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UNGAP best practice for improving solubility data quality of orally administered drugs

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UNGAP best practice for improving solubility data quality of orally administered drugs

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Abstract

An important goal of the European Cooperation in Science and Technology (COST) Action UNGAP (*UNderstanding Gastrointestinal Absorption-related Processes, www.ungap.eu*) is to improve standardization of methods relating to the study of oral drug absorption. Solubility is a general term that refers to the maximum achievable concentration of a compound dissolved in a liquid medium. For orally administered drugs, relevant information on drug properties is crucial during drug (product) development and at the regulatory level. Collection of reliable and reproducible solubility data requires careful application and understanding of the limitations of the selected experimental method. In addition, the purity of a compound and its solid state form, as well as experimental parameters such as temperature of experimentation, media related factors, and sample handling procedures can affect data quality. In this paper, an international consensus developed by the COST UNGAP network on recommendations for collecting high quality solubility data for the development of orally administered drugs is proposed.

Keywords

Oral drug absorption, Solubility, Terminology, Exerimental Methods, Best Practice, UNGAP

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1. Introduction

A major issue when considering published solubility data for a specific compound is the variability of reported values. For example, the average inter-laboratory reproducibility of aqueous equilibrium solubility data of drug-like chemical structures, has been determined to be 0.17 log unit, at best (Palmer and Mitchell, 2014; Llinas and Avdeef, 2019).

For orally administered compounds, solubility in gastrointestinal fluids, and, depending on the type of administered product, in the excipients of the dose unit, controls the dissolution of the dose and, therefore, affects the rate or even the extent of drug absorption (Reppas and Augustijns, 2010; Rosenberger et al. 2018). For practical reasons, solubility in gastrointestinal fluids is usually estimated by data collected in media simulating the composition of luminal contents to various degrees (e.g Markopoulos, Andreas et al. 2015). Increased complexity and limited stability of relevant media and, also, high intra- and inter-subject variability in the luminal composition make the collection of reliable and reproducible solubility data more difficult than in simple aqueous media (e.g. Riethorst et al . 2016)

In this paper the characteristics and limitations of the available experimental methods are summarized firstly. Then, factors affecting data quality across experimental methods, such as the purity and the solid state of the compound, the temperature of experimentation, the characteristics of media, and sample handling procedures are discussed. Due to the limited relevant literature, special emphasis is given to the solid state characterization of residual solids and to solubility media that are relevant to the development of absorption-enabling oral drug products. Finally, general guidelines for collecting high quality solubility data of orally administered drugs and decreasing intra- and inter-laboratory data variability are proposed.

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1.1 Solid States

To the best of our knowledge, the most inclusive classification scheme for organic compounds (drugs) solids has been proposed by Stahly (Stahly, 2007) (Figure 1). In a first step, solid-state forms are classified as either crystalline or non-crystalline. Crystalline materials possess a long-range order of the molecules in their solid form which is not the case for non-crystalline materials. In between these two scenarios are liquid-crystalline solid-state forms, also called meso-phases. They show a long-range order in fewer than all three dimensions. Meso-phases are very rare for pharmaceuticals

and we do not discuss them in depth herein. Stahly introduced a term “form” in order to spread the proposed scheme not only to single-component solid state systems, but also to multi-component ones. This term has been fully accepted in the crystallographic and pharmaceutical literature. A great demand of systems that can improve the solubility of poorly soluble compounds has initiated a vibrant discussion around multicomponent crystals in pharmaceutics and the correct definition of such systems (Aitipamula et al., 2012) especially during the last decade. However, multi-component solid-state forms have been used for several purposes in pharmaceutical materials for a long time, with the most well-known being pharmaceutical salts and pseudo-polymorphs. Excellent summaries of these topics can be found in Hilfiker (2006) and in Stahl and Wermuth (2011). In recent years the field of cocrystals have gained impact. Cocrystals consist of two or more components forming a particular crystalline structure having unique properties. The term cocrystal requires a clarification of the definition of a “component”, or as more commonly denominated for co-crystals, the coformer. Cocrystals consist of two or more neutral components forming a particular crystalline structure having unique properties. Accordingly, cocrystals and pseudopolymorphs such a hydrates and solvates have a similar principle in the their crystal structure. The difference between them is that the second component beyond the drug is a liquid – if in the pure phase – for a pseudopolymorph and a solid for a co-stystal. Note that salts can also exist in non-crystalline form. Formation of the salts from organic compounds involves the proton transfer from an acid to a base. It is enlightening to think of organic salts as the multi-component species where the components are individual ions. Studies on the crystal structures revealed that whether a proton is transferred from one component to another in a crystalline solid is dependent on the crystalline environment and cannot be predicted from pKa values alone, although the pKa is a good indicator as described by Cruz-Cabeza (2012). Thus, it is reasonable to consider the crystalline salts and cocrystals as species existing at either end of a continuum of multicomponent crystal structures. At the salt end the proton transfer is complete, and at the cocrystal end the proton transfer is absent. The single-component crystals and cocrystals can be polymorphic (revealing the ability of a compound to crystallize in more than one crystal structure).

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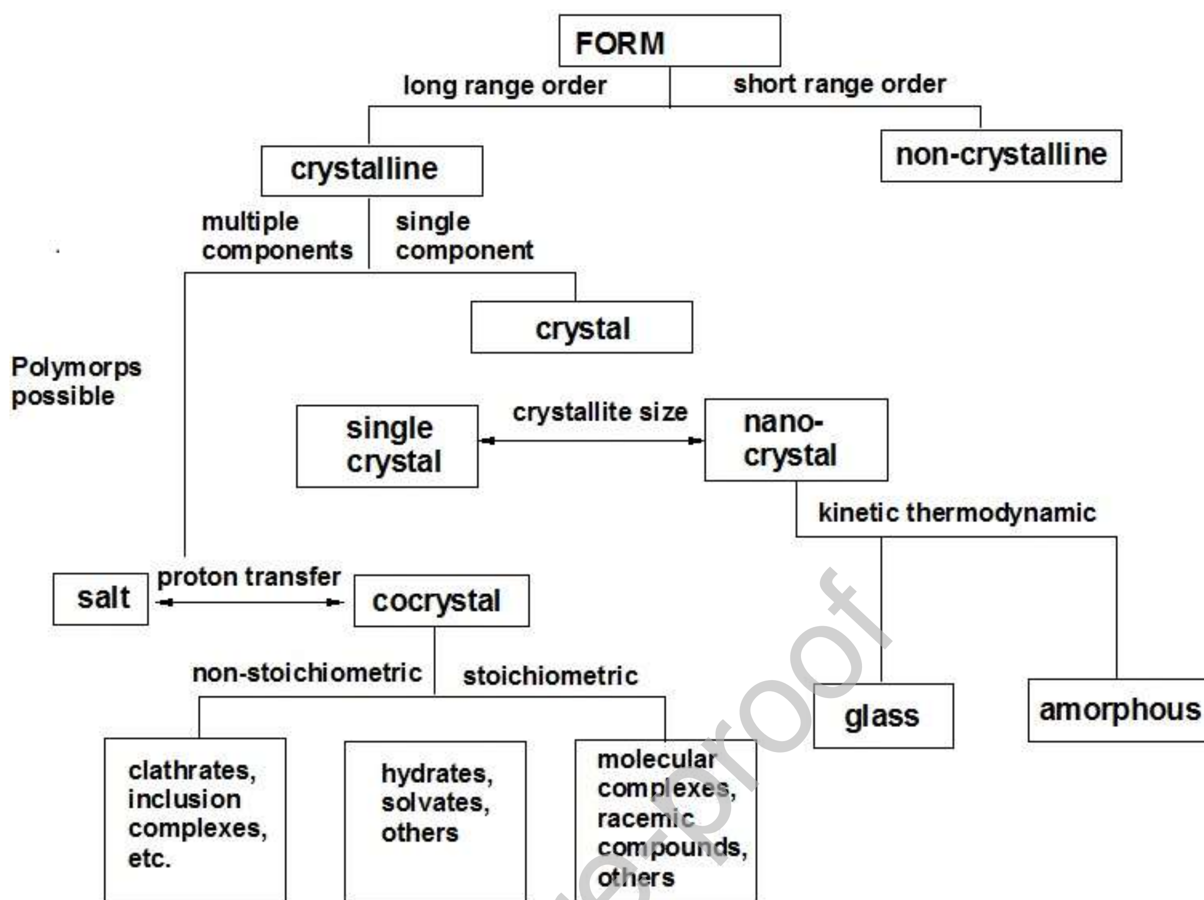

Figure 1. Classification scheme for organic solids [Reproduced from Stahly (2007) with permission].

Recently, Grothe et al (2016) proposed a new classification system for multi-component crystals (Figure 2). Multi-component molecular crystals were here initially sorted into three main classes: solvates (yellow color), salts (red) and cocrystals (blue), with possible overlapping between the three sets. The sets are defined based on three fundamental definitions: ion is a residue with a nonzero formal charge; solvent is a neutral residue that is liquid at ambient conditions (T = 293.15 K, P = 10^5 Pa); co-former is a neutral residue that is not a solvent and is a solid in its pure form at room temperature. Therefore, the following definitions of the main classes were proposed: (i) salt is a crystal containing at least two ions; (ii) solvate is any crystal with one or more solvents, two or more ions plus/or one or more co-formers;; (iii) cocrystal is a crystal with a co-former molecule plus either another co-former or at least two ions. The seven subclasses are defined as follows: (1) true solvate is one or more solvents and exactly one co-former (no ions); (2) true salt is only ions; (3) true cocrystal is only co-formers; (4) salt solvate is one or more solvents and two or more ions (no co-formers); (5) cocrystal solvate is one or more solvents and two or more co-formers (no ions); (6) cocrystal salt is one or more co-formers, two or more ions (no solvents); (7) cocrystal salt solvate is one or more solvents, two or more ions, one or more co-formers. So, as a result of the dissolution,

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each of the seven defined subclasses can be detected as a precipitate in the dissolution medium. It is important to realize this situation, as for the investigation (identification / characterization) of each of subclasses the use of specific method(s) is required (see 3.1).

As especially the definition and regulatory situation for cocrystals represented a challenge and led to some confusion during the beginning of this millennium, regulatory authorities as the United States Food and Drud Administration (FDA) [FDA, 2018; http://www.icdd.com/assets/ppxrd/presentations/11/P24-Andre_Raw-Regulatory_Considerations-Polymorphs_and_CoCrystals.pdf (accessed 26.07.2020)] and the European Medicines Agency (EMA) (EMA, 2015) issued guidance how to treat co- crystals. For pharmaceutical salts and polymorphs such guidance has been available for a much longer time (Saal 2021).

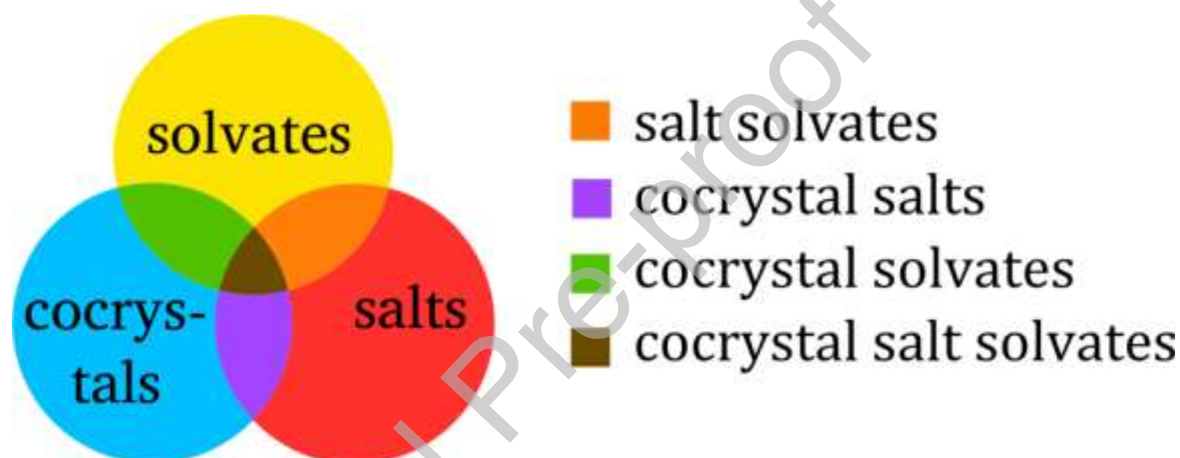

Figure 2. Visualization of the new classification scheme for multicomponent crystals according to the principles of set theory (Grothe et al 2016) [Reproduced with permission].

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1.2 Solubility, Solubilization, Supersaturation

The **(thermodynamic) equilibrium solubility** of a compound in a certain medium and at a certain temperature is defined as the concentration of the compound in solution while in equilibrium with an excess of the solid form of the compound (i.e. in a saturated solution). In equilibrium means that both the concentration of the compound in solution and the solid phase do not change over time, implying that the solid phase should be the most stable crystalline form (Sugano et al. 2007). For ionizable compounds (acids, bases or ampholytes) in aqueous media, the solubility depends on the degree of ionization and thus on the pH of the medium. In this respect, the **intrinsic solubility** of a compound refers to the solubility of the unionized form, which can only be measured at a pH where the acidic and/or basic groups do not dissociate.

In simple aqueous media, the solubility of a compound only includes molecules that are freely dissolved in the solvent. In more complex media, however, additional interactions between the compound of interest and the constituents of the medium may challenge the correct assessment and interpretation of solubility values (Bergström and Avdeef 2019). In gastrointestinal fluids, the presence of bile salts, phospholipids and postprandial lipid digestion products generates additional phases, consisting of micellar or vesicular colloids, or even lipid droplets. For lipophilic compounds, **solubilization** occurs as a result of association with these phases and is identified by an increased concentration without increasing the concentration of molecules freely dissolved in the aqueous solvent phase (Buckley et al. 2013).

Solubilizing excipients in absorption-enabling formulations may further increase compound solubilization in the gastrointestinal tract. Such excipients include fairly well described surfactants (creating additional micelles), cyclodextrins (creating inclusion complexes) and cosolvents (altering the polarity of the solvent), but also highly concentrated hydrotropes, which may solubilize hydrophobic compounds by a mechanism that is not yet fully understood (Booth et al. 2012). With respect to absorption, solubilized molecules behave differently as compared to freely dissolved molecules: while freely dissolved molecules are readily available to permeate the intestinal mucosa, permeation of solubilized molecules may be hampered by their interaction with the solubilizing agents (reduced thermodynamic activity) (Porat et al. 2018). Therefore, it is important to distinguish between the **true molecular equilibrium solubility** of the compound including only the freely dissolved molecules, and its **apparent equilibrium solubility**, including both freely dissolved and solubilized molecules (Buckley et al. 2013). It should be noted that the term '**apparent solubility**' is also being used in scientific literature to describe solubility values that are not fully in accordance with the definition of a thermodynamic equilibrium solubility e.g., solubility of a metastable solid form (Almeida et al. 2015) or solubility determination without confirmation of equilibrium by a time course measurement (Sugano et al. 2007, Murdande et al 2011a).
From a thermodynamic point of view, the equilibrium solubility of a compound in a certain medium is the maximum concentration of this compound in this medium. However, in certain cases, higher concentrations can be achieved; in such cases precipitation will occur. Since precipitation does not always occur immediately, however, a temporary, metastable solution may exist in which the concentration of a compound in a certain medium exceeds its equilibrium solubility in that medium (**supersaturation**) (Brouwers at al. 2009). When evaluating the food- or formulation-induced enhancement of compound concentrations in complex gastrointestinal fluids, it is important to distinguish between (i) supersaturation, which temporarily increases the concentration of freely

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dissolved molecules above the molecular equilibrium solubility, and (ii) solubilization, which only increases the apparent equilibrium solubility without affecting the freely dissolved molecular concentration (Frank et al. 2012).

Strictly speaking, solubility is a physicochemical property of a compound in a certain medium that is only defined at a thermodynamic equilibrium between solute and solid phase. In the pharmaceutical industry, however, the term solubility not always refers to a solution at equilibrium. In this respect, the **kinetic solubility** of a compound in a certain medium can be defined as the maximum concentration of the compound that can be added to the medium from a highly concentrated solution without precipitation in a relatively short time course (Sugano et al. 2007; Alsenz et al. 2007). Kinetic solubility thus refers to the precipitation tendency of the compound. Since formation of metastable precipitate is possible, kinetic solubility values are often higher than the equilibrium solubility and highly dependent on the experimental conditions, including the time course (Alsenz et al. 2007; Saal et al. 2012).

By definition, the equilibrium solubility of a compound refers to the most stable crystalline form. No 'true' solubility can be defined for higher energy solid forms (e.g., high-energy polymorphs or amorphous forms), since those can only exist in a metastable (and not thermodynamic) equilibrium with the solute. Yet, the term solubility is widely accepted for metastable polymorphs (Pudipeddi and Serajuddin 2005; Nicoud et al. 2018). In addition, the term **amorphous solubility** is used in pharmaceutical sciences to indicate the maximum supersaturated concentration of a compound that can be achieved from an amorphous form (Murdande et al. 2011b). Obviously, the inherent instability of an amorphous form complicates the accurate assessment of amorphous solubility values (Almeida et al. 2015).

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2. Experimental Methods for Measuring or Estimating Solubility of Drugs

2.1 Shake-flask methods for measuring apparent solubility

2.1.1 Solvent evaporation shake-flask methods for measuring apparent solubility

Measurement of (thermodynamic) equilibrium solubility would be desirable for compound selection and optimization. However, at this stage there are two issues. Firstly, robotic handling of low milligram amounts of solids is still a challenge (Alsenz, 2011; Comley, 2009; Bahr et al., 2020). As a workaround, "solids" generated by evaporation from stock solutions of compounds were introduced. Since the residual solid state form is usually unknown, it is not possible to correlate measured solubility with solid state properties in this type of assay. The second issue relates to the fact that solubility is measured using impure compound batches (Pudipeddi and Serajuddin, 2005; Urwin et al., 2020). These impurities result from starting materials, chemical synthesis (byproducts, interaction products, catalysts, residual solvents, etc.), and/or compound degradation during synthesis and storage. Also, since the stable crystal state may not have been fully characterized, or even discovered, potential change of the solid phase over time as identified from the residual solid should be compared with the initial solid phase characteristics in the beginning of the solubility study (Avdeef, 2016).

In early discovery, solvent evaporation-based shake-flask methods (solvent casting) are very popular for solubility screening (Alsenz and Kansy, 2007; Alsenz, 2012). These assays are adapted to already available high-throughput instrumentations and workflows. They avoid the labor-intense weighting step by distributing compound to microtiter plates as solutions followed by solvent evaporation to generate the starting solids for subsequent studies. A frequent solvent is dimethyl sulfoxide (DMSO) since stock solutions of compounds in DMSO are used as starting materials in a variety of discovery assays; however other solvents such as water, methanol or methanol/ 1,2-Dimethoxyethane (DME) have also been reported (Heikkilae et al., 2011; Tan et al., 2005). A typical example for this type of assay is the LYOphilization Solubility Assay (LYSA) used at Roche (Alsenz and Kansy, 2007). The scope of LYSA is to produce a fast solubility ranking for compounds in the early phase. Thirty microliter of a 10 mM stock solution of the compound in DMSO is added to a V-shape 96-well microtiter plate. DMSO is removed with a centrifugal vacuum evaporator (Genevac Technologies)(38-40°C, 1,700 rpm (~500g), 60 min, full vacuum) and 0.15 mL solvent is added. In each well, sample is sonicated for 15

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min at maximal power using an ultrasonication system (Covaris C-1000). Samples are shaken for 2h at 1,500 rpm and filtrated through a 96-well Multiscreen plate (Millipore, MSSLBPC50) for 5 min (2000 rpm (~600g)) at 22°C. After filtration the compound is typically diluted 1:10 and 1:100 in DMSO in a 384 plate and then further diluted 1:100 in a Water/Acetonitrile mix containing a standard for LC-MS/MS analysis. Experiments with DMSO solutions suggest that dryed compound may finally still contain up to 2% of DMSO since DMSO is not fully removed even if when the evaporation time is extended to more than 2h (Guo et al., 2008, Zhou et al., 2007). Although this DMSO concentration is expected to have only minor effects on compound solubility, even for lipophilic compounds, protocols with lower boiling solvents were established. Examples are (1) Tan et al., 2005 who applied 0.25 mL of 1 mg/mL compound solutions/well in 50/50 Methanol/DME (w/w) and removed the solvent with a centrifugal evaporator, (2) Roy et al., 2001 used 0.3 mL of a 10 mg/mL compound solutions/well in acetontrile and a nitrogen stream for 2h for solvent removal, (3) Barillaro et al., placed 2% compound concentrations in acetone in 10 mL vials and used overnight heating at 40°C for solvent removal, and (4) Shanbhag et al., 2008 applied 0.3 mL of a 0.2 mg/mL compound solution in aceton:ethanol (1:11) per well and removed the solvent by vacuum centrifugation (Genvac® HT-4X) for 2h at 40°C.

A variety of modifications of the solvent evaporation method have been reported. They address some known liabilities of the technique such as residual solvent in wells after evaporation, simple characterization of residual solids, extention of solubility range or generation of more stable polymorphic forms. Nonetheless, the majority of solvent evaporation methods applied in early stage high throughput (HT) discovery environments typically do not characterize the generated solids. Reasons are (1) the often too small amounts of solids generated per assay that do not allow the use of standard analytical methods such as X-ray diffraction (XRD), (2) incompatibility of methods with plate/vessel materials or (3) time-considerations if time-consuming methods such as microscopy are applied. In this stage it is generally accepted that unknown amounts of residual solvent and unknown solid state properties may generate measurement uncertainties. Therefore, the solubility values from these assays are typically only used for compound ranking and for strategic decisions. They are not taken for granted and, as development of the compound progresses, they need to be confirmed by later stage assays under better controlled conditions.

The Partially Automated Solubility Screening (PASS) assay (Alsenz et al., 2007) is a medium-throughput solubility assay in microtiter plates applied in drug discovery phases. Compound is dispersed in a non-solvent (typically, heptane) to a total amount per volume of 25 mg/mL and 40-80 µL of the suspension is transferred to 96-well microtiter plates. Heptane is removed by evaporation

(e.g. by evaporation for 1 h at RT using a SpeedVac at 2000 rpm), 40-80 µL of medium and a stir bar are added, the wells are capped and samples are stirred for (21±3) h at room temperature (25±5) °C. Samples are filtered through ≤ 0.45 µm hydrophilic polyvinylidene difluoride (PVDF) filters and dissolved compound in the filtrate is quantified using an appropriate UPLC (or HPLC) method. In aqueous media, the final pH of the filtrate is determined. The typical compound consumption in a PASS assay is 0.5 to 2 mg per medium and the solubility range covered is 0.001-100 mg/mL.

Distribution as suspension in the PASS assay facilitates flexible dispensing of variable amounts of solids per well by simply adjusting the dispensed volume. The vertically inserted stir bars allow effcient mixing even in highly viscous media. PASS assay is limited to compounds that do not completely dissolve in heptane. Due to the mixing procedure, there is a potential risk that compounds are milled, amorphous material is formed and supersaturated solutions are generated. Furthermore, the usually small amounts of residual solid are not sufficient for powder- XRD (P-XRD) analysis and other methods such as Raman spectroscopy or polarised light microscopy have to be applied for solid state characterization. Nonetheless, PASS assay solubility data provide an excellent basis for compound ranking in discovery optimization phases and for the identification of appropriate formulation strategies early on.

In solvent evaporation solubility protocols, the polymorphic status of the reconstituted solid and of the residual solid at the end of the experiment is usually unknown. In part this is attributed to the generally low amount of solid per well or to well materials that do not directly allow e.g P-XRD or microscopic analysis. Another reason is that the often time-consuming solid state characterization methods do not fit into high-speed solubility protocols or are not regarded usefull by discovery scientists.

In almost all solvent evaporation assays comparing several compounds, constant volumes and compound concentrations in a single volatile solvent are used. Therefore, the amount of compound per vessel is limited by the selected starting concentration of the compound in the volatile solvent and by the maximal volume of the vessel. For higher compound loading, several volatile solvents with maximal solubility for individual compounds may be used, volumes added per vessel may be varied according to needs and/or compound solutions may be added several times to individual vessels followed by evaporation (Heikkilae et al., 2011). Alternatively, lower volumes of the final medium may be added to increase the tested solubility range or to reduce the consumption of biofluids (Guo et al., 2008).

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As solvent evaporation assays do not typically lead to equilibrium solubility measurements, incubation times can be an important aspect (Alelyunas et al., 2009; Bard et. al., 2008). Nonetheless, solubility values resulting from solvent evaporation methods may still be closer to the “true” thermodynamic solubility of a compound than kinetic solubility data resulting from compound precipitation in a medium (Zhou et al. 2007).

2.1.2 Shake-flask methods for measuring apparent equilibrium solubility

Most assays designed to determine (thermodynamic) equilibrium solubility are based on the shake-flask method. To apply this method, excess amount of compound with high purity in the thermodynamically stable crystal state is added to a liquid medium in a flask/vial/microtiter plate and the suspension is mixed at a specific temperature until equilibrium of solid phase with the molecularly dispersed compound is reached. Compound concentration is measured in the saturated liquid phase, typically, after removal of residual solid.

The Solubility and Residual Solid Screening (SORESOS) assay is another medium throughput solubility assay that allows the simultaneous, small scale screening of compound solubility in various pharmaceutical vehicles and identification of changes in solid state by HT-XRD (see section 3.1.1). It is a microtiter plate based, miniaturized shake-flask method fullfilling many criteria for a simple, standardized workflow, proposed by Wyttenbach et al. (2007). It is usually applied in late discovery/early development when compound is already available in larger quantities. Ten to fifteen milligrams of compound are dispensed volumetrically into filter plates with a Titan 96 Well Resin Loader™, 100 µl of medium and a stirring bar are added and, after sealing, samples are mixed by head-over-head rotation of the plate for (21$\pm$3) h at room temperature (25$\pm$5) °C. Liquid is separated from residual solid by centrifugation through the 0.4 μm polycarbonate track edged (PCTE) membrane of the microtiter plate, the compound in the filtrate is quantified and, if an aqueous medium is used, the pH is measured. Wet substance pellets on the filter membrane in the plate are directly analyzed by HT transmission P-XRD (or RAMAN microscopy). If residual solids contain mixtures of polymorphic forms, the higher soluble polymorph dominates measured solubility even if present in less than 10%, as shown for piroxicam anhydrate / monohydrate mixtures, by Kirchmeyer et al. (2016). The SORESOS has a couple of advantages: First, in addition to aqueous media, also organic solvents compatible with the membrane/plate and viscous vehicles such as oils and liquid detergents can be tested. Second, head-over-head rotation with inserted stirring bars bears a low risk of milling of particles with potential generation of amorphous material and/or formation of

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supersaturated solutions. Third, compound adsorption to filters can be neglected since filters are presaturated with compound during incubation. Fourth, removal of liquid from solid is easy, since the incubation “flask” is already the separation device. Fifth, the solubility range covered spans from a few µg/mL up to about 100 mg/mL. However, while solubility test with one compound in many media in a single SORESOS plate can be easily done, measurement of several compounds in few media in one plate is challenging with the 96-well Resin loader. Alternatively, wells can be individually loaded with solid compound using the “Powder picking” method (Alsenz, 2011), a suspension of the compound in a volatile non-solvent, e.g. heptane (Alsenz et al., 2007). Commercially available robotic powder dispensing system such as the Chemspeed SWING FLEXYWEIGHER PLUS (chemspeed.com) or Capsugel Xcelodose (capsugel.com) may also be cosidered, however, instrument costs are in the range of one million US dollars. For some organic solvents with low boiling points, sealing of wells can be problematic. Here, incubation in a separate plate with tighter sealing and transfer to the filtration plate just before liquid-solid separation can solve the problem. If compound availability allows, the number of replicates in solubility studies should be at least 3-4 and individual solubility values along with descriptive statistics should be reported. To avoid biased assays (especially, if on a high-throughput setting), special distribution patterns for replicates may have to be considered if solubility studies are performed in microtiter plates (MTP). In MTPs uneven distribution of variability across the plate may result in so-called “edge effects”, “plate effects” or “positioning effects”. Sources of location dependent factors can be temperature distribution, MTP composition, faster solvent evaporation around the perimeter, channel-specific bias of liquid handling equipment, uneven stirring/sonication/shaking, and/or reader variability (Roselle et al., 2016). An example might be Caffeine, where different polymorphic forms were found in triplicate measurements on MTPs after incubation for 24h at room temperature (Wyttenbach et al., 2007).

A simple miniaturized version of the classical shake-flask method using Whatman UniPrep filter chambers was proposed by Glomme et al. (2005). After addition of the API and 2 mL of liquid medium, the chamber is closed with a plunger that has a filter membrane on one end and a pre-attached cap/septum on the other. After shaking at 450 rpm and 37°C for 24 h, the plunger is pressed down, forcing the filtrate into the reservoir and compound is quantified in the filtrate. Although possible, analysis of the residual solids was not reported. Mini-UniPrep filters with slit caps and a vial shape that fits directly into HPLC 2 mL autosamples are also commercially available (https://www.gelifesciences.com/en/ch/solutions/lab-filtration). Their working volumes are 0.1-0.4 mL and various filter and vial materials are available to circumvent potential compound adsorption issues. Minor modifications could certainly improve this method in the future, e.g. analysis of

residual solid after removal of the plunger or exposing filters to the medium-compound suspension already during the incubation or for some time at the end of the experiment to reduce the risk of compound sorption to filters.

The THErmodynamic Solubility Assay (THESA) used at Roche is an example of a low throughput parallel screening assay used in lead optimization phase (Alsenz and Kansy, 2007). Approximately one milligram of the compound is manually placed in a glass tube with 300 μl of medium. The tube is placed on an ultrasonic bath for 1 h to speed-up dissolution, then shaken for 2 h and stored at RT (22±2) °C overnight. Then the pH of the solution is measured, the solution filtrated through Millipore filter plates (MSGVN2250, 0.22 μm, hydrophilic PVDF), and the compound is typically quantified by HPLC measurement. Similar protocols have been published by other companies (Tan et al., 2005; Qiu and Albrecht, 2018; Varma et al., 2012) and are now also used by service providers (e.g. www.pion-inc.com).

Frequently, few milliliters of solubility medium in about 10 mL (ca.) glass vials are employed. This allows pH measurement before and after the solubility study and pH adjustment during the experiment, if required. Since the vials can be easily capped, vigorous agitation and incubation at various elevated temperatures can be done even for extended periods of time.

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2.1.3 Factors affecting apparent solubility data collected with shake-flask methods

Amount of compound in excess

The amount of compound in excess should be sufficient to maintain the presence of solid particles at the end of the experiment. In discovery / early development phase, however, in case of very high solubility and/or insufficient availability of compound, less compound may be added and solubility is reported as “higher than” a specific value (typically of the order of mg/mL or higher).

A more than 2-4 fold excess of solid over equilibrium solubility is not recommended since this may enhance the effect of soluble impurities and may also pose other problems as pointed out by Avdeef (2007). For example, if the compound is (partially) ionized or it is a salt, the buffering capacity of the solution may be exceed, and solubility may either increase or decrease depending on the type and amount of compound added (Murdande et al., 2011b). To keep the excess of compound added less than 4-times of its solubility, a preliminary small scale solubility study may be useful (Kawakami et al., 2005; Mohammadi et al, 2018). This can be done, e.g. by adding a DMSO stock solution to the

solvent of interest until precipitation occurs or by addition of increasing amounts of solvent to a known amount of compound until all compound is dissolved.

Mixing conditions during experimentation

Mixing can be done by various methods, ranging from simple agitation without (Bergström et al., 2002; Glomme et al., 2005) or with glass beads (Roy et al., 2001), stir bars (Alsenz et al., 2007; Tan et al., 2005; Guo et al., 2008), multichannel cartridge pumps (Chen and Venkadesh, 2004), sonication (Oldenburg et al., 2005) or head-over-head mixing with a stirring bar (Wyttenbach et al., 2007). Homogenous mixing in viscous liquids, for example in Tween 80, is more challenging than mixing in low viscosity ones; however, the latter may also be problematic due to the volume loss through evaporation in non-sealed vessels or liquid condensation in upper parts of sealed vessels (Alsenz and Kansy, 2007; Alsenz et al., 2007). The effect of mixing is also related to the physical geometry and size of the vessel used during the experiment, and therefore, the decision on mixing technique needs to be made in accordance with the information around the plate or vial system used for the experiment.

Introduction of an additional sedimentation step after mixing also affects resulting solubility values (Mohammadi et al., 2018). In a previous consensus recommendation paper, sedimentation was recommended as the first choice to separate solids from solution (Avdeef et al., 2016). Sedimentation has also been recommended to use prior to filtration to reduce filter clogging (Ono et al., 2019). Applying the step of sedimentation may also prevent the formation of a supersaturated solution caused by intensive stirring (Avdeef et al., 2016; Baka et al., 2008).

However, direct sampling from the supernatant requires technical skills, especially when using small vials or microtubes. Nanoparticles and fine powders may be re-suspended even by slight stimulation. Therefore, sedimentation may not be a viable method in drug discovery since particle size is not controlled and assays need to be done in a high-throughput mode.

Depending on the volume and liquid, Brownian movement alone may already be sufficient for mixing (Chan and Katzarian, 2005). If energy input is needed for mixing, e.g. ultrasonication, the solution may become heated and solid state transformations may be triggered. The increased temperature may result in altered (typically increased) solubility compared with that of the material under controlled temperature conditions, whereas solid state transformation may trigger formation of both more or less soluble polymorphs than the one introduced. Stirring can result in grinding of particles and generate larger surface areas and potentially amorphous surfaces. For sonication, special equipment is needed with one ultrasonic pin per vessel. Energy input with those instruments can be quite high resulting in medium heating and drug particle degradation (Oldenburg et al., 2005); temperature hence need to be controlled in the solution when taking the sample and potential

degradation products need to be followed. If any form of degradation is apparent this information needs to be added to the solubility value reported, since these components may signifcantly impact on the resulting concentration measured. It is critical to perform an in situ measurement with a thermometer in the solution since the surrounding temperature is not reflecting that of the ultrasonicated solution. Overall, ultrasound water baths are inappropriate for mixing since ultrasound waves are inhomogenously distributed which will result in uneven mixing, heating and/or particle destruction (Nascentes et al., 2001).

In most protocols, the recommended, average incubation time to attain equilibrium solubility is about 24h, however, incubation times of 48 h up to 2 weeks are also reported (Loftsson et al., 2005,, Avdeef et al., 2016, Bergström and Avdeef, 2019). The latter may be needed for poorly wettable or slowly dissolving, uncharged, low solubility solid drugs. Particularly amorphous solids, less stable polymorphs or oils often need more time to essentially convert into the thermodynamically most stable polymorph. In traditional shake-flask protocols, a minimal medium volume of at least a few milliliters and an incubation time of >24h were regarded necessary to obtain reliable equilibrium solubility results (e.g. Glomme et al, 2005). However, Bergström et al., (2002 and 2004) demonstrated that a reduction of volume from 1 mL to 0.05 mL did not result in statistically considerable discrepancies and that an incubation time of 24h was sufficient for the majority of compounds to reach saturation solubility. It should be noted that the time-frame spent is dependent on the quality needed for the final measurement, the material used for the assay and the assay itself. If an absolute value has to be obtained, time-versus-concentration profiles need to be established to prove that the equilibrium solubility has been reached. The 24h rule was established based on that most of the compounds explored then had reached their equilbrium value at this time point in the medium explored (water)—several of them significantly earlier than the 24h time point—and for those that were not at equilibrium, the thermodynamic solubility was close to the 24h time point, although the data points as such were significantly different from the final data point at which equilbrium was reached. For the latter, these were typically poorly soluble commpounds with slow dissolution. Hence, both the agitation speed used and the particle size of the material studied will influence the final time needed to reach the thermodynamic solubility value. The advantage of taking samples during the solubility study is that it can be assessed during the experiment how long time it takes for apparent equilibrium solubility to be achieved. Generally apparent equilibrium solublity is achieved when two subsequent samples have drug concentrations that are less than 5% apart, perhaps less than 10% when measuring solubility in lipid excipients (Persson et al 2013).

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2.2 Estimating apparent equilibrium solubility from dissolution data collected under sink conditions

According to the Nernst-Brunner approach, the dissolution rate is connected to the difference between the actual concentration in the bulk solution and the equilibrium solubility of the compound in the same solution (e.g. Dokoumetzidis and Macheras, 2006). If the bulk concentration of the solute is kept close to zero (sink conditions apply) and the surface area of the dissolving species is controlled, equilibrium solubilities of compounds could be ranked, and even quantitatively estimated, provided that diffusion layer thickness is known [usually it is set to be 30 μm for particles > 30μm in diameter (Hintz and Johnson, 1989)] and the diffusion coefficient is estimated (usually by the Stokes-Einstein Equation considering the molecular weight (Aljanabi, 1990)). The idea for specific experimental setups to control the surface area exposure during the dissolution process dates back to Takeru Higuchi in the 1960s (Higuchi, 1963). Two geometric systems have been considered: (a) unidirectional leaching or extraction from a simple planar surface, and (b) three-dimensional leaching or extraction from a spherical pellet or crystals.

2.2.1 Dissolution experiments from a planar surface

Using cylindrical zero-porosity compacts that are covered with inert material everywhere but on one flat face, intrinsic dissolution rates (IDR) can be measured: the surface area of the solid remains unchanged during the (initial part of the) dissolution process, and dissolution rate is a function of solubility. The method employes tablets (typically 1-10 mm in diameter) that are slowly compressed at high pressures to make zero porosity compacts. The compression assembly is designed as to allow the tablet to stay in the die after compaction, and to expose the flat face of the tablet to the medium. Under controlled experimental conditions (temperature, hydrodynamics, etc.), linear dissolution rate curves are revealed as long as the solute concentration is low. Typically, official validated dissolution test apparatus (e.g. European Pharmacopoeia) is used, with up to 900 mL of medium. For poorly soluble compounds, smaller volumes are advantageous, or particle-based methods are preferred.

Typically, well-reproducible values are found, which makes this approach very useful to rank and to compare substances regarding intrinsic dissolution rate and, thus, equilibrium solubility. Polymorphs and pseudo-polymorphs can also be distinguished even if their solubilities are practically identical (Surov et al., 2012). However, if there are medium-mediated changes on the surface of the solid

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during the course of the experiment, the dissolution curve will not be strictly linear throughout. Typical examples are polymorphic transition reactions or the formation of hydrates and solvates and IDR can even be useful for determining the solubility of unstable phases (Milosovich , 1964).

2.2.2 Dissolution experiments using pellets, single particle, or multiple particles

Based on the Nernst-Brunner approach, Hixson and Crowell (1931) derived the dissolution kinetic equation for round particles / non-disintegrating beads of isotropic properties, and zero porosity. Single particles (zero-porosity pellets) have been observed during the dissolution process by on-line optical monitoring of the pellet volume decrease using 8 model drug substances of widely different solubilities (Svanbäck et al. 2015). The study revealed that from the initial dissolution kinetics in water, solubility data can be derived and the quantitative values were in good agreement with shake-flask methods (average solubility difference approx. 8%). The experimental approach is limited by challenges regarding the preparation and isolation of the pellets and their initial mass determination. On the other hand, only small amounts of material are needed (10 µg) and it is a fast method: depending on dissolution rate, an experiment takes between minutes and few hours (Svanbäck et al. 2015). Another study used the optical monitoring of single crystals of a drug substance (indomethacin) in different media, including biorelevant media. Solubility could still be measured rapidly, however, solubility values as compared to the shake-flask method deviated up to a factor of approx. 8 (which still is regarded reasonable as compared to common deviations within the shake-flask results) (Strukelj et al. 2019). It has also been attempted to observe dissolution of samples containing multiple particles. Suspension techniques are advantageous to avoid wetting challenges. Obviously, suspensions need to be standardized in terms of particle size distributions to allow for a valid connection between dissolution kinetics and solubility. In order to start the dissolution process, such suspensions would be diluted by a medium. On-line monitoring of dissolved material is necessary to follow the rapidly starting dissolution process. This can be a challenge in suspensions. Optical fibre ultra violet (UV) probes have been successfully used (Andersson et al. 2017; Teleki et al. 2020).

If the particle sizes of the suspension are not controlled beforehand, particle size distributions can be estimated by curve fitting (Andersson et al., 2016); however, the quantitative data for intrinsic dissolution rates normalized by surface area become more uncertain.

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2.2.3 Restrictions of the kinetic methods for estimating equilibrium solubility

The background of the kinetic approach to equilibrium solubility requires a single solid phase and simple dissolution mechanism. Therefore, the following restrictions apply to all the above-mentioned kinetic methods:

a. Disproportionation of the molecules on the surface of the solid or in the diffusion layer as well as precipitation - due to "microenvironmental pH" that changes over the diffusion layer - will alter the dissolution kinetics (Lin et al. 1972; Wong et al. 2005). Such phenomena have been thoroughly studied and discrepancies found in literature values discussed (Serajuddin and Jarowski 1985).
b. If surface reactions of the solid, eg. hydrate formation, are fast they do not lead to observable non-linear parts of dissolution curves because exclusively the dissolution rate of the reaction product is measured.
c. After the experiment, it is useful to confirm that the solid state of the sample has not changed. However, bulk methods to investigate solid state (such as P-XRD and DSC) will not catch solid state transformations that only take place at the surface and not in bulk.
d. A specific problem can arise for non-isotropic crystals if the properties of the faces are widely different (e.g. in terms of polarity or wettability). Crystals of widely different habit and thus face area ratios will show different dissolution kinetics (Modi et al. 2014).

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2.3 Methods for measuring kinetic solubility

2.3.1 Methods applied in drug discovery stage

From early 1990s and for about two decades kinetic solubility assays were a quasi-standard in early drug discovery programs, since they are highly automation compatible, fast, cost effective, and fit well into the Absorption Distribution Metabolism and Excretion (ADME) evaluation of compounds. In these methods, compounds are dissolved in an organic solvent, added to the aqueous medium of interest and precipitation of compound is detected. Therefore, kinetic solubility assays measure the maximal solubility of the fastest precipitating species of the compound in a medium and solubility obtained is often much higher than measured equilibrium solubility (Sou and Bergström, 2018). However, the results of kinetic solubility assays are highly time- and method-dependent, since they are affected by a variety of factors. Among them are mixing conditions, solid state of fastest

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precipitating species, impurities, and sensitivity of analytical method. The latter was in the range of 1-300 µM which was sufficient for decision making in early drug discovery but not sufficient for later stages of drug development programs (Lipinsky et al., 1997; Sou and Bergström, 2018). Therefore, reproducible results between different laboratories using different protocols are not expected. Kinetic solubility assays typically use DMSO as organic solvent; rarely other solvents such as ethanol or dimethoxyethane are applied (Alsenz, 2012; Alsenz and Kansy, 2007). Most published protocols start from 10 mM stock solutions of the compound in DMSO that are added to the buffer of choice to give a final DMSO concentration of 2%. After mixing by shaking, stirring, ultrasound or combinations thereof, samples are analyzed after 2-48h, with or without liquid/solid separation, by various analytical techniques such as nephelometry, UV/VIS, LC-UV, LC-MS, Chemiluminescent Nitrogen Detection (CLND) or Evaporative Light scattering detector (ELSD). Tables summarizing representative kinetic assays have been published previously and the interested reader is referred to papers by Alsenz (2012), and Koenczoel and Dargó (2018).

The DMSO stocks have some known liabilities to be considered when setting up kinetic solubility study protocols. First, not all compounds are soluble in DMSO at 10mM concentration (Li and Kerns, 2006). Tetko et al (2013) studied a dataset of 163,000 compounds and showed that approximately 4% of the compounds were not soluble at 10 mM concentrations. Protocols used by some CROs (e.g. Bioduro: https://bioduro.com/adme-solubility-assay/) and groups (Kozikowski et al., 2003, Lin and Pease, 2016) work with 20-50 mM stocks and hence are likely to have even higher percentages of nonsoluble compounds at those target concentrations. Second, freeze/thaw cycles may result in significant compound loss for various reasons, e.g. precipitation of thermodynamically more stable/less soluble polymorphs or decrease of compound solubility in DMSO by water-uptake during vial opening (Kozikowski et al., 2003, Li and Kerns, 2006). Third, oxidation- and/or hydrolysis-sensitive molecules may be degraded during storage in DMSO (Li and Kerns, 2006). Therefore, only nitrogen or argon degassed and dried DMSO should be used for stock solutions. Fourth, the final DMSO percentage in a medium affects solubility in a highly compound-dependent manner, in particular for low solubility compounds - even at concentrations a slow as 0.1% DMSO - a solubility enhancing effect may be observed for highly lipophilic compounds (Lin and Pease, 2016, Chen et al., 2002; Hoelke et al., 2009). Fifth, the maximal solubility in a typical kinetic solubility assay (10 mM DMSO stock, 2% final DMSO) is limited to about 0.2 mM. This may be sufficient for early drug discovery purposes but not for later stage formulation development.

Except for a few studies that apply polarized light microscopy to classify precipitates either as crystalline or amorphous, kinetic solubility assays do not characterize solids and hence measured

solubility cannot be attributed to specific polymorphs (Sugano et al., 2006; Saal and Petereit, 2012). This clearly bares the risk of misdirection in compound selection and ranking in early drug discovery and fosters the risk to progress amorphous, bad crystallizing compounds. Moreover, the final pH in aqueous media is typically not determined. Therefore, ionizable compounds with reasonable solubility may shift the pH in media with insufficient buffering capacity and favor misinterpretation of solubility data. In general, increased incubation time promote the formation of more stable polymorphic forms and is beneficial for generating higher quality solubility data (Lin and Pease, 2016; Sugano et al., 2006; Saal and Petereit, 2012). Other factors affecting kinetic solubility results are solid/liquid separation and the sensitivity of analytical methods applied. Solid/liquid separation by filtration may result in significant compound losses of low solubility compounds by compound adsorption to filter membranes (Chen et al., 2002; Alsenz and Kansy, 2007). The sensitivity of methods increases in the order of nephelometric, UV-spectroscopic and HPLC assays for low solubility compounds, with typical limits of detection of 2×10^{-5} mol/L, 5×10^{-7} mol/L, and 8×10^{-9} mol/L, respectively. In contrast, no major difference between methods was found for high solubility compounds (> 2×10^{-5} mol/L) (Hoelke et al., 2009).

2.3.2 Methods applied in the drug development stage for evaluating kinetic solubility in the fasted upper intestinal lumen

In recent years, the in vitro evaluation of tendency of drug precipitation in the upper intestinal lumen, i.e. the time period during which luminal contents are supersaturated with the compound, has generated special interest. Relevant efforts to date have focused mainly to the conditions in the upper gastrointestinal lumen of healthy adults. The increased number of orally administered lipophilic weak bases and absorption - enabling drug products, such as lipid-based formulations and amorphous drug solid dispersions are two main reasons. In both situations, the likelihood of precipation in the upper small intestine can be critical to the oral drug absorption process. Various methods have been proposed in an attempt to evaluate drug precipitation in the fasted state (O’Dwyer et al. 2019; Butler et al 2019). In early stages of drug development, when drug quantities are typically limited, small-scale tests facilitate an early evaluation of the potential precipitation risk in vivo and allow for initial screening of prototype formulations (Table 1). At later stages of formulation development, full-scale methods are needed to simulate the behaviour of formulations at clinically relevant doses (Table 1). In all relevant methods, the changing environment from the bulk gastric contents to the bulk contents of the upper small intestine is simulated (O'Dwyer et al 2019; Butler et al 2019). However, the degree of simulation of key physiological various processes such as

- the gastrointestinal drug transfer process
- the drug transport to the mucosa of the upper small intestine, and
- the drug transport to lower regions of the small intestine

varies substantially. To date, only few of full scale compartmental models addressing intestinal drug absorption employing the open loop configuration have been evaluated for their usefulness in simulating (supersaturated) drug concentrations in the upper small intestine after administration of weak bases or enabling drug products (e.g. Psachoulias et al. 2012; Kourentas et al. 2016; Van Den Abeele et al. 2020).

Table 1: *In vitro* methods for evaluating the tendency of drug precipitation in the fasted upper intestinal lumen (O'Dwyer et al. 2019; Butler et al 2019).

Small-scale methods to assess drug precipitation
Medium shift (solvent shift) tests
Medium & pH shift tests
Two-stage tests
Methods addressing intestinal drug absorption
Full-scale methods to assess drug precipitation
Medium & pH shift tests (Dumping test)
Medium & pH shift tests or two-stage tests using the compendial apparatus II
Compartmental (transfer) models employing the closed or the open loop configurations
Compartmental models addressing intestinal drug absorption and employing the open loop configuration

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2.4 Methods for measuring amorphous solubility

Amorphous solubility is the concentration achieved in solution after equilibrium between the solution phase and the amorphous material. Knowledge of amorphous solubility can be important for several reasons, e.g. to develop absorption-enabling oral formulations for low solubility compounds (Biopharmaceutics Classification System (BCS) class II or IV, Developability Classification System (DCS) class IIa, IIb or IV) or for the development of formulations representing highly concentrated solutions, which are supersaturated with regards to crystalline solid-state forms. However, amorphous solid-state forms are a specific example of metastable solid-state forms. The tendency to convert into crystalline solid-state forms can be very pronounced for amorphous solid materials. Crystallization

tendency will depend on several parameters. In general smaller molecules and molecules with a high polarity or pharmaceutical salts, have a higher tendency to crystallize compared to larger molecules or very unipolar molecules. For example, many oligopeptides, oligonucleotides or oligonucleosides with a molar mass well above 1,000 g/mol have a very low crystallization tendency. Consequently, there will be no conversion of the amorphous solid-state form to a crystalline solid-state form during incubation with an aqueous medium during solubility determination for these molecules. For smaller molecules, e.g. within the rule-of-five space this is generally not the case, and amorphous to crystalline phase transitions are pronounced during incubations, e.g. to establish equilibrium for solubility determination. For such compounds, amorphous solubility in water can be predicted if the thermodynamic equilibrium solubility is known (e.g. Taylor and Zhang, 2016). However, measurement of amorphous solubility can be very challenging or even impossible to determine, e.g. using shake-flask methods. This crystallization might lead to a metastable solid-state form, according to Ostwald's rule, or it might lead to the thermodynamically stable solid-state form. If at the end of the incubation the thermodynamically stable solid-state form is observed, this does not mean that Ostwald's rule is not followed. The conversion of the amorphous form to the thermodynamically stable solid-state form might have metastable solid-state forms as intermediates, which might remain undetected as they quickly convert further.

Another approach to shake-flask methods is to increase the concentration of the compound for which the solubility has to be measured stepwise. For this purpose, one adds the amorphous compound in small portions to the medium. After each addition one has to wait until the added portion is dissolved completely, as this avoids the presence of seeds which might lead to crystallization (Saal 2020). As long as the concentration of the compound is below the solubility of the amorphous phase, complete dissolution will occur. If the solubility of the amorphous phase is reached, the amorphous phase will remain also as a solid residue. The solubility of the amorphous phase will be between the concentration which corresponds to the concentration of the last portion which as been dissolved completely and the concentration where a solid residue remained the first time. Frequently as soon as the added amount of amorphous phase is not dissolved and exists as a solid residue, this triggers conversion of the solid residue to a crystalline phase and this triggers precipitation of dissolved compound as the respective crystalline solid-state form. Accordingly, the concentration of the compound in solution drops drastically.

Amorphous solubility can also be measured by amorphous dissolution experiments (Zhang et al. 2018; Plum et al. 2020; Ueda et al. 2020). Applying the fiber optic UV probes in the microDiss Profiler (Pion, US) a direct determination of drug in solution during dissolution of an amorphous compound

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can be achieved (Plum et al., 2020). The maximum concentration of of dissolved amorphous compound is the directly determined as the amorphous solubility. This can also be achieved by adding drug in solution in an organic phase to a buffer, here the liquid-liquid phase separation concentration, determined by fluorescence probes, was found to correlate with the predicted amorphous "solubility". However, a crystallization inhibitor may need to be employed to prevent crystallization of the molecule during the experiment (Almeida et al. 2015).

Recently, determination of amorphous solubility based on dissolution of amorphous solid by combining the fields of optics and fluidics, the single particle analysis, has been proposed (Štukelj et al. 2019a). The method consists of a flow-through setup and an image-analysis algorithm, which enables imaging of the fixed drug particles under constant flow conditions. The method only accommodates one study at a time, and can thus be considered as low-through-put. The method is optically based and measures particle size reduction as the direct measure of dissolution; amorphous solubility is then estimated using the Noyes-Whitney equation. The constant flow continuously displaces drug molecules from the surface of the dissolving particle, thus, enables calculation of the concentration of the solute to be at the particle solution interface , which is the solubility of the amorphous compound, according to Noyes-Whitney theory (Štukelj et al. 2019b). The method is based on 2D imaging of a 3D event, which can give some shortcomings, however, the study found a good correlation measurements in the micro-Diss profiler (Plum et al 2020).

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3. Factors Affecting Solubility Data Across Experimental Methods

3.1 Solid state form of the compound

The link between measured solubility and residual solid-state form is crucial in interpreting solubility data. In the simplest case of a single compound in a solvent or in an aqueous solution, the solid phase may contain: 1) an individual solid substance in the most thermodynamically stable or a metastable solid-state form, e.g. polymorphic modification which represents an anhydrate or ansolvate; 2) crystalline hydrate or crystalline solvate (depending on the medium used) which might also be thermodynamically stable or metastable; 3) an amorphous solid-state form (this will always represent a metastable solid-state form).

3.1.1 Analytical techniques for solid-state characterization

A plethora of methods exists for assessing solid residues from solubility experiments. One general aspect which must always be considered is sample pre-treatment. After separation of the solid and the liquid phase, e.g. by filtration or centrifugation, the solid phase usually still contains some solvent or water. One should not try to remove this completely, e.g. by heating, vacuum drying, purging with air or nitrogen or other techniques. Such treatment might lead to a conversion of the solid-state form to another one, e.g. a conversion of a hydrate to an anhydrate, a higher hydrate to a lower hydrate, a solvate to an ansolvate. Instead, the solid-residue should be contemporary used for the analytical method without any sample pre-treatment as this makes sure that the solid-state form which is investigated by the analytical technique is still the same as obtained from establishing the equilibrium over the incubation used during the solubility measurement.

Certainly, the simplest analytical method to characterize the solid residue obtained from a solubility experiment is just having a look at it. This can easily be done by optical microscopy and especially by polarized light microscopy. Even if this method does not reveal the exact solid-state form as polymorph, pseudo-polymorph, pharmaceutical salt etc., the method will tell, if the solid-state form is crystalline or amorphous (Petereit et al 2012). As differences in solubility between an amorphous solid-state form and a crystalline solid-state form are larger compared to differences between different crystalline solid-state forms, this information already represents a very useful information for medicinal chemists working on optimization of compounds with regards to solubility. If together

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with the information about solubility – expressed e.g. in µg/mL – the medicinal chemists also get the message if this number refers to a crystalline or an amorphous residue, this will tell if "optimization" is really done to improve the solubility or if "optimization" is done to hinder crystallization of the compound. The latter can end up in a nightmare, e.g. if in a project measured solubility refers to the amorphous form and later a crystalline residue is obtained with substaintially lower solubility. This will lead to a significant decrease in solubility. A detailed discussion of this topic has been published by Saal and Petereit (2012). This approach by polarized light microscopy can easily be carried out from measurements of thermodynamic solubility, which just require 2-3 mg of compound. Sugano et al. (2006) introduced an automated birefringence crystalline/amorphous diagnosis system to identify crystalline, partially crystalline, and amorphous solids by using a glass bottom plates, an inverted microscope and a photographic imaging processing software. The method is rapid, cheap, and generates valuable information for solubility data interpretation.

X-ray diffraction (XRD) is one of the most reliable and powerful methods to characterize solid-state forms. At early discovery programs it has also been applied at high-throuput setting. A detailed descrition of high-throughput XRD can be found in Wyttenbach et al. (2007). Briefly, 96-well filtration plates are mounted in a 96-well plate sample stage and residual solid on the filters is analyzed without prior preparation or additional processing with a STOE Stadi P Combi diffractometer equipped with primary Ge-monochromator (CuKα1 radiation) and imaging plate position sensitive detector (IP-PSD). The imaging plate detector is exposed for 5 to 10 min to X-ray radiation for each well.

If the diffractogram of the initial substance coincides with the diffractogram of the substance after the experiment (e.g. same positions of peaks in the diffractogram (2Θ) and same intensities) then there were no changes during the experiment, and the obtained value of solubility unambiguously corresponds to the studied solid-state form. XRD can be carried out either as P-XRD or single-crystal XRD (SC-XRD). P-XRD is very useful for investigating solids residues obtained from solubility measurements. Samples can be prepared on a film or in a capillary. Again, one should make sure that harsh conditions for sample pre-treatment are avoided. Beyond telling that the residue is either amorphous or crystalline, P-XRD will provide a characteristic, highly specific pattern which allows to distinguish different crystalline solid-state forms, e.g. polymorphs, pseudo-polymorphs, pharmaceutical salts or co-crystals. In contrast to SC-XRD, which allows the exact determination of the structure of a crystal, P-XRD data does not reveal this information. However, the link between the P-XRD pattern of a solid residue and the structure of the solid-state form can be drawn indirectly by two methods: (1) If the single-crystal structure of a solid-state form is known, the P-XRD pattern

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can be calculated from the single crystal structure. The calculated P-XRD pattern can be used for comparison with the P-XRD pattern obtained from the solid residue. (2) If the single crystal structure of a solid-state form is not known, one can collect other data to elucidate the structure of the solid-state form. This can be data from NMR-spectroscopy, ion-chromatography, gas chromatography, thermogravimetry which might be coupled to IR-spectroscopy or mass-spectrometry. From the data it can be concluded if the respective solid-state form represents an anhydrate, a solvate, a hydrate, a co-crystal, a pharmaceutical salt, a hydrate of a pharmaceutical salt, and so on. Experimentally, one will prepare a larger sample, e.g. about 50 mg for such characterization, measure P-XRD from this and use this diffractogram for comparison with the P-XRD pattern obtained from the solid residue from a solubility experiment. If several solid-state forms exist for a certain compound, such characterization should be carried out for all the relevant forms. Another strength of X-ray diffraction is its ability to tell, if a solid residue represents a pure phase or a mixture of several phases. If a P-XRD pattern has been calculated from a single-crystal structure, it is obvious that the P-XRD pattern refers to a pure phase. If the single crystal structure is not available, the P-XRD pattern might refer to a single phase or to a mixture of two or more phases. In the latter case interpreting and using the solubility data can be confusing and misleading. However, even in the absence of a single crystal structure one can get information about phase purity be indexing the P-XRD pattern. If indexing - which means ascribing an elemental cell with an exactly defined geometry - to the P-XRD pattern is successful and the elemental cell has a reasonable size, one can be sure to have a pure phase. If this is not successful, or the elemental cell is very large and doubtful, one must take into account that the solid-residue might also be a mixture of two or more solid-state forms (USP Chapter 941). Typically, the limit of detection of other phases by P-XRD will be about 10 %.

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Beyond P-XRD being used as a fingerprint technique for identification of the solid residue, there are different other analytical methods acting as fingerprint techniques for such identification. IR-spectroscopy, NIR-spectroscopy, Raman-spectroscopy or solid-state NMR-spectroscopy can be used for the same purpose. All of them have certain advantages and disadvantages. A common disadvantage of all of them is that it is difficult to obtain information about phase purity. In Raman-spectroscopy the sample is exhibited to a high energy density. Accordingly, phase conversion can be problematic and always must be assessed carefully. NIR-spectroscopy is very easy and quick to be carried out but requires a significant amount to sample. In addition to these spectroscopic techniques, also thermo-analytical methods such as Thermogravimetry (TG) and Differential Scanning Calorimetry (DSC) can be used for solid-state form characterization. TG can yield information about the stoichiometry of pseudo-polymorphs, e.g. whether a solid-state form represents a mono-hydrate, di-hydrate, hemi-hydrate etc. The techniques get still more specific if couple with a second

analytical technique such as IR-spectroscopy and mass-spectrometry to identify the nature of the exhaust gas. In solubility experiments, evaporation of residual solvent can be a problem in TG analysis since it may interfere with the detection of pseudo-polymorphs. DSC yields information about phase transitions between different solid-state forms and between solid-state forms and the melt. Accordingly, it can also be used to a certain extend for identification of solid-state forms obtained from solubility experiments. However, DSC does not represent the strongest analytical technique for this purpose.

Based on the above, one can choose amongst several analytical techniques for characterization of the solid residue obtained from a solubility experiment. However, P-XRD should be the preferred one if this is available, as it gets along with a small amount of sample, is relatively quick and delivers highly specific information about the solid-state form using about 1-10 mg of the compound.

Finally, one has to bear in mind, that there might be cases where there is not just one amorphous phase, but different amorphous phases can exist. This phenomenon is called “poly-amorphism”. It refers to the fact that an amorphous phase represents a disordered phase without long range order (Guinet et al 2016, Zhu and Yu 2017). Reader with kids certainly will have realized that there is not only one way to have the children’s room in a disordered state, but there are many ways how kids can realize disorder in their rooms. The same applies for amorphous solid-state forms. In such poly-amorphic systems, the different amorphous phases are generally difficult to distinguish, e.g. all of the will exhibit a halo in P-XRD. There are other features like IR-spectra or the glass temperature which can allow a distinction of such phases. But, as mentioned such distinction is generally difficult. Of course such poly-amorphic phases might also show different solubilities.

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3.1.2 Examples of solid-state characterization

Figure 3 illustrates the dissolution profiles for two polymorphic forms of bicalutamide (Perlovich et al. 2013). Form I is thermodynamically stable and there is no difference between the initial solid-state form and the solid-state form at the end of the experiment. P-XRD and DSC obtained from the original solid and from the solid residue as obtained from incubation during the solubility determination are identical. The same holds true for other analytical techniques which can be used for solid-state characterization such as Raman- or IR-spectroscopy. As a result, there are no characteristic points (maxima, “humps”) on the dissolution profile.

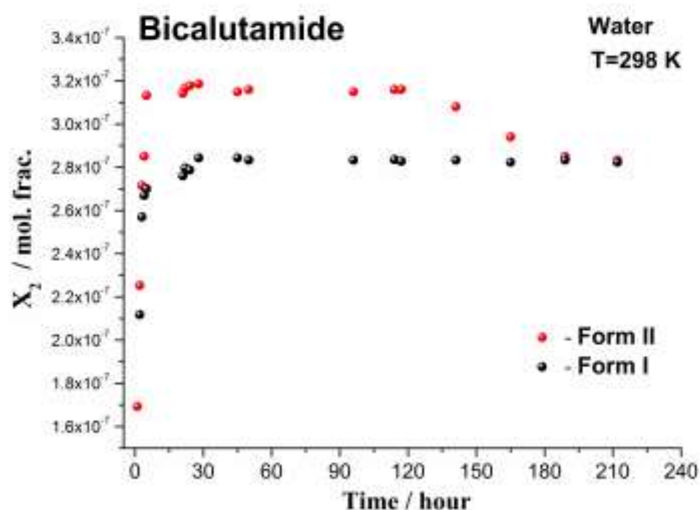

Figure 3. Dissolution profiles for bicalutamide (Forms I and II) at 298 K in water (modified from Perlovich et al 2013).

The situation becomes more complicated if the initial substance is in a metastable solid-state form, and the solid phase after the experiment is a different – typically thermodynamically stable - polymorph of the compound. However, there are also cases, where the solubility experiment starts with a metastable solid-state form, and there is a conversion from this metastable form to another metastable form which is lower in energy compared to the original one. In this case, the solubility value corresponds to the more stable polymorph which can be the thermodynamically stable one or another metastable form. In Figure 3, the discussed situation refers to bicalutamide form II, which after 190 hours of the experiment completely transforms to form I. Such a long period of the transformation of one form to another upon the dissolution is explained by a very low aqueous solubility of the compound as the transition process from form II to form I is solution mediated: The metastable form II shows a higher solubility compared to form I. Therefore, form II dissolves and the resulting solution is supersaturated with regards to form I. Consequently, form I precipitates from the supersaturated solution which yields in total a conversion of the metastable form II to the thermodynamically stable form I. As the solubilities of both form II and form I in this case are low, there is only a small amount of bicalutamide which can pass this process during a certain time. This leads to a slow kinetic of the form II – form I conversion. If a similar experiment is performed in another medium (in which the bicalutamide solubility is high), then the phase transformation would accelerate significantly. The question arises: "Is it possible to estimate the difference in solubility of different polymorphic forms in one dissolution experiment?" Obviously, this can be done by measuring the dissolution profile of the dissolution of a metastable polymorph.

Figure 4 demonstrates an example of such dependence for two polymorphic forms of the Salicylamide + Oxalic Acid (2:1) cocrystal (Surov et al., 2017), where form II is less thermodynamically stable compared to form I. A characteristic feature of the dissolution curve of a metastable form is a "hump". Since the less stable polymorphic modification dissolves faster than the stable one, the first part of the curve corresponds to the properties (incl. solubility) of just the metastable modification. As a result, a supersaturated solution is formed, from which a thermodynamically stable polymorphic form which is less soluble precipitates, leading to a decrease in solubility. As a rule, the maximum of the dissolution profile is attributed to the solubility of the metastable form, and the “plateau” of the curve - to the solubility of the stable form. However, there are numerous cases, where precipitation of the thermodynamically stable form from the supersaturated solution is very rapid. In this case, dissolution of the metastable form and precipitation of the thermodynamically stable form occur to a large extent in parallel, and the concentration reflecting the solubility of the metastable form is never reached.

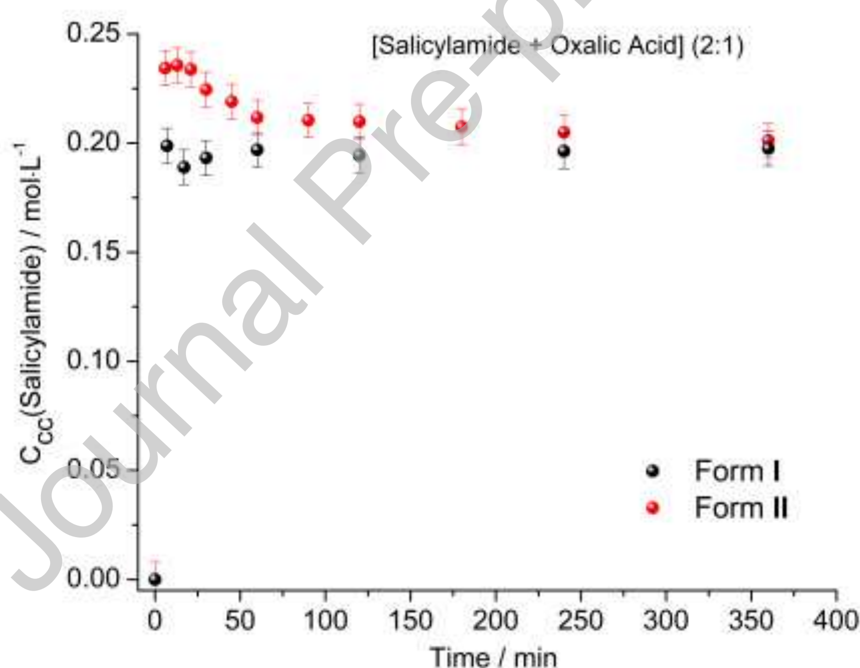

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Figure 4. Dissolution profile of title crystal forms in acetonitrile at 298 K plotted as Salicylamide concentration in solution against time [Reproduced from Surov et al (2017) after permission].

It is interesting to estimate the extent of the difference between the solubilities of various polymorphic forms under similar conditions. Pudipeddi and Serajuddin (2007) performed such analysis, the result of which for 80 pairs of polymorphic forms is presented in Figure 5. Evidently, for 78 % of compounds the solubility ratio lies in the range from 1 to 2 for true polymorphs. However, it

should be emphasized that for the remaining 22 % this parameter is more than 2 indicating the quite large differences. Therefore, the description of the solubility experiments should contain information about the solid-state form to which the solubility value refers.

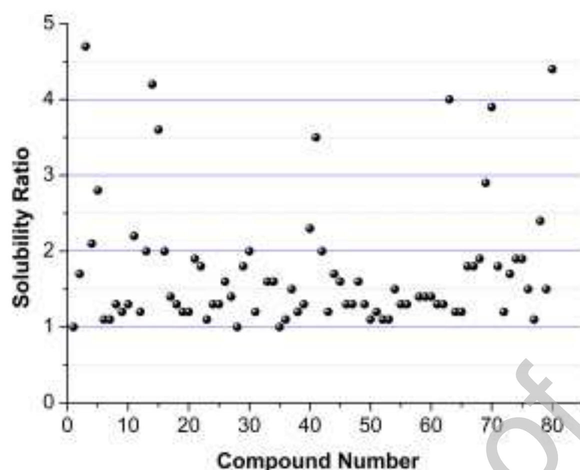

Figure 5. Solubility ratios for 80 pairs of polymorphic form [Reproduced from Pudipeddi and Serajuddin (2007) with permission].

The next objects of studying the solubility of individual substances are the compounds which, as a result of the dissolution, form hydrates or solvates. This means that the crystals of a hydrate or a solvate – also called pseudo-polymorphs - are more thermodynamically stable in solution than the crystals of anhydrates. In this case, the solubility value of the compound refers not to individual compound, but to the hydrate or solvate. This is an important issue, since the values assigned to the individual compounds are entered into the databases used, but these values are essentially underestimated. The kinetic dissolution profiles for the systems forming hydrates or solvates do not differ much from the similar curves for different anhydrate or ansolvate forms. As an example, Figure 6 shows the dissolution profile for ciprofloxacin (Surov et al., 2015a) and arbidol (Surov et al., 2015b), also demonstrating the supersaturation effect. In contrast to the study of polymorphic modifications, the solid phases of solvates and hydrates can be identified also using the TG method, as hydrates and solvates lose their water or solvent which leads to a step in the TG curve occuring at a characteristic temperature and showing the loss of a characteristic percentage of mass.

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(a) (b)

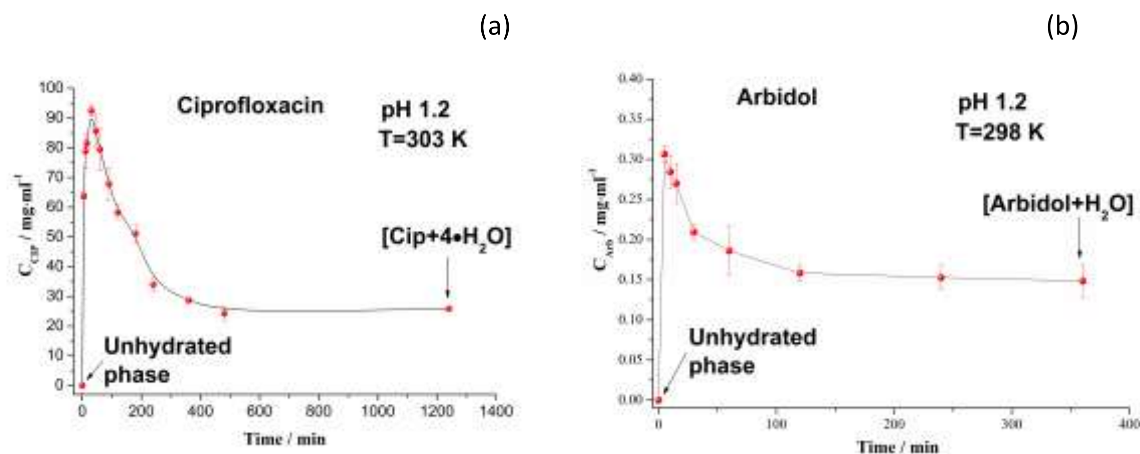

Figure 6. Dissolution profiles for Ciprofloxacin (a) [Reproduced from Surov et al. (2015a) with permission], and Arbidol (b) [Reproduced from Surov et al. (2015b) with permission].

Besides the solubility ratio for the polymorphic phases, Pudipeddi and Serajuddin (Pudipeddi and Serajuddin, 2007) analyzed the solubility ratio of anhydrate to hydrate phases for 23 pairs. The results are presented in Figure 7. Evidently, 26 % of the considered compounds have the solubility ratio of more than 5 with the hydrate form generally representing the lower soluble solid-state form. There are only very few exceptions where an anhydrate form exhibits a lower solubility in aqueous solution compared to a hydrate form, as described by Saal et al. (2015) and in references therein. This proves once again that the dissolution characteristics are directly determined by the solid-state forms.

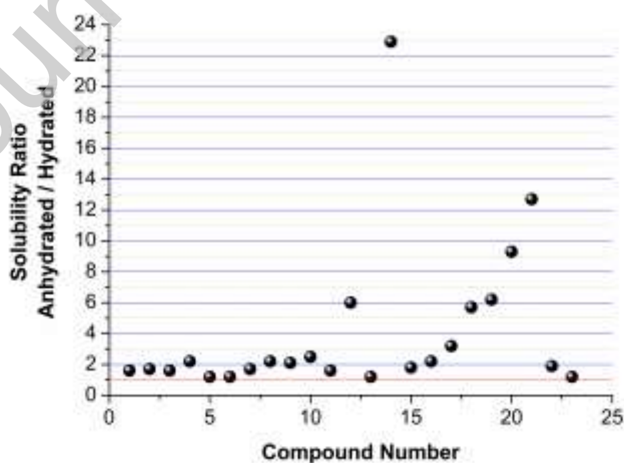

Figure 7. Solubility ratios for 23 pairs of anhydrates/hydrates [Reproduced from Pudipeddi and Serajuddin (2007) with permission].

However, anhydrates can transform to hydrates (or solvates) e.g. by medium-mediated transformation. If this transformation takes place only on the surface of the crystals, the measured solubility is that of the hydrate, while bulk analytical methods, such as P-XRD or Raman-spectroscopy, to characterize the solid phase will still reveal the anhydrate. Theophylline and Nitrofurantoin and their respective hydrates are prominent examples (de Smidt et al. 1986; Aaltonen et al. 2006). The kinetic and extent of the transformation is typically dependent on experimental conditions, such as composition of the solution, temperature, stirring velocity etc.

Finally, the third case of the solid-state form analysis is the existence of an amorphous substance in equilibrium with a solution. As a rule, such situations occur when studying large molecules, for which the processes of homogeneous and heterogeneous nucleation or crystal growth are highly complicated and slow. An example is the the antifungal compound I (Figure 8). Obviously, concentration reaches a "plateau" after a long period of time, but a metastable amorphous phase remains as solid residue. Correlation of the thermodynamic equilibrium solubility value to the metastable amorphous phase is an incorrect action from the thermodynamics point of view. However, due to the impossibility of obtaining a thermodynamically stable solid phase, one must conventionally assume that a “hypothetical” thermodynamic equilibrium is achieved in such a system. This solubility value is overestimated. If we try to estimate the solubility using predictive models, the calculated values can significantly differ from the experimental values towards underestimation. This situation is also very typical for larger molecules such as for example antibodies, oligo- or polypeptides, substances of natural origin such as some antibiotics as well as oligonucleotides and, generally, for new biological entities. It is also very common in the arena of polymeric excipients which have a very low tendency to form crystalline solid residues.

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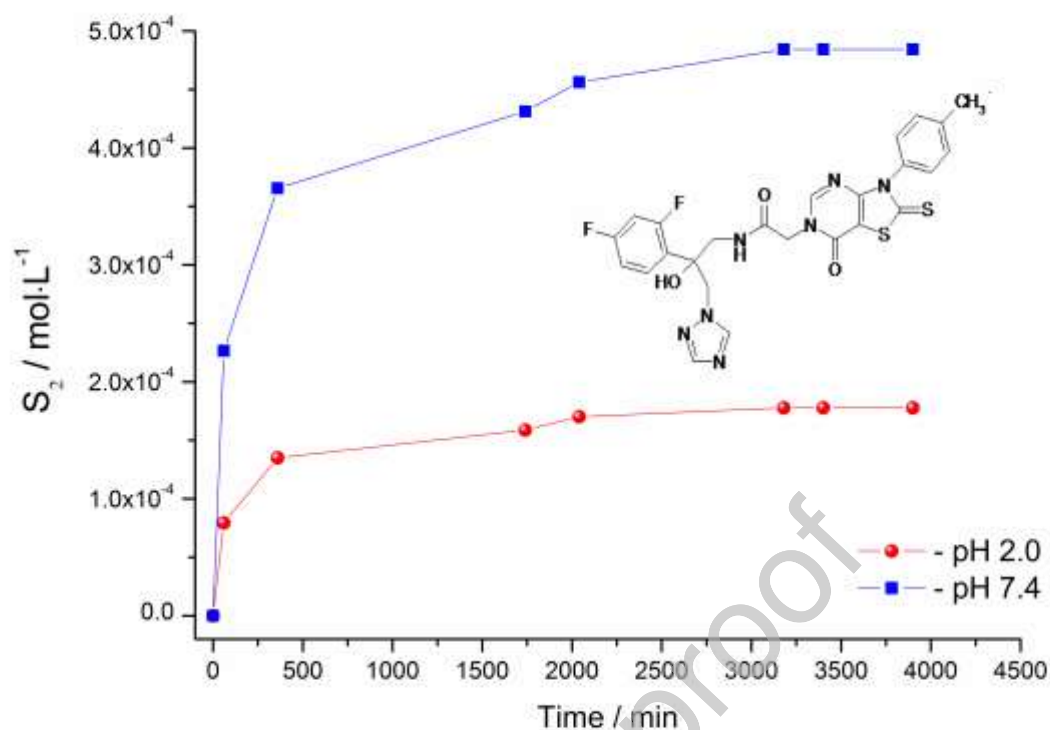

Figure 8. Dissolution profiles for compound I at 298 K with amorphous state at the solid phase after experiment (Perlovich et al. unpublished data).

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3.2. Media related factors

3.2.1 Water

Water should be freshly distilled/purified and equilibrated at ambient air and at the study temperature before use. Ionizable compounds, in particulat those with multiple pKa values, may form self-buffered solutions (Ozturk et al, 1998; Avdeef et al., 2016). For practically insoluble free bases (pKa>9), dissolved carbon dioxide in water may significantly affect measured solubility as discussed in detail by Avdeef et al. (2016); the publication also adresses the handling and pH-adjustment of self-buffered ionizable compounds in water. Hydrolysis may be an issue at the extremes of pH or over long study times (Waterman et al., 2002).

3.2.2 Buffered aqueous solutions

Buffered aqueous solutions resist pH changes upon addition of small amounts of acid or base and, thus, facilitate adjustments to specific pH values. Buffering capacities between up to 25mM are preferred, as too high concentrations may cause complications such as unintended compound-buffer interactions or interference with analytical methods (Avdeef et al, 2016). Commercially available standard buffers, e.g. Titrisol buffers from Merck (Merck ready-to-use buffers) or United States Pharmacopeia buffers (USP Buffers) may facilitate comparison of solubility values generated by different labs. Alternatives that cover broader pH ranges (if needed) are “Universal buffers” consisting of mixtures of several individual buffers and “Minimalist Universal Buffers (MUB)”. The latter contain the same buffer species over the pH range of 3-11, avoid weaker salt formers of drugs such as chloride, borate or phosphate, have sufficient buffering capacity, and can be titrated to the desired pH with sodium hydroxide alone (Avdeef et al., 2016). Determination of the pH after achieving equilibrium solubility is necessary, when ionizable compounds are tested. Depending on the amount and on the solubility of the compound, the buffering capacity of a solution might be exceeded and significantly shift the pH. Highly soluble ionizable compounds are more likely to affect final buffer pH and impurities will affect solubility less than in case of lower soluble compounds.

Buffer species can also influence equilibrium solubility, of ionizable compounds. It can either raise solubility by forming more soluble compound-buffer complexes or decrease it by forming compound-buffer precipitates (e.g. salting out of bases below pH_{Max} or of acids above pH_{Max}). Buffer concentration and final ionic strength may further affect compound solubility (Avdeef et al., 2016) and even differences in cationic counter ions in buffers (potassium versus sodium) at the same buffer capacity (50 mM) can make a difference (Figure 9A) (Hamed et al. 2015). Furthermore, solubility enhancing excipients such as surfactants or cyclodextrins may also significantly alter the final pH in solubility experiments with ionizable compounds in both water (Figure 9B) and buffered solutions (Figure 9C).

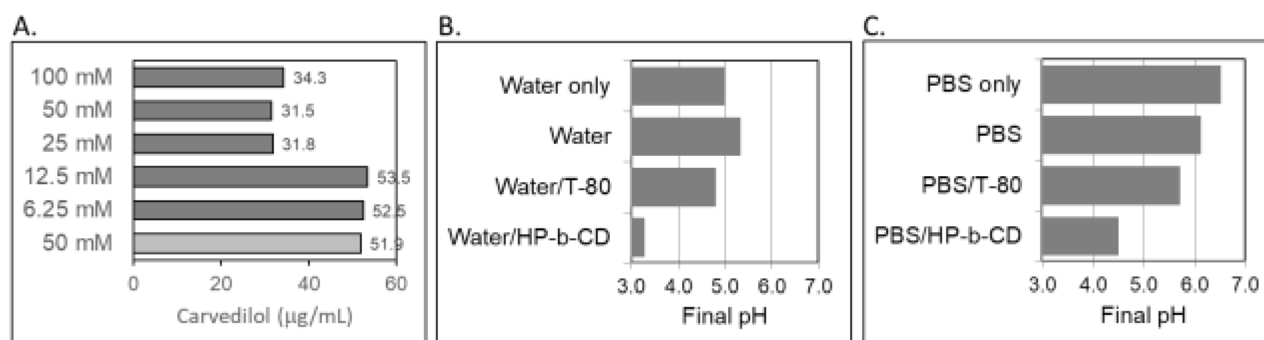

Figure 9. (A) Carvedilol solubility in phosphate buffer, pH 6.8 with various sodium phosphate concentrations (1:1 ratio of NaH_2PO_4 and Na_2HPO_4 , final pH measured). Data in the bottomcolumn (grey) are in 50 mM IntPh3 (International Pharmacopeia) buffer, pH 6.8, that contains both KH_2PO_4 (3.4 mg/mL) and Na_2HPO_4 (3.53 mg/mL) [This figure was constructed using data from Hamed et al. (2015)].
(B) Final pH of water-only and of ibuprofen (pKa 4.11) suspensions in water, in 10% aqueous solution of Tween 80 (Water/T-80), and in 10% HP-beta-cyclodextrin (Kleptose®) (Water/HP-b-CD), after shaking for 24h at room temperature (Alsenz J, unpublished data).
(C) As in (B) but water was replaced by 50mM NaH_2PO_4 phosphate buffer, pH 6.5 (PBS) (Alsenz J, unpublished data).

Level I biorelevant media are aqueous media that, in addition to pH, simulates luminal conditions with regard to buffer capacity at a specific region of the gastrointestinal lumen. At this level, distinction between fasted state and fed state conditions is made (Markopoulos et al. 2015, Pentafragka et al 2020a). Particularly, when simulating fasted state conditions in the small intestine, maintaining constant pH during equilibration can be problematic when the compound is ionizable. Physiologically, it would be more appropriate to use bicarbonates for simulating the conditions in the fasted intestine. In this case, the use of special equipment is required (e.g. Garbacz et al., 2013; Merchan and Basit, 2014). Recently, a simple bicarbonate buffer system by emplying a floating lid method was proposed (Sakamoto et al. 2021)

3.2.3 (Level II) Biorelevant media

Level II biorelevant media are aqueous media that in addition to simulating luminal conditions with regard to pH and buffer capacity simulate also include bile components, dietary lipids, lipid digestion products, and osmolality to simulate the composition at a specific region of the gastrointestinal lumen in the fasted or fed state (Markopoulos et al. 2015). In the pharmaceutical literature these are frequently termed “biorelevant media”.
The colloidal structures present in these media will greatly impact the solubility of lipophilic, poorly water-soluble compounds (e.g. Fagerberg and Bergström, 2015). Characterization of colloidal structures is useful to understand solubilization potential, the possible change in colloidal structures during storage and hence, a potential impact on solubility. Useful techniques to characterize colloidal, lipid-rich structures include, but are not limited to, dynamic light scattering (DLS), diffusion

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ordered spectroscopy nuclear magnetic resonance (DOSY-NMR), asymmetrical flow field-flow fractionation (AF4), multi angle laser light scattering (MALLS), cryo-transmission emission microscopy (cryo-TEM), atomic force microscopy (AFM), small angle x-ray scattering (SAXS) and small angle neutron scattering (SANS) (Elvang et al. 2017; Clulow et al. 2017). These techniques allow structure, size and colloidal volume to be determined and contrasted to e.g. aspirated fluids. The obtained data can further be used to calculate solubilization in the colloidal volume fraction and simple measures such as Solubilization Ratio (Mithani et al. 1996).

Commercially available products are available to facilitate preparation of Level II biorelevant media (www.biorelevant.com). Relevant products allow for rapid preparation of various standardized biorelevant media and it is suggested to be used within 48 after their preparation (www.biorelevant.com). However, regardless of the method of preparation, it should be kept in mind that micelles and other supramolecular assemblies need time to form and to equilibrate. It is good practice to prepare such solutions well in advance of the experiment (e.g. 2-24 hours before).

One should keep in mind that solubility of a compound in a single Level II biorelevant medium cannot give full insight into the solubility of a drug in different individuals. Especially in the fed state, both intersubject and intrasubject variability in pH and colloidal species concentrations is high (e.g. Riethorst et al. 2016).

To get a better understanding of the influence of the variability in composition, and thereby colloidal structures on drug solubility, the Design of Experiment (DoE) approach has been suggested (Khadra et al., 2015, Madsen et al., 2018). Based on a selected design space, covering a relevant interval for each of the selected factors (e.g. buffer capacity, bile salts, phospholipids, pH), a number of media are prepared. After determining the solubility in these media, a mathematical model can be fitted to the data, thereby giving a complete overview of the compound solubility in the selected design space, as shown by Madsen et al., 2018. Here, it was concluded that the important factors for the solubility was pH and bile salt and phospholipid concentration. By only combining these three factors, it is possible to limit the DoE to 11-13 different media. As triplicate determinations are not needed in a DoE, this means that in the case of a 12 media DoE, more information on the effect of gastrointestinal composition on drug solubility can be achieved with the same work load as when using four popular media in triplicate. Thus by applying a DoE approach, the number of experiments can be reduced, thereby saving time and money, but still achieving an understanding of how different factors, and interactions between these, can affect the solubility within the entire design space by developing a fitted model.

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3.2.4 Luminal Aspirates

To date, luminal aspirates have been used for evaluation intraluminal drug equlibrium solubility. The methodology involves two main steps: (a) the aspiration of samples of luminal contents from the region of interest and sample handling and storage procedure and (b) the measurement of drug equilibrium solubility in luminal aspirates ex vivo.

Aspiration of luminal contents and sample handling and storage

Aspiration of gastric contents or contents of the upper small intestine can be performed after nasal or oral intubation. Aspiration of contents from the lower intestine can be performed via colonoscopy (Augustijns et al 2020).

For the aspiration of contents form the upper gastrointestinal lumen, two sterile tubes (one positioned into the stomach and one positioned in the duodenum) or one sterile two-lumen duodenal tube are introduced through the mouth or nose of a human volunteer, the final position of the tube(s) is confirmed fluoroscopically and any nasal, oesophageal and/or gastric secretions in response to the intubation process should be aspirated out of the stomach. (Vertzoni et al 2012, Petrakis et al 2015, Augustijns et al 2020). In the fasted state, samples of contents from the stomach and/or the upper intestine should be aspirated manually over a period of one hour after the administration of a 240 mL of water i.e. mimicking conditions to which drugs/drug products are administered during bioavailability / bioequivalence (BA/BE) studies in healthy adults (FDA 2002). In the fed state, samples of contents from the stomach and/or the upper intestine should be aspirated over a period of four hours, ideally, 30 min after the ingestion of the high-fat, high-calorie meal (reference meal) proposed by regulatory agencies (FDA 2002). To date, only commercially available liquid meals have been used to measure solubility in gastric and intestinal contents (Dressman et al 2007, Augustijns et al 2014). Liquid meals (e.g., NuTRIflex®, Ensure Plus®, Scandishake Mix®) have similar composition, origin of calories, calorie content and/or volume to that of the reference meal (Persson et al 2005, Kalantzi et al 2006, Clarysse et al 2009, Clarysse et al 2011). The reference meal has been used in aspiration studies only recently (Rubbens et al 2019; Pentafragka et al 2020a; Pentafragka et al 2020b).

Collection of samples of colonic contents (i.e. ileum, caecum and ascending colon) requires prior cleaning of the distal colon and any effect(s) of this procedure should have been reversed by the time sampling started. Diakidou et al has proposed a protocol for direct sampling of colonic fluids (Diakidou et al 2009a; Reppas et al 2015) in order to be used for their physicochemical

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characterization and for drug solubility studies (Vertzoni et al 2010). Colonic contents should be collected five hours after the administration of a glass of water or after the consumption of reference meal (FDA 2002) i.e. about the time drugs administered as conventional products or multiparticulate modified release products are expected to reach the lower intestine after oral administration. Handling of contents upon collection is important in order to generate useful and reproducible data. Immediately upon collection, care should be taken to prevent any post-sampling alteration in the aspirated contents until the solubility experiments (Reppas and Vertzoni 2019, Augustijns et al 2020). It is necessary to deactivate enzymes immediately, before storing the sample under deep-freeze conditions (−20 °C or lower). Especially in the fed state, enzymatic digestion should be inhibited by adding lipase and protease inhibitors (Hernell et al 1990, Clarysse et al 2009). Precautions should be taken to limit exposure to the atmospheric environment, since this may alter the pH and the buffer capacity of the aspirates (Litou et al 2016, Litou et al 2020). If the aim of the study is to correlate solubility values with the physicochemical parameters of the contents, then pH and buffer capacity should be measured immediately upon collection. In the fed state, assuming that the micellar phase concentration drives absorption, solubility values can be over or underestimated if only the solubility in the total luminal contents is reported and, it would be preferable to report solubility values determined in the micellar phase to predict food effects (Dressman et al 2007, Vertzoni et al 2012). The micellar phase of the contents can be obtained using ultracentrifugation at 410,174g for 2 h at 37 °C (Hernell et al 1990, Vertzoni et al 2012). After ultracentrifugation, four phases are obtained, that is, triglyceride phase, interphase, primarily micellar phase, and pellet. In the case of contents collected from the lower intestine, the sample is ultracentrifuged (30,000 g, 15 min, 25°C) under anaerobic conditions and the aqueous content is separated and used for solubility measurements. By applying these ultracentrifugation conditions, elimination of both solids and bacteria is ensured without affecting the structure of bacteria, i.e. without liberating intracellular components which could contribute to degradation in the supernatant (Reppas et al. 2015). Samples should be divided in sub-samples before storage to avoid second freeze—thawing cycle of samples (Vertzoni et al 2012, Litou et al 2016, Reppas et al 2015). It has been shown that application of one freeze-thaw cycle to lipase-containing biorelevant media prior to using them, lowered the observed solubility values for dipyridamole and ketoconazole by 18-34% and 13-45%, respectively (Diakidou et al 2009b). Individual or pooled samples can be used for solubility estimations. Individual samples give the advantage of solubility values' correlations with levels of specific components in each sample and thus the opportunity to determine the most important factors affecting luminal solubility of the tested compound (Vinarov et al 2011). On the other hand, a pooled sample from a large number of volunteers offer greater volumes and solubility measurements of many numbers of APIs in contents having the same composition and thus enables comparisons across a set of compounds. For the

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creation of a pooled sample, the volume taken from each individual sample should be held constant, to ensure that the pooled sample is equally representative of all subjects (Dressman et al 2007).

Equilibirum solubility measurements in luminal aspirates ex vivo

To date, equilibrium solubility in luminal aspirates is measured with the shake-flask method at 37 °C (Dressman et al 2007, Augustijns et al 2014). The equilibration time should be as short as possible to minimize composition changes in the aspirated samples but long enough to allow the system to reach equilibrium. Usually, preliminary experiments in biorelevant media can be performed in order to identify the appropriate equilibration time for the tested compound. At the end of equilibration the pH must be measured.

3.2.5 Liquids used for formulating oral drug products

Equilibrium solubility measurements in water-miscible cosolvents

Water-miscible solvents, e.g. ethanol, glycerol, propylene glycol (PG), polyethers (glycofurol), macrogols (e.g. PEG400) are typical ingredients in aqueous formulations to increase compound solubility.

From a mechanistic point of view, cosolvents are very similar to hydrotropes. In many cases the addition of even small cosolvent fractions may increase the solubility significantly. In many cases, solubility values of compounds in cosolvent systems reported in the literature differ even more than such values reported for single solvents. In the following some aspects are discussed that may elucidate possible reasons. Obviously, ternary solvent mixtures are even more difficult; however, the principles discussed for binary mixtures will still apply, and ternary mixtures are not specifically discussed here.

Typically, solubility does not correlate with composition in a linear way; maxima and minima are frequently observed. A widely studied example is acetaminophen in binary ethanol+water blends as depicted in Figure 10 (Jiménez and Martinez 2006). Solubility of acetaminophen shows a maximum, which is due to changing compositions of the compound solvation shells: differences between the bulk composition and the local fractions of solvent/cosolvent in the solvation shell around the solute are formed. In addition to the polarity difference of the two solvents, the hydrophobic hydration of acetaminophen at the aromatic ring and the methyl group (by ethanol in water-rich blends), and acidic behavior of water molecules interacting with the hydrogen-acceptor moieties (carbonyl group) leading to preference of water for solvation shells in ethanol-rich blends, play a major role. For another example, ketoconazole, the solubility in different water-cosolvent mixtures increases 4

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orders of magnitude (Jouyban et al. 2020). Similar reasons, namely changing compositions of the solvation shells, as well as non-linear enthalpy-entropy compensation, have been found.
Such mechanistic understanding makes it reasonable to assume that even small deviations in cosolvent blend composition may have a comparably large effect on solubility. Thus, in general, careful mixing of the respective solvent fractions needs to be carried out, and evaporation needs to be controlled.

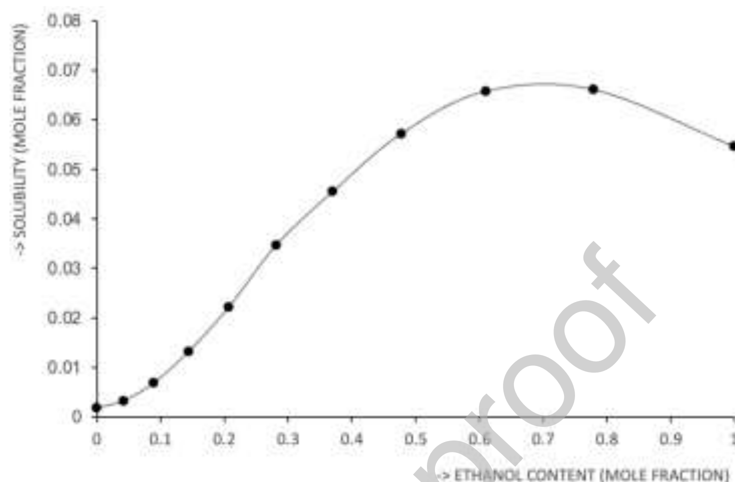

Figure 10. Equilibrium solubility data of acetaminophen in ethanol-water blends vs. mole fraction of ethanol at 25 °C [Data are from a table published by Jiménez and Martinez (2006)].

It should be kept in mind that the addition of a cosolvent intended to increase solubility of the compound may at the same time decrease the solubility of other ingredients (e.g. buffer salts).
After solvents are mixed thoroughly, it is good practice to wait for any temperature equilibration and volume effects. If volatile liquids are involved, the composition of the solvent blend may change by preferred evaporation of one of the components. Therefore, evaporation should be avoided, even the void volume in a flask may have an influence on composition and thus on the measured solubility value, especially when cosolvents contain organic solvents with low boiling points (e.g. methanol, ethanol). In those situations, tightly closed containers during the study are required, to avoid solvent evaporation with the risk to generate supersaturated solutions with time. Moreover, handling of such cosolvents may require positive air displacement pipettes for accurate manipulation of samples volumes. These pipettes are also useful for more viscous or oily solvents such as Tween 80 or Peceol, in particular in dilution steps (Alsenz et al., 2007).
In cosolvent systems, both the definition of the pH values itself as well as its experimental measurement can be difficult. Here again, for explanation of the challenges, ethanol is used as an example of a widely used cosolvent. For pure ethanol (EtOH), different pH values have been defined in the literature, ranging from pH 7 (because ethanol is neutral, which may pragmatically defined as

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being pH 7) up to pH 9.8 (because EtOH dissociates much less than water, its $(H^{+})_{solv}$ concentration is $10^{-9.8}$ mol/L). Another typical literature value is pH 7.3, which is obtained when the pH of ethanol is measured using a standard pH electrode that has been calibrated with aqueous buffers. In this case, the proton activity in the sample is compared with the proton activity in the aqueous standard buffers. When EtOH and water are blended, definition of pH becomes even more complicated. First of all, EtOH dissociates to a much lesser extent than water. In a first approximation, H^{+}_{aq} ions present in the water component may simply be diluted by the ethanol component. There will be no pH shift as the OH^{-}_{aq} ions are diluted correspondingly. However, the dissociation of EtOH should not be totally neglected, because the dissociation behaviours of water and respectively EtOH interact in a nonlinear way. Up to EtOH concentrations of approx. 80%, the deviation of such data from the simple dilution model is typically small, above this concentration it may become significant (Kotbra & Schilling, 2017).

However, because ethanol molecules are less polar than water molecules, they are less involved in building solvation shells around ions, e.g. dissociated compound molecules. This regards EtO^{-}_{solv} and H^{+}_{solv} ions of the weakly dissociating EtOH itself, and thus the compound molecules will also dissociate to a lesser extent. This is the reason why acids are said to be less acidic in ethanol. It needs to be mentioned that in any case, by definition, a higher pH (i.e. lower H^{+}_{solv} concentration) is the result of lower degree of ethanol dissociation.

For the practical experiments, there is yet another major challenge in terms of pH of water ethanol blends. As is common knowledge, water (even double distilled water) may show different pH values, mostly due to amount of dissolved carbon dioxide. Different water pH values translate into widely different pH values of the respective cosolvent mixtures, as can be seen in Figure 11 for a set of consecutive series of mixtures and their respective measured pH values.

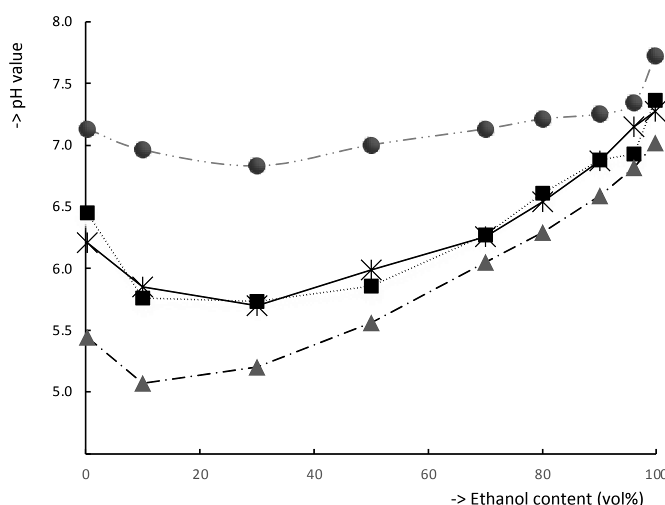

Figure 11. Measured pH values of distilled water samples of different pH in blends of rising EtOH concentrations; 4 independent series of measurements [Data were selected from Kotbra et al. (2017)].

These examples suggest that the pH of the water transfers into the cosolvent mixtures and thus use of pure water without pH control as a component in cosolvent mixtures cannot be recommended. Frequently it is recommended to use a buffer instead of water; however, it is well known that the increased ionic strength by the addition of buffer salts to the water (and even more so in cosolvent mixtures) has a large effect on the activity of the hydronium ions, and thus pH, as well as on solubility.

Considerable experimental error is created if pH in ethanol–water mixtures is measured using electrodes calibrated with aqueous standard buffers. If the internal electrolyte solution of the electrode is purely aqueous, an additional electric potential is produced at the junctions between the aqueous system and the (partially) organic solution. This leads to a deviation of up to 0.3 pH units for ethanol fractions below 90%. Furthermore, pH measurements with glass electrodes are based on the hydrated gel layer at the outside of the glass bulb. Organic solvents and cosolvent mixtures may dehydrate this gel layer. This results in drifting readings, long response times and poorly reproducible values. In many cases the organic (co)solvents are poorer conductors as compared to water. Special low resistance glass pH electrodes are then preferably used, together with high impedance pH meters. It has also been suggested to add a small amout of neutral salt (e.g. quaternary ammonium salt) to the sample to increase conductivity (Thermo Scientific Application Note 007 (2014)).

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Equilibrium solubility measurements in lipidic (water-imiscible) excipients

The compound solubility (or miscibility) needs to be determined to understand the level of possible compound loading in a liquid dosage form. When the excipients are cosolvents, surfactants and liquid lipids at room temperature, the methods to use are relatively similar to those of aqueous solubility determination. The amount dissolved, referred to as either solubility in the excipient or loading capacity of the formulation, is reported as w/w or w/v of the excipient or formulation studied. Since these excipients typically show greater compound solubility than that of water, less sensitive analytics is required and are commonly based on UV-detection. Hence, analytical platforms in use are UV-cuvette for single determinations, UV-plate reader for multiple determinations turning the analytical platform into more of a screening mode, and HPLC-UV for those in need of higher sensitivity. For the latter, also UPLS-MS/MS may be used if solubility is limited in the studied

excipient (Alskär and Bergström 2015). Furthermore, a chromatographic methodology may be needed to resolve problems with excipient UV absorption interfereing with that of the API.

An example of solubility determination in a single excipient is briefly described below [more information is provided by Persson et al. (2013)]. The same method applies also to complex lipid based drug delivery systems (LbDDSs), with the diference being that LbDDSs are mixed and equilibrated at least over night prior to being used as the medium for the solubility assay (Alskär and Bergström 2015).

An excess amount of compound is added to replicate glass vials containing the excipient. The amount is either decided upon as an absolute number based on the expected needed dose that is to be formulated or simply added in excess to the vial; the reason for undertaking the solubility measurement determines this. If dose is known, this amount is added to the vial and studied in an approriate volume of the excipient. If instead the absolute compound solubility is warranted, potentially to make use of the obtained experimentally measured value to decide on future formulation strategies and need for more complex formulation design, an excess material is added to the volume chosen. Common volumes to use are 1-5 mL of the excipient and that the solubility assessment is performed in glass vials rather than in plates or volumetric flasks. However, smaller volumes and plate formats can also be used, in particular if the explored excipient is studied after being dispersed into water forming water:cosolvent mixtures, micellular solutions or emulsions. For the highly viscous excipients, e.g. some oils and surfactants, the plate reader system may become more cumbersome to use. Automatic pipetting of such media is more dificult and need specific calibrations. Further, the compound particles tend to settle over time making the surface area of the compound that is in contact with the solvent significantly reduced. While this can be handled by rigorous shaking, magnetic stirring or manual vortexing every now and then for a glass vial system, this is more difficult in the plate format, potentially making the dissolution slower and the time needed to reach equilibrium longer. That said, both systems have their place in the drug development setting.

After adding an excess amount of solid compound to the excipient or formulation, the resulting slurries are placed on a plate shaker or a magnetic stirbar is added to increase dispersion of the solid material and reduce the effect of particle sedimentation. The slurries are then continously sampled over time where centrifugation is used to separate the dissolved material from the undissolved. Filtering is difficult due to the high viscosity of many of the studied vehicles (see 3.4.1). The vials are then vortexed again, placed on the plate shaker or magnetic stirring device and the experiment is continued till equilbrium is reached. Hence, the same slurry is sampled multiple times to determine the thermodynamic solubility. While some of these excipients may achieve the equilibrium solubility

within a few hours others may take days. Therefore, a common approach is to sample the suspensions every 24h until the solubility plateau is reached and two consecutive samples do not differ more than 10%. To streamline the process and reduce sampling the first sample can be taken after 72h followed by the 96h sample; for the majority of drugs and excipients the equilibrium solubility is reached by then. The excipients produce relative high solubility as compared to the corresponding water experiment and, therefore, these samples need to be diluted with a proper solvent prior to concentration determination. The solvent used needs to dissolve the range of excipients studied and still be a good solvent for the compound to not trigger compound precipitation when the excipient is diluted. The most common solvent to use is methanol, which may be used in combination with a stronger organic solvent such as chloroform to dissolve highly lipidic excipients, e.g. soybean oil. An alternative solvent is NMP (N-Methyl-2-Pyrrolidone). It is easier to handle due to its high boiling point, dissolves a broad range of excipients and compounds at high concentrations, and is compatible with most UPLC separation protocols (Wyttenbach et al., 2007; Alsenz et al., 2007). These dilution steps are commonly performed in small volumetric flasks into which a defined mass of the sampled supernatant is added to e.g. 5 mL of solvent. Alternatively, positive displacement pipette tips (www.gilson.com) may be used, in particlular for highly viscous, oily or volatile liquids (Alsenz et al., 2007). Similar to syringes, these tips use pistons for precise volume adjustment. Dilutions may then be performed in an organic solvent (e.g. NMP) in 1.5-2 mL HPLC autosampler glass vials with screw or crimp caps and a septum. The samples are then ready for concentration analysis with any of the methods reported above. The solid material is collected after the final centrifugation step and analysed for its solid state form with any of the commonly used techniques (P-XRD, DSC and/or Raman spectroscopy; see section 3.1.1). The first step is typically addition of solid material of known crystalline form and separation is performed with centrifugation. In all other steps, the same range of equipment and methodology as applied for solubility determination in water are applicabe also for determination of solubility in lipidic excipients.

For the development of LbDDSs a design of experiments (DoE) approach can also be applied for identifying the combination of excipients with the highest possible drug solubility, and also other desired properties, e.g. droplet size distribution upon dispersion (e.g. Ren et al. 2013).

The purity of lipidic excipients may affect both chemical stability and solubility of compounds. Light, heat, certain trace metals and impurities from synthesis can initiate oxidation; examples are Tween 80 and PEG 400, which may contain peroxides. Oxidation of sensitive compounds may be reduced or blocked by protection from light, storage under nitrogen or argon or addition of antioxidants. The hydration state (water content) of long- and medium-chain triglyceride oils (e.g. olive oil, Miglyol 812) can also have a significant effect on their ability to solubilize compounds (Land et al., 2005).

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Therefore, only well-defined media should be used for solubility studies and stability of compound in mediums needs verification by appropriate analytical methods.

3.3. Temperature of experimentation

In the majority of cases, the solubility of a compound in aqueous media increases as the temperature increases. However, in certain cases, if the dissolution process is largely exothermic (i.e. if the enthalpy of the solvation process of the molecules overrides the enthalpy of the solid state), the opposite may be the case (e.g. Van den Mooter 2012). Cyclosporin A is a prominent example of higher solubility at lower temperatures (Ismailos et al. 1991). In any case, it is of importance to maintain the temperature constant during a solubility study to prevent dissolution / precipitation processes. In late stages of pharmaceutical development and on, the solubility at body temperature (37 °C) is of interest, as this can inform the solubility of the compound in the gastrointestinal contents and how much may, thus, be available for absorption. This is usually the case, also, when LbDDSs are studied for their digestability by exploring lipolysis caused by pancreatic extract or lipases *in vitro* (Porter et al. 2008; Williams et al. 2012).

It should be noted that from a self-life point of view, room temperature or 25°C is more suitable as a study temperature for measuring solubility in the LbDDS. However, in the case of supersaturated LbDDS, the compound is dissolved above the saturation solubility at elevated temperature and therafter precipitation is kinetically hindered when cooling down. By dissolving a compound at elevated temperature (e.g. 60 °C for three hours), followed by storage at 37 °C, a stable supersaturated LbDDS can be obtained for some compounds. For some compounds, supersaturated LbDDSs have been proven stable for at least 8 months for some compounds (Thomas et al 2012).

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3.4 Sample handling procedures

3.4.1 Separation of solids from solution

Except for the MicroDiss Profiler (UV detection) and Sirius T3 (Checksol, potentiometric method for ionizable compounds only) from pION Inc., most solubility assays require removal of solids.

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The most popular method is filtration. When using filtration the pore size of a filter should be smaller than the minimum particle size of the particle size distribution. Potential issues with filters are clogging by particles, high backpressure for viscous liquids, and media incompatibilities with filter materials (Alsenz and Kansy, 2007; Veseli etal., 2019; Vöglyi et al., 2018; Avdeef, 2016).

The second most popular method is centrifugation. Depending on the original particle size or the one generated by the mixing method (see above), relatively low (~1000g) or very high centrifugation forces (>20,000g) needed to be applied for separation (Avdeef, 2016; Bergström etal., 2002; Robertson et al., 2016). For low volumes, supernant collection may be difficult. Moreover, non-wettable particles or oily phases formed may float at the surface, and/or, similar to filtration, adsorption effects to equipment may occur. In specific cases, centrifugation and filtration can be combined (Alsenz et al., 2007). When using centrifugation for separation, errors in solubility studies can occur when e.g. the rate of centrifugation is not high enough and small (nanosized) compound particles are not spun down. This will then result in an incorrect higher solubility, which can be realized by e.g. observing the sample using polarized light microscopy, where crystalline particles will be visible or detected by using dynamic light scattering. The risk of remaining solid is especially high also when solubility is determined in viscous liquids, e.g. lipidic excipients. A recent study showed that the solubility of brick dust compounds in viscous lipid excipient was overestimated even using a centrifugation of 17,000g for 15 min, also in this case crystalline compound could be observed by polarized light microscopy in the supernatant. For grease ball molecules this was not the case (Liu et al 2020).

When using luminal aspirates, samples are frequently centrifuged at 37 °C given that, in most of cases, aspirates cannot be filtered through 0.45 μm filters. Adequacy of centrifugation could be partly evaluated from the variability of estimated values or from preliminary experiments in biorelevant media. It should be noted that in the case of solubility measurements in gastric and/or intestinal contents aspirated in the fed sate, when experiments are performed in the total luminal contents, it is difficult to find the appropriate speed of centrifugation in order to separate the excess solid drug from the rest of the sample prior to analysis (Riethorst et al 2018).

With either filtration or centrifugation, separation of nano-sized particles from solution is a tedious objective (Nothnagel et al. 2018). The non-linear increase in compound solubility that has been associated with compound particles sizes below 10 nm, based on hypothetical calculations using the Ostwald-Freundlich equation (Grant and Brittain, 1995; Kaptay, 2012) is another factor that may complicate the evaluation or experimental data. However, drug nanosuspensions are rarely in this

size range and further, this increased solubility has not been experimentally proven, and is likely to be related to the difficulty in separating nanosized particles from the compound in solution. Van Eerdenbrugh et al., found that non-separation based techniques is preferered to obtain a correct solubility of drugs in nano-suspensions. This is e.g. stepwise addition of nanosuspension to the liquid of interest, followed by assessement of the presense of nanoparticles using dynamic light scattering or turbidity followed by extrapolating back to no presences of nanoparticles. Using this method the presence of micelles in the medium has to be corrected for (Van Eerdenbrugh et al. 2010).

3.4.2 Sample processing after solids separation

Adhesion (adsorption) of solute molecules to any surfaces or interfaces is due to interactive forces. Typically for drug-like compounds, these are low intermolecular forces including hydrophobic forces, van der Waals forces, and hydrogen bonding. They lead to segregation of the solute by increasing its concentration at interfaces and thus decreasing the concentration in the bulk solution. For solubility determination, adsorption is not a particular problem at the stage of the suspension in equilibrium, because any surfaces will be saturated by the excess of the solid and leave the bulk concentration of the solution unaffected. The problem starts and persists at all subsequent stages of sample handling where there is no excess solute material present and the solution is exposed to new surfaces. It is our experience in accordance to the most common adsorption models (e.g. Langmuir and BET isotherms), that adsorption is most pronounced (in %) for large surface/volume ratios, and for low concentrations (poorly soluble drugs and diluted solutions). Any contact surfaces of the entire experimental set-up need to be considered, such as the flasks, vials, closures, pipette tips, filters, etc. Obviously, the degree of adsorption depends not only on properties of surface material and the solute, but also widely on solution composition. There are no general rules that guide towards the prediction of the degree of adsorption nor its kinetics of equilibration.

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Once the solution has been separated from solids, the sample must be processed immediately to avoid potential decrease in compound concentration that may be induced by contact to other surfaces, temperature changes, evaporation, etc. Tips for minimizing such risk are provided in Table 1.

Table 1: Tips to minimize potential decrease in compound concentration after separation of saturated solution from solids until analysis

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✓ Use vials stored at temperature close to the temperature at which the solubility experiment was performed
✓ Pre-fill vials with aliquots of a suitable medium for *all* components of the saturated solution. A suitable medium may be the mobile phase of the chromatographic method to be used for the assay. If the medium is a blend, pre-blend before pre-filling the vials. Depending on the temperature of the vials high-evaporating media may need to be handled cautiously
✓ Transfer aliquots of saturated solution as soon as possible into the pre-filled vials, especially if different solid phases can be formed
✓ Keep samples at room temperature, if possible, i.e. if there are no chemical instability issue.
✓ If needed, use a freezer. When thawing the samples, thorough shake/stir is needed to get a homogenous matrix again.

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4. Recommendations for Improving Solubility Data Quality of Orally Administered Drugs

Based on the factors affecting solubility data quality and the characteristics of available in vitro methods, general recommendations and method-specific recommendations for improving soluiblity data quality are made in Table 2 and Table 3, respectively. It is anticipated that relevant recommendations will complement previous relevant attempts focusing on aqueous equilibrium solubility (Avdeef et al. 2016; Ono et al. 2019) and apparent equilibrium solubility (Andersson et al. 2016), and reliability and reproducibility of solubility data for orally administered compounds will increase.

Table 2: General recommendations for improving solubility data quality of orally administered drugs

Before the solubility experiment
• Compound, solvent, and chemicals used for preparing the solubility medium should be of highest available quality; source and purity should be documented
• The solid-state form of the compound should be known
• Quantitative composition of the medium to be employed in a solubility measurement should be documented
• Chemical stability of the compound in the solubility medium should be confirmed
• Stability of solubility medium during the experiment should be ensured
• The procedure for separating solids from the solubility medium should be justified; the procedure can be challenging, especially when employing nanoparticles of the compound or luminal contents as solubility medium
• Sample processing after solid separation and until analysis must be documented
• Unless the solubility medium is a pure lipid or organic solvent, the pH of the medium should be measured; in case of cosolvents measurement can be challenging
• If a biorelevant medium simulating the fasted state conditions is employed inter-subject variability in luminal composition may worth considering
• If samples from the gastrointestinal lumen are employed, details on collection and sample treatment methodology as well as storage conditions must be documented
During the solubility experiment
• For estimating luminal solubility, experiment should be performed at 37 °C; other temperatures may be appropriate when solubility in excipients is measured
After the solubility experiment
• Compound's adsorption from the solubility medium onto surfaces of containers should be evaluated and minimized, especially when the compound is lipophilic
• Unless the solubility medium is a pure lipid or organic solvent, the pH of the medium should be measured and documented; in case of cosolvents measurement can be challenging
• The solid-state form of the compound should be characterized

Table 3: Method-specific recommendations for improving solubility data quality of orally administered drugs

Shake-flask methods for measuring apparent equilibrium solubility
• Less than two to four-fold solid amount required to saturate the solubility medium should be used
• Sample shaking is preferred over stirring or ultrasonication
• Equilibration time should be confirmed by sampling during the equilibration period or with preliminary experiments by measuring concentration in vials incubated for different time periods
• Measurement of apparent equilibrium solubility requires confirmation of the presence of the most stable crystalline form at the end of the experiment.
Kinetic methods for estimating apparent equilibrium solubility
• Useful only when the most stable crystalline form of the compound is available and purity of the sample used is high
• If particles are used, ideally, they should be isotropically round with zero porosity and known particle size distribution
Methods for measuring kinetic solubility in aqueous media
• If applicable, non-aqueous media should be - evaluated for potential limitations before the experiment, and - used at total concentration of less than 2% (v/v) and its impact on compound solubility should be known
• If estimation in the fasted upper intestinal lumen is aimed, it is recommended to employ methods for which their usefulness in relevant estimation has already been documented
Methods for measuring amorphous solubility
• Duration of the experiment and mixing conditions can be challenging
• When amorphous drug solid dispersions are subjected to amorphous solubility measurements, data should be accompanied by the identity and concentration of polymer(s) in the formulation

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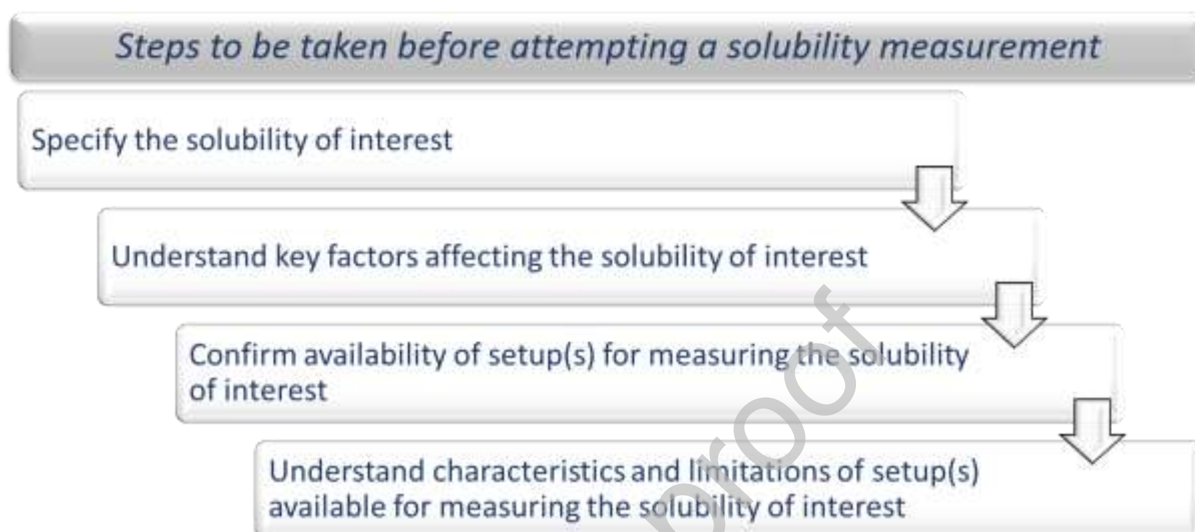

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