Annex G. Solubility

How do we measure solubility?

- Solubility Determination
  - Visual inspection
  - Nephelometry
  - UV/VIS (absorbance)
  - UV/VIS following filtration step
  - Separation using High Performance Liquid Chromatography (HPLC) or Liquid Chromatography (LC) coupled with Mass Spectroscopic (MS) or UV detection (e.g., LC/MS, HPLC/MS)
- Several time points could be considered to make sure equilibrium is reached.

How does insolubility affect the concentration in an in vitro method?

- IC_{50} values can be shifted up if the test item precipitates as the effective concentration will be lower than the nominal concentration prepared.
- Precipitates may also affect read-outs of the *in vitro* method and lead to impaired reproducibility

For some test items achieving suggested maximal target test concentration is difficult due to lack of solubility in test media.

- The exact threshold depends on the test item and the nature of the media used in the specific *in vitro* method.
- The highest concentration in OECD TGs for mammalian cells for genotoxicity testing is 10 mM test item concentration in the tissue culture medium. Therefore, solubility needs to be achieved at a 1M in DMSO (so that the final concentration of DMSO on the cells is not higher than 1%).
- There are exceptions which do not require full solubility e.g., OECD TG 442D, TG 442E and TG 487, where the highest test concentration is at the border of solubility, showing turbidity or slight precipitation (if not limited by other factors, such as cytotoxicity, pH, osmolality, etc.).

For these test items should there be guidance on how to establish the top concentration to test? For example if guidance indicates substances should be tested at top concentration of 1 mg/mL or 1mM but a substance does not go into solution at that concentration, is there some fractional concentration factor that

should be used to determine what next lower concentration to try?
• The spacing between concentrations is *in vitro* method-specific and can be used for solubility assessment as well. It is desirable to stay as close as possible to the precipitating concentration with the top concentration. • A preliminary test is often carried out to determine the appropriate concentration range of the test item to be tested, and to ascertain whether the test item may have any solubility and cytotoxicity issues (OECD TG 455) often using log-serial dilutions starting at the maximum acceptable concentration (e.g., 1 mM, 100 µM, 10 µM, maximum solubility, etc.) to find a concentration-response curve. Further runs, using smaller serial dilutions (e.g., 1:2, 1:3) are then used to focus in on the concentration-response curve, usually using six to eight concentrations (e.g., OECD TG 442E).
If it is difficult to get a test item in solution, at what point is the test item set aside as non-testable in the *in vitro* method?
• Test items are generally dissolved in a solvent (e.g., DMSO, ethanol, purified water). As a general rule, the final solvent concentrations should be as low as possible to avoid any potential interference with the *in vitro* method. Additional treatment(s) such as employing longer time frame, vortexing, sonication and/or heating may be if required. • In general test items should also be evaluated at low non-precipitating concentrations (if dissolved).
Is there a standard method or methods that could be used to accurately establish the solubility limits of test items so appropriate concentrations could be selected for testing?
• There are very accurate methods to determine the saturation point: e.g., analytical determination by HPLC and/or LC-MS/MS of concentration sampled from the supernatant, see OECD TG 105 for examples. • Nephelometric measurement of turbidity is much more accurate than visual evaluation (also possible in 96-well microtiter plates). • Precipitation can be identified with the eye quite easily. • It is important that the test facility has defined procedures (ideally SOPs) in place that describe how to conduct measurements and how to calibrate the procedure with known compounds depending on the intended applications. • Kinetic aspects should consider that there are compounds that need significant time to reach equilibrium.
Are there methods for determining solubility that work for some types of test items and not others?
• The choice of the solubility is dependent on the test item characteristics. UV/VIS methods must absorb light at the selected measurement wavelength to be employed. Nephelometry may suffer from interference when strong coloured items or items that fluoresce (e.g., contain a benzene ring) are

measured, e.g., phenol red which has light absorption in 430 and 560 nm and is excited by these wavelengths which results in fluorescence emission.

- It is important to stay, when determining solubility, as close as possible to the real test conditions, where temperature and medium components such as pH, salts and proteins can influence solubility as most organic compounds absorb light in the UV range.

What are the set of acceptable solvents that are compatible with in vitro assays?

- As a common practice, organic solvents (e.g., DMSO, ethanol, methanol and acetone) are generally used to prepare the stock concentration even if the test item can also be dissolved in purified water. One of the reasons is that organic solvents prevent or minimise the growth of microorganisms which can then impact the test item stability over time. In case of a chemical/analytical method without living organisms/cells/tissues acetonitrile or methanol may be useful.
- It is important to use a high purity of the solvent (95% to 100% purity). The final solvent concentration depends on the nature of the *in vitro* method but it needs to be less than 5% in most cases and can be as low as 0.1% [v/v].

If a test item is not soluble in the preferred solvent for a given *in vitro* method, how many of the other potential solvents should be tried? Will the compatible solvents be specific to each *in vitro* method and should the *in vitro* method define which are acceptable solvents and at what concentrations they are acceptable in the final test media?

- This is an *in vitro* method-specific question. Compatible solvents have to be defined by the *in vitro* method developer or user. And it has to be clearly demonstrated that the chosen concentration of the solvent has no adverse impact on the data.
- There are recommendations published for specific *in vitro* methods (also in the OECD TG or related SOPs or scientific literature for new *in vitro* methods).

If a substance is highly soluble in an aqueous solution, can the substance be dissolved in the *in vitro* method media directly and tested without solvent carrier?

- Yes, it is the most preferred practise to test without adding any additional compound, so the absence of a solvent is highly welcome.