A calibration curve was prepared by integrating the area under the curve from the ELSD detector (Figures 4-6). The liquor above the precipitate in selected Diversity Set wells was carefully removed and analyzed using the same conditions and instrumentation as before. After integrating these peaks, they were plotted against the calibration curve, and a relative concentration determined for the compound solution. Drug concentrations in solution between 7 mM and 0.001 mM were measured.

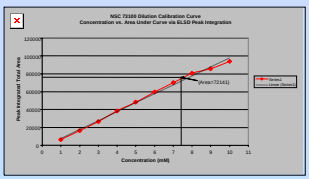

Figure 4

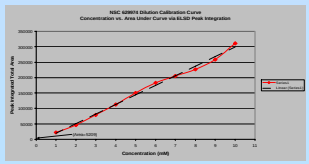

Figure 5

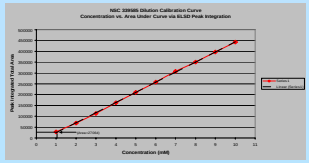

Figure 6

Experiment #2 – How quickly do chemicals at 10 mM in DMSO begin to precipitate?

Additional dry material was obtained from the NCI repository and solubilized at 10 mM in DMSO. The solutions were placed in a 96-well microtiter plate, heat-sealed, and frozen at -70º C. A portion of the material was analyzed by the above method prior to freezing. The frozen samples were thawed once a week for four weeks and analyzed as before (Figure 7). It does not require long-term storage for some compounds to precipitate out of solution. A reduction in concentration can be detected in as little as two weeks, with additional precipitation over time. Ongoing studies with long-term storage plates confirm this observation, with some compounds precipitating quickly, while others show a very gradual change. The material must be resolubilized if it is to be tested at the correct concentration. This may be difficult, depending upon the compound itself. Some will readily re-dissolve, while others will not.

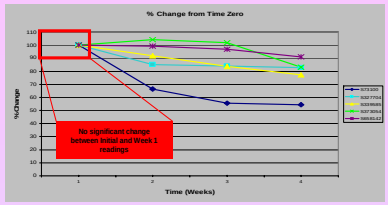

Figure 7

Experiment #3 – Once a compound has precipitated, can it be brought back into solution?

After a compound has precipitated, it can be very difficult to re-solubilize. Shaking, sonicating, and heating failed to bring all of the precipitated Diversity Set compounds back into solution (data not shown). One method investigated to address this problem was to add a co-solvent to the wells and remove the DMSO, leaving a "slurry" of compound "smeared" to the bottom of the well. Glycerol was used in this experiment due to its relative non-toxic nature and very high boiling point. A portion (200 µL) of the solubilized material from Exp. 2 was added to two 96-well microtiter plates. To one plate, 2 µL of glycerol was added. Both plates were dried under vacuum and re-solubilized in 200 µL DMSO, with Figure 8 showing the dried material both with and without glycerol, before and immediately after DMSO addition. Comparing the wells after drying and after re-solubilizing in DMSO shows a distinct difference in appearance. One set of plates was agitated for approximately one hour and analyzed using the above method. A second identical set was prepared as before, but was allowed to sit for 16 hours at room temperature and mixed via hand pipette prior to analysis. Quantification of drugs in solution is shown in figures 9 & 10. Resolubilization was much more rapid with several problem compounds when glycerol was present. Full solubilization required as much as 16 hours of agitation. Re-solubilizing the wells in DMSO after drying showed slightly better results for those compounds with glycerol over a short mixing cycle (Figure 9). However, there was essentially no difference when the plate was allowed to sit at room temperature for a long period of time (Figure 10). Each method has its advantages and disadvantages, leaving the choice up to individual preference.

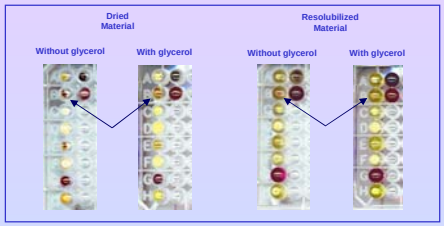

Figure 8

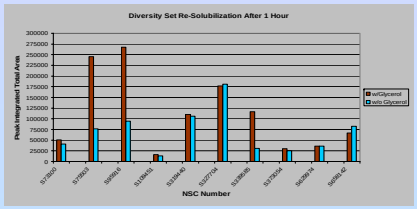

Figure 9

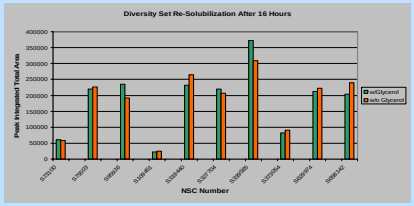

Figure 10

Experiment #4 – Do problem compounds in long-term freezer storage in DMSO at 1 mM also precipitate?

Dry material was obtained from the NCI repository, solubilized at 1 mM in DMSO, and analyzed as before. Plates containing the 1 mM Diversity Set were obtained, and the wells containing the compounds used in the experiments above were analyzed to determine the relative concentration of the plated material (Figure 11). Visible precipitation did not occur in the 1 mM Diversity Set "problem compound" wells, with analysis of the subset confirming this.

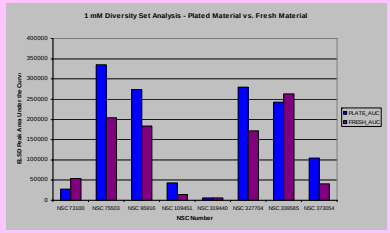

Figure 11

Experiment #5 – After long-term freezer storage in DMSO, are "non-problem" compounds still in solution at 10 mM?

Dry material of some "non-problem" compounds from the Diversity Set was obtained from the NCI repository, solubilized at 10 mM in DMSO, and analyzed using the above conditions. Solution from the Diversity Set 10 mM plates was also analyzed as above and the results compared (Figure 12). Non-problem compounds remain at 10 mM in DMSO for a long time. Analysis showed virtually no difference between a freshly prepared solution and that which was stored frozen for an extended period of time. This was also true of the analysis of the 1 mM Diversity Set. The differences between the fresh and frozen concentrations were at most 3-fold, with all being very close to each other (Figure 11). Differences between results from the 10 mM and 1 mM Diversity Set analysis could be attributed to the concentration difference. The "problem" compounds precipitate at 10 mM, but not at 1 mM. This raises the question of whether there is an optimal storage concentration for all compounds at which they will remain in solution during freezer storage.

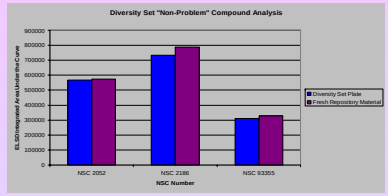

Figure 12

Experiment #6 – What effect do varying amounts of water and glycerol have on solubility of compounds in long-term freezer storage in DMSO at 10 mM?

A number of the "problem" compounds from the Diversity Set were solubilized in DMSO and added to a 96-well microtiter plate containing a water/glycerol matrix (Figure 13). These plates were heat-sealed and placed in a -70º C freezer for long-term storage and observation. A higher degree of precipitate is visible in wells with more water in them.

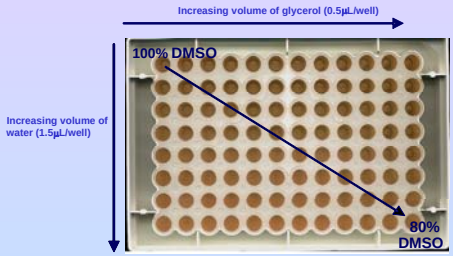

Figure 13

The presence of water in DMSO, even in small amounts, can enhance the precipitation rate for some compounds. As seen in Figure 13, the wells with a higher water content show a much greater degree of precipitation than those with less water. The presence of glycerol seems to alleviate this somewhat, as can be seen by the gradual decrease in precipitation in the wells with more glycerol. Some compounds (data not shown) exhibited precipitation in all of the wells after only two weeks, while others started displaying some small degree of precipitation after 6 months in freezer storage (experiment ongoing). The wells in which the precipitation is present are the wells in the lower-left portion of the plate, with more water and little or no glycerol. The importance of keeping vials or plates sealed during storage and having a pre-made solution at hand versus drying the material and having to re-solubilize when needed are factors that need to be considered on an individual basis.

Conclusions

- Using DMSO for long-term storage of compounds can lead to a number of problems.
- Compound storage in DMSO/water at -20ºC is not the optimal storage condition.
- Compound polarity and inherent stability, coupled with storage and handling issues, can influence how the compound will behave over long-term storage.
- Using the HPLC/MS/ELSD system allowed for a quick and reproducible method for compound analysis.
- The concentration of some compounds in solution on the Diversity Set plate ranged from ~7 mM down to ~0.001 mM, with some even lower. This represents a two- to three-log difference in the stated concentration and the probable testing concentration if only the liquor above the precipitate were used for testing.
- There is no one easy, all-encompassing answer to compound storage.

Recommendations

- Compound storage in a DMSO solution should be at -70ºC or lower.
- Compound storage in dry DMSO solution in a dry atmosphere at room temperature for a short time (<1 week) is preferable to freezer storage for the same time period, for those compounds likely to precipitate.
- Compounds solubilized in DMSO should ideally be used within one to two weeks after solubilization. Longer periods of storage, regardless of temperature, are likely to cause compound precipitation.
- If multiple freeze/thaw cycles are anticipated, multiple replicate plates should be considered, rather than one master plate.
- Storage as a "slurry" of glycerol may aid in re-solubilizing, but different preparation conditions may render this unnecessary.
- Keeping the samples well sealed to avoid water absorption is of great importance.
- Precipitation is least likely to be induced when frozen plates are thawed quickly and used promptly.

Reference

*Modified from "**Phase Diagram for the System Water-Dimethylsulphoxide,"** D.H. Rasmussen and A.P. MacKenzie, Nature, 220, 1968.

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