*ADMET & DMPK 3(2) (2015) 84-109; doi: **10.5599/admet.3.2.193***

Open Access : ISSN : 1848-7718

http://www.pub.iapchem.org/ojs/index.php/admet/index

Review

Suggested Improvements for Measurement of Equilibrium Solubility-pH of Ionizable Drugs

Alex Avdeef

In-ADME Research, 1732 First Avenue #102, New York, NY 10128 USA

Corresponding Author: E-mail: Alex@in-ADME.com; Tel.: +1-646-678-5713

Received: June 14, 2015; Revised: June 22, 2015; Published: July 01, 2015

Abstract

The accurate prediction of solubility of drug-like molecules is difficult, and perhaps a satisfactory general model is not yet available. The most cited challenge to good prediction has been the lack of enough high-quality and drug-relevant solubility data that adequately cover the chemical space of drugs. This review addresses data quality in solubility measurement. Specifically, the "gold standard" shake-flask and related methods used to measure equilibrium solubility of ionizable drug-like compounds as a function of pH were reviewed. Over 800 publications were examined. Many factors affecting the quality of the measurement were recognized, and a number of suggestions are offered to improve the experimental methodology. Some of the suggestions focus on improving methods for future measurements, and some refer to improvements in data mining, i.e., to ways of extracting more reliable information from existing data. By normalizing data for pH (i.e., deriving intrinsic solubility, S_0) and for temperature (by transforming measurements performed in the range 20 – 40 °C to 25 °C), it is suggested that the 0.6-0.7 log unit currently expected interlaboratory reproducibility can be reduced to near 0.15. It is the aim of the review that the improvements in data quality would lead to better predictions of drug solubility using in silico methods.

Keywords

Shake-flask solubility; intrinsic solubility; Henderson-Hasselbalch equation; aggregates; oligomers; micelles.

Introduction

Since the mid-1990s there has been a heightened effort in drug discovery to predict drug-relevant aqueous solubility, described in at least a hundred publications (e.g., Huuskonen *et al.* [1,2]; Abraham and Le [3]; Jorgensen and Duffy [4,5]; Bergström *et al.* [6]; Hou *et al.* [7]; Delaney [8]; Dearden [9]; Balakin *et al.* [10]; Taskinen and Norinder [11]; Jain and Yalkowsky [12]; Shayanfar and Jouyban [13]; Wang and Hou [14]; Elder and Holm [15]; McDonagh *et al.* [16]). The typical errors in drug solubility prediction are 0.7 – 1.0 log unit, and for low-soluble compounds, errors are considerably greater than a log unit (Jorgensen and Duffy [5]; Palmer and Mitchell [17]). According to Faller and Ertl [18], whose sentiment may be broadly shared, "no really satisfactory approach to (drug) solubility prediction is available yet," in spite of the large number of prediction studies.

Two large aqueous solubility databases have been the sources for many of the *in silico* studies. First, the 1608-page *Handbook of Aqueous Solubility Data, Second Edition* (Yalkowsky *et al.* [19]) contains over

18,000 solubility values covering 4661 molecules, collected from many chemical classes, including pharmaceuticals. Second, the PHYSPROP database (Howard and Meylan [20,21]) from Syracuse Research Corp. (http://www.srcinc.com/) had accumulated over 6000 measured solubility values, with substantial coverage of agrochemicals and potential environmental pollutants. In the two curated compilations, the pH of saturated solutions had not been reported for most of the ionizable compounds. In addition, smaller databases of crystalline drug-like molecules have been published (*Analytical Profiles of Drug Substances* [22], McFarland *et al.* [23]; Rytting *et al.* [24]; Bergström *et al.* [25]; Faller and Ertl [18]; Llinàs *et al.* [26]; Hopfinger *et al.* [27]). These smaller databases consist largely of intrinsic solubility values, S_0 – i.e., the solubility of the neutral species.

The most cited challenge to good prediction has been the difficulty to access enough high-quality, drug-relevant, and sufficiently-diverse solubility data that adequately cover the chemical space of drugs and hopefully that of research compounds. In this context, the focus on *drug* molecules is important, since it is consistently shown that the best prediction models are devised from training sets most similar to the test sets (Walters [28]). However, Palmer and Mitchell [17] posed a contrarian view that the challenge to good prediction might rest in the deficiency of current QSPR methods.

Measuring high quality data is expensive and analytical-resource consuming. Even with great costing, quality is not ensured when results are determined from poorly-designed assays. Selecting training set molecules which are ionizable, but ignoring the effect of pH, can mitigate accurate prediction. Drawing on data from a range of temperatures without adjustments also can be problematic. There are other factors that affect data quality.

To address many of the above concerns, this review draws on our past experiences and also on the examination of over 800 publications to suggest ways (a) to improve the quality of future measurement of equilibrium solubility and (b) to normalize existing data for pH and temperature effects to extract intrinsic solubility values with improved accuracy. We focus on the shake-flask solubility measurement *as a function of pH* which is still the "gold standard" methodology in the minds of most experimentalists. Also, two potentiometric methods are considered. The characterization of solid forms (crystalline, amorphous, nanoparticle, etc.) and their impact on the measured solubility are beyond the scope of this review, although some aspects (i.e., solvate, polymorph, racemate effects) are noted.

In part, this review serves as background preparation for the "panel of experts" solubility session at the *4th World Conference on Physico Chemical Methods in Drug Discovery and Development* (PCMDDD-4) in Croatia, 21-24 September 2015 (http://www.iapchem.org/page.php?page_id=56).

Methods

Briefly stated, in the course of gathering published aqueous solubility training data, 803 publications have been examined and a largely fresh intrinsic solubility database containing 4557 entries for 2413 compounds has been assembled. In addition to this, 666 publications reporting pK_a values have been processed, to add to the *in-ADME* Research Wiki-pK_a database, now consisting of 2651 qualified entries (www.in-adme.com/wiki_pka.php). It has been nearly a full-time project at *in-ADME* Research since 2011. The collection of training data is nearing a state suitable to support a new solubility prediction effort, which is planned to be the subject of another publication. We are energized to improve the accuracy of the prediction of intrinsic solubility from 2D structure, particularly of sparingly-soluble (or practically-insoluble) ionizable drug-relevant molecules. Preliminary Random Forest regression modeling (Walters [28]) has been tried using the 193 descriptors calculated by the open-source chemoinformatics and machine learning

RDKit library of programs (Landrum *et al.* [29]; http://rdkit.readthedocs.org/en/latest/), combined with the Abraham five solvation descriptors (Abraham *et al.* [30]), and Lang and Bradley [31] predicted melting points in the QsarDB open repository of data and prediction tools (http://qsardb.org/repository/handle/10967/104). The most sensitive molecular descriptors will be further tested in Partial Least Squares models, to better understand the impact of specific descriptors on the predicted solubility.

In the database construction, intrinsic solubility (S_0) values were mostly derived from (a) S_w aqueous solubility determined in distilled water, often with pH not reported, (b) S_{pH} single-pH buffer values, and (c) multiple-pH buffer S_{pH} values (log S vs. pH). In cases (a) and (b), the Henderson-Hasselbalch equation (discussed below) was assumed to be valid. The *p*DISOL-X program (*in-ADME* Research; *www.in-adme.com/pdisol_x.html*) was used to determine the S_0 values from the reported S_w and S_{pH} data, as described in Völgyi *et al.* [32] and Avdeef [33,34].

Data

The 4557 set of measured solubility values used to deduce the intrinsic solubility (S_0) database were collected from four secondary sources (57 %) and the rest from primary sources (43 %):

- PHYSPROP database (Sep. 1999 version: 6356 measured water solubility, S_w) [20,21]: 1327 shake-flask values were selected of molecules which were not appreciably ionized at pH~7. Filters used to *exclude* compounds were: (a) melting point < 40 °C, (b) log S_w < -8 or > 0, (c) surfactants or compounds with long aliphatic chains, (d) multi-ring aromatic hydrocarbons, (e) peroxides, (f) carboxylic acids, (g) salts/complexes with chloride, bromide, iodide, sulfate, phosphate, sodium, potassium, lithium, calcium, magnesium, zinc, copper, arsenic, mercury, lead, antimony, silver, silicon, tin, etc., (h) dyes or names containing color, and (i) herbicides, pesticides, insecticides, rodenticides, and acaricides (as indicated by "tags" at the Royal Society of Chemistry ChemSpider website: http://www.chemspider.com/). Of the selected compounds, the S_w values of 1210 nonionizable/nonionized molecules were taken to be S_0. The remaining 117 compounds were processed by *p*DISOL-X to calculate S_0 and pH_{sat} (pH of saturated aqueous solution) from the given S_w and pK_a.
- *Handbook of Aqueous Solubility Data* [19]: 1130 S_w data were selected, with 776 values subjected to *p*DISOL-X analysis to determine S_0 values. A few of the same preceding filters were used in the selections.
- *Analytical Profiles of Drug Substances* [22]: All 39 volumes of the series of monographs were searched for quantitative solubility data. Monographs on 155 molecules were found to be useful. Most of the reported solubility values of ionizable molecules were measured in pure water with unspecified saturation pH. For those ionizable molecules which were not salts, the intrinsic values were calculated by *p*DISOL-X. Unfortunately, the solubility accompanied scant experimental detail (e.g., temperature not always reported), and many entries were referenced as 'personal communication.' But there are several high-quality log S - pH data sets in the monographs.
- Miscellaneous publications (shake-flask data, some from secondary sources): 852 S_0 values (taken as reported).
- CheqSol S_0 Data (potentiometric): 201 values for 151 molecules collected from several publications (Stuart and Box [35]; Sköld *et al.* [36]; Llinàs *et al.* [26,37]; Box *et al.* [38]; Hopfinger *et al.* [27]; Narasimham *et al.* [39]; Hsieh *et al.* [40]; Comer *et al.* [41]; Palmer and Mitchell [17]; Schönherr *et al.* [42]).

- Dissolution Template Titration (DTT) S_0 Data (potentiometric): 75 published values were collected (Avdeef [43,46]; Avdeef *et al.* [44]; Avdeef and Berger [45]; Faller and Wohnsland [47]; Bergström *et al.* [48]; Fioritto *et al.* [49]; Ottaviani *et al*. [50]).
- Rytting *et al.* [24] free-base and -acid (no salts used) shake-flask S_w: solubility of 122 molecules were gathered, all measured in one laboratory, with S_0 calculated by *p*DISOL-X.
- Shake-flask measurements in *two or more* pH buffers (primary sources): 697 molecules with *S*-pH data were analyzed by *p*DISOL-X to determine S_0. Figure 1a shows the frequency distribution of the multi-pH data. There were 101 studies (14 %) with 2 pH/assay and 164 studies (24 %) with 3-5 pH/assay. Eight studies were reported, each with more than 40 pH points defining the log *S*-pH profile. The median in the set of 697 compounds was seven *S*-pH points per molecule. Most of the published data were presented graphically as log *S* vs. pH plots. The process of digitizing data from plots introduced some small error. It was not possible to digitize the graphs entirely if the molecules were low soluble and the plots were in non-logarithmic *S* units vs. pH. A large fraction of the primary source data originated from five journals (in rank order): *Int. J. Pharm.*, *J. Pharm. Sci.*, *Pharm. Res.*, *J. Chem. Eng. Data*, and *Eur. J. Pharm. Sci*. It is primarily from this group of 697 "gold standard" shake-flask multi-pH measurements that many of the suggestions below were formulated.

The assembled 4557 intrinsic solubility set ranges in log S_0 from -11.6 to +1.8 (log molarity). Figure 1b shows a frequency distribution for the set. About 40 % of the compounds have log S_0 between -6 and -3, the typical range of values for research compounds (Faller and Ertl [18]; Walters [28]). The median log S_0 of the distribution is -2.8. About 5 % of the molecules have log $S_0 < -6$. The least-soluble molecules (log $S_0 < -8$) in the set are amiodarone, cosalane, halofantrine, clofazimine, itraconazole, quinclorac, probucol, brodifacoum, epristeride, silafluofen, carbenoxolone, tamoxifen, fluotrimazole, moteretinide, esfenvalerate etofenprox, etretinate, and NPC-1161C (which includes four agro-chemicals). The most soluble (log $S_0 > 0$) substances are amino acids, simple carboxylic acids, and carbohydrates.

The compounds in the 4557-set are solids at room temperature, with propofol and nitroglycerin at the border line with the melting points 19 and 14 °C, respectively. The shake-flask values were mainly clustered around room and physiological temperatures: 23 ± 3 °C (78 %) and 37 ± 5 °C (22 %). Figure 1c shows the temperature frequency distribution. Surprisingly, a large number of sources don't state the temperature used in the assay. Contextually, room temperature is a reasonable guess in most instances. Other publications cite "room temperature." Where the precise temperature was not reported, it was taken to be 23 °C in the assembled database.

Figure 2 shows plots of six high-quality examples of multi-pH solubility data analyzed by *p*DISOL-X. Most of the compounds can be represented by simple Henderson-Hasselbalch equations (cf., below). The data from verapamil was best fit with a tricationic aggregate below pH 7. Above pH 9 the free-base form of verapamil may participate in the formation of uncharged water-soluble aggregates or micelle-like structures, consistent with the discussion by Surakitbanharn *et al.* [51].

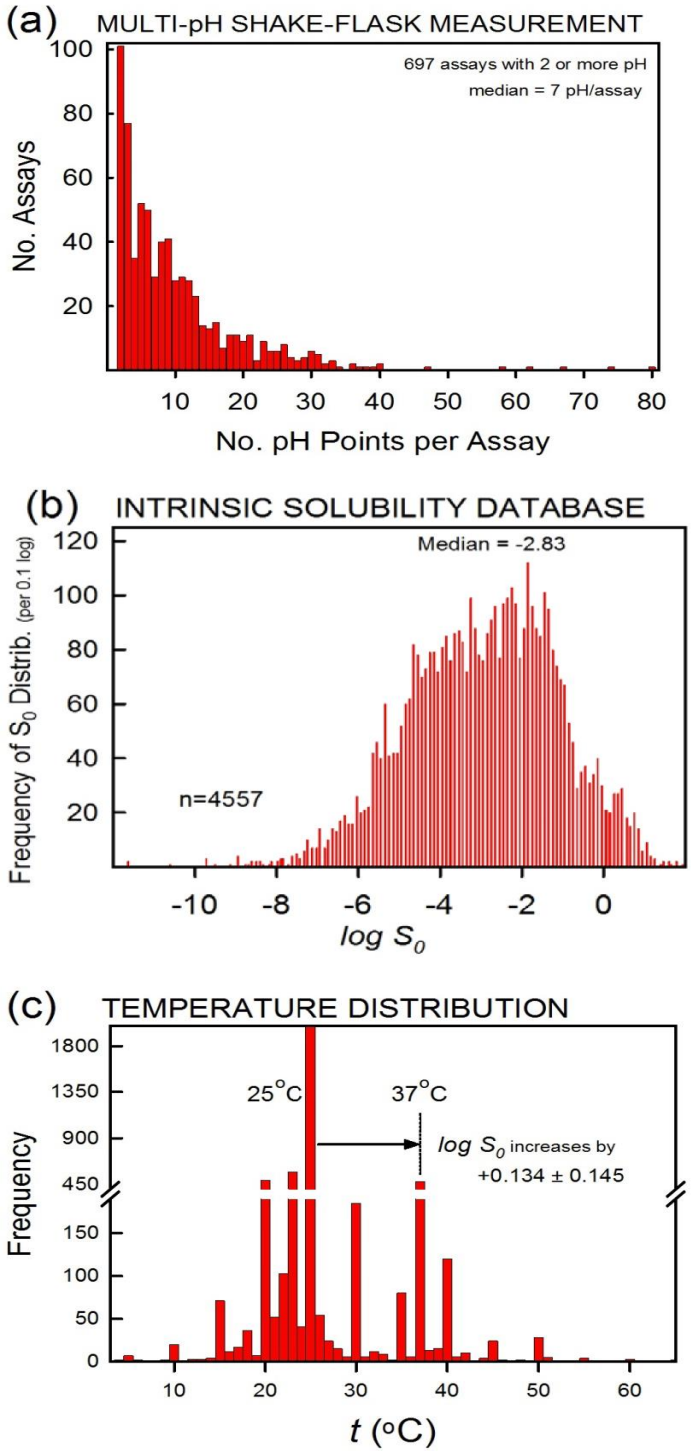

Figure 1. (a) Distribution of the number of multiple-pH shake-flask measurements per assay. (b) Intrinsic solubility, log S_0, distribution, as counts in 0.1 log unit intervals. (c) Distribution of assay temperature in the 4557-set. On the average, log S_0 increases by 0.134 log unit as temperature goes from 25 to 37 °C.

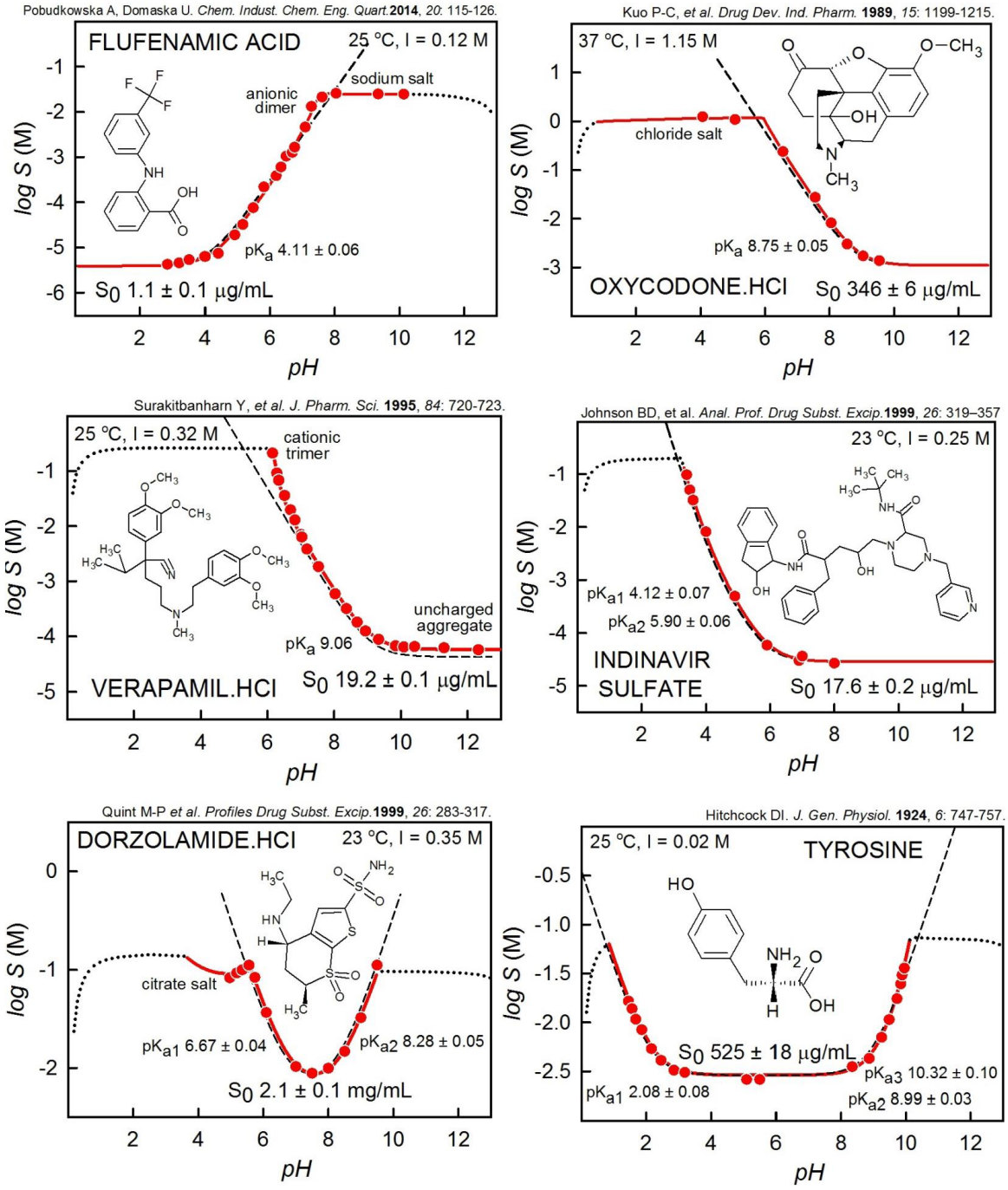

Figure 2. Examples of log *S* – pH profiles from well-designed assays for an acid (flufenamic acid), three bases (oxycodone, verapamil, and indinavir), and two ampholytes (dorzolamide and tyrosine). Note that the tyrosine is based on measurements done in 1924. The solid (red) curves represent the best-fit *p*DISOL-X model which rationalizes the measured solubility values (filled circle symbols). The dotted curves depict the pH interval where the compounds were fully-dissolved. The dashed curves were calculated from the Henderson-Hasselbalch equations. Flufenamic acid, oxycodone, and dorzolamide were studied at concentrations high enough to effect salt precipitation. In all cases but verapamil, it was possible to refine the pK_a values. Verapamil and flufenamic acid show some evidence of formation of aggregates.

Results and Discussion

Solubility Units and Conversion Issues

Solubility measurements have been reported in a multiplicity of concentration units: mol/L (molarity, M), mM, µM, mol/kg (molality, m), mole fraction, mass fraction, weight/mL (e.g., mg/mL, µg/mL, ng/mL, pg/mL …), mg/100 mL, mg/dL, %w/v, g%mL, mg/mL%, mg%, "1 in 40 of water," "soluble in 2 parts of water," "3% soluble in water," units of IU/mL, etc. Mole fraction, mass fraction, and molality units are almost always used when solubility is determined over a wide range of temperatures, since the units do not depend on the density of the solutions. It is not clear why units such as mg% would be preferred. In certain publications such units are not explicitly defined. Usually (but not always), mol fractions are precisely defined in the publication, so that conversion to molarity is straight forward if the solution density is known (which is slightly higher than that of pure water). The same cannot be said of the very frequently used weight/mL "practical" units. In the clearly presented papers, the *equivalent* molecular weight to use to convert the practical values to molarity is stated (e.g., "concentration is expressed as *free base equivalent*") or is evident, but too often, the reader has to guess what the authors intended. For a given molecule, often the uncertain units became evident when compared to results from different publications.

As different units are in common usage, consequently a useful sense of comparisons between different studies is a challenge. On a practical note, it is *all too easy to make a mistake* in converting the units to the preferred molarity scale of the database. It could be argued that solubility should be presented in log units (preferably based on molarity), since (a) direct values span over 12 orders of magnitude and cannot be accurately depicted in *S*-pH plots at the low end of the scale, and (b) since errors in log values do not depend on the magnitude of the log solubility (whereas they do when direct units are considered). For example, it should be possible to claim that shake-flask log *S* values have an average reproducibility of 0.1 log unit for a given molecule between values reported in different publications; it makes no obvious sense to present an average reproducibility value of *S* measurements that can span so many orders of magnitude. In the 4557-set database, values reported in molality units were noted, but not converted to those in molarity (by correcting for density), since the differences are small near the temperature range of interest (20-40 °C), and the since the actual solution density is seldom reported.

Impact of Accuracy in the Type of Data used for Prediction Training Sets

Most studies aimed to predict S_0 values, whether explicitly stated or not. However, most studies used S_w values in the training sets, which resulted in increased errors for low soluble ionizable molecules. The exception is with nonionizable molecules, where water and intrinsic solubility are the same: $S_w = S_0$.

The majority of the aqueous solubility values, S_w, were measured by adding excess solid (ideally, the free-acid or free-base, not the salt) to distilled water. Frequently, pH of the saturated solution, pH_{sat}, was not reported (and probably not measured).

When a moderately soluble weak acid or base is added to distilled water, the aqueous pH is altered by the ionizing molecule, in the direction where the molecule remains largely in the uncharged form: $S_w \approx S_0$, provided the compound is added as a pure free acid or free base. For compounds added as salts, it is frequently not possible to deduce S_0 and pH_{sat} from just S_w and pK_a, since the total added amount of compound can affect the disposition of the saturated solution. For example, if not enough salt form of the

drug is added, the solid disproportionates to the free acid/base in the saturated solution, with pH_{sat} depending on the weight of salt added.

If the ionizable compound is practically insoluble, then the value of S_w can be quite different from S_0, since not enough of the compound dissolves to alter the pH in the direction of maintaining a nearly uncharged molecule. In such cases, it is possible to calculate the pH_{sat}, as well as S_0, provided the pK_a is known and that the Henderson-Hasselbalch equation accurately describes the solubility-pH relationship for a one-pK_a molecule ('±' in Eq. 1: acid when '+' and base when '-'):

$$\log S = \log S_0 + \log \{ 10^{\pm(pH-pKa)} + 1 \} \quad (1)$$

More complex Henderson-Hasselbalch equations for ampholytes and multiprotic acids and bases have been tabulated elsewhere [52,57].

A feature of *p*DISOL-X allows the calculation of pH_{sat}: when the pH is not reported with the S_w value, a pH_{sat} of 7.0 is initially assumed, and the regular mass balance regression analysis is performed [32]. (Fixed values of carbon dioxide may be included.) The pH titrant volume is calculated. If the volume is non-zero, the value of pH_{sat} is adjusted iteratively by the regression procedure until the calculated volume is driven to zero. At the same time, S_0 is refined.

Variance Increase due to Pooling of Training Set Solubility Data Determined at Different Temperatures

It appears that many of the published prediction studies have drawn data from two large secondary sources, as noted above. These two compilations have data collected mostly in the range 20 – 40 °C, roughly in a bimodal distribution (cf., Fig. 1c). Some prediction papers state that only 25 °C data were used. Many prediction papers are less clear, and it might be that some pooling from different temperatures takes place, which would contribute to increased variance in the experimental training set data. If the temperature dependence of solubility could be predicted, then the number of available training set values in the former case would increase, and in the latter case the variance in the measured data would decrease. That is, solubility values could be normalized to a single reference temperature, e.g., 25 °C. As far as we are aware, there are no publications where temperature dependence of solubility is predicted solely from 2D structures. We have collected a database of 626 values of enthalpies of solution, with 77 determined by calorimetric methods, and the rest by solubility methods (van't Hoff slopes from log *S* vs. 1/*T* plots). The calorimetric data are producing the most promising results, with $r^2 > 0.5$ using just the Abraham solvation descriptors. This procedure will be described in a separate publication.

As a preliminary observation, on the average, measured log S_0 values increase by 0.13 log unit (cf., Fig. 1c), as the temperature is raised from 25 to 37 °C. In cases where the training set data are pooled from multiple temperatures, the variance can be expected to increase by about 0.13 log unit. This can be avoided if the data mining process were to convert measurements to a single reference temperature.

Limitations of Intrinsic Solubility, S_0, Determined from a Single Measurement of S_w

When measured compounds contain protogenic impurities, the pH_{sat} may be affected, which could lead to a change in the measured S_w. For this reason, it is highly recommended that pH_{sat} be measured and not just calculated. Otherwise, the conversion of S_w to S_0 may be inaccurate.

Abraham and Le [3] discussed the relationship between the measured S_w and the underlying S_0 for ionizable molecules, and identified under which circumstances large errors could result for S_w used (in place of the calculated S_0) as the training set values. The authors derived useful plots of S_0/S_w vs. log S_w for acids and bases over a range of pK_a values. For example, $S_w \approx S_0$ for acids with pK_a 5 and S_w > 0.001 M, or with pK_a 3 and S_w > 0.1 M. With bases, $S_w \approx S_0$ for pK_a 10 with S_w > 0.01 M, or pK_a 8 with S_w > 0.0001 M.

There are further considerations. When the aqueous solubility of practically insoluble free *bases* (pK_a > 9) are measured in distilled water, the pH is only slightly affected by the extent to which the base dissolves. Most notably, the observed pH often is regulated by a much stronger buffer present in water: ambient dissolved *carbon dioxide*. This is often overlooked. The pH of the drug-saturated solution can vary from 6-10, depending on how much CO_2 is dissolved in water and how insoluble the basic compound is. For example, Rytting *et al.* [24] reported log S_w = -4.87 (molarity) for terfenadine dissolved in water. If CO_2 content in solution were ignored, the calculated log S_0 = -5.7 and pH_{sat} = 9.2. However, if [CO_2] = 20 µM (a common ambient level), the calculated log S_0 = -8.3 and pH_{sat} = 6.6. The error in determining S_0 of molecules like terfenadine in distilled water is expected to be enormous (as much as 3 log units), since it is very difficult to eliminate CO_2 entirely simply by bubbling an inert gas through solution. In fact, any protogenic drug impurity in solution under the circumstances would lead to large uncertainties in the intrinsic solubility of the drug substance of interest. The simple remedy might be to measure the pH of the saturated solution. However, measuring the pH accurately when the solution is essentially unbuffered is problematic, due to the effects of uncontrolled electrode junction potentials and other factors [52].

Added Buffer Improves Accuracy when Measuring Aqueous Solubility (S_{pH}) at a Single pH

The above terfenadine water solubility example illustrates that high errors can result when the pH of the saturated solution is not known or whose measurement is problematic. The remedy is to buffer the solution (but not excessively) *and* actually measure the pH when the saturated solution reaches equilibrium. However, when only a single aqueous solubility at a known pH, S_{pH}, is measured, it is still necessary to assume that the Henderson-Hasselbalch equation accurately describes the log *S* - pH profile, in order to calculate the S_0, given an accurate independently-determined pK_a.

Measurement of Aqueous Solubility at Several Buffered pH, below and above the pK_a, for Best Accuracy

By measuring solubility at several different values of pH (cf., Fig. 2) below and above the pK_a of an ionizable molecule, it's possible to overcome several of the above sources of error. The log *S* data as a function of pH can be analyzed, to determine the value of S_0. The validity of the Henderson-Hasselbalch equation can be easily tested in such analysis, using mass-balance based solubility simulation software (e.g., *p*DISOL-X).

A universal buffer mixture (e.g., whose pH is nearly linearly controlled by added aliquots of standardized NaOH solution) or several independent buffers may be used, *but the pH still needs to be measured when the saturated solution reaches equilibrium*. It can be misleading to assume that the buffer pH will remain unchanged in the course of the drug dissolution. Nor is it a good idea to use excessively high concentrations of buffers, since drug-buffer complexes and precipitates may form, affecting the measured solubility (Shoghi *et al.* [53]).

In simply-behaving systems, even the pK_a can be determined from the log *S* – pH data (Zimmermann [54]; cf., Fig. 2), but this is not generally recommended [32-34]. It is far more reliable to use purpose-designed pK_a measurement techniques (e.g., potentiometric, spectrophotometric, or capillary

electrophoretic), under conditions where ionic strength is well controlled and complications due to sample complexation, self-aggregation, or precipitation are avoided.

Calibration of pH Electrode

Glass pH electrodes are not all equal. There is no single standard method for calibrating electrodes. In solubility publications, the electrode calibration is virtually never described. The make of the electrode is hardly ever stated. In contrast, researchers who determine pK_a values using commercial instruments are well acquainted with the routinely-used four-parameter procedure (Avdeef and Bucher [55]). Measurements of pH are sensitive to ionic strength effects, especially in poorly buffered solutions. In measurements of salt solubility, it is not uncommon to have the ionic strength reach 1-3 M. Methods for electrode calibration to address these challenging conditions have been discussed in the literature (Völgyi *et al.* [32]; Wang *et al.* [56]). Errors in the pH scale can easily exceed 0.1-0.2 units in the mid-scale region. For measurements at pH < 1 or pH > 12, the pH scale can be in error by as much as 0.5 log unit. The pH electrode practices routinely used in modern pK_a methodology would benefit solubility measurement and lead to improved quality of results.

Separating Solid from Saturated Solution

An advantage of the potentiometric methods is that they do not require phase separation. Also, certain fiber optic probe methods can determine concentrations in turbid solutions (cf., Appendix). However, most traditional methods require some sort of phase separation.

Of the 4557 entries in database, there were only 583 indications of the type of phase separation used. It's surprising that not all primary sources identified the means of separation, although one might surmise that most of these employed filtration. None of the secondary sources lists such detail. Of the indicated values, 44 % used some means of filtration, 14 % used centrifugation, and 9 % used sedimentation solely. The majority of those using filtration seemed to be aware of the problem of filter adsorption and discarded the initial filtrate solution or performed "double filtration" [52,57]. A few of those using filtration did not take heed of the Chen *et al.* [58] recommendations against using *nylon* filters.

A solid drug substance in equilibrium with its saturated solution is dynamically dissolving and precipitating at equal rates. Since that equilibrium is maintained by the presence of the solid, the act of separation by filtration disrupts the process, to the extent that the solution concentrations may be altered by reactions of the substance with the container and filter surfaces. Thus, avoiding filtration, if possible, can be recommended. However, it is such a popular procedure, that most non-potentiometric protocols use it.

Especially noteworthy, 13 % of separations were done by *centrifugation, followed by filtration*. This can be problematic with low soluble compounds, for the above reasons. If a saturated solution is devoid of excess solid (which centrifugation removes), then passing the weakly-poised (i.e., nearly subsaturated) solution through a filter can produce a significantly *sub*saturated solution. Vessel surface adsorption may contribute to a further lowering of the amount of dissolved sample. In such a combined procedure, solubility may be significantly *under*estimated. The above practice is best avoided, especially with low soluble compounds.

Centrifugation is needed in cases of samples that form (a) opalescent saturated solutions (e.g., colloid systems), or (b) stable suspensions that do not sediment easily, or (c) small agglomerate particulates that can pass through commonly-used filtration membranes. It was shown by McGovern *et al.* [59] that certain types of low soluble research compounds, dubbed "promiscuous inhibitors," form agglomerates of the order of 0.1 µm in size. These can pass through most filters used for phase separation, leading to *over*estimates of the solubility. If such compounds are suspected, it may be a useful *first* to filter the suspension and then to ultracentrifuge it (*in that order*).

Equilibration Time and Identifying the Final Form of the Solid in the Saturated Solution

Most of the equilibrium protocols reviewed here strive to determine the solubility of the most stable form of the solid, most likely crystalline (and sometimes hydrated). Assay times are selected to be long enough to ensure that the measured solubility is no longer changing and that it has reached its lowest value. It is a common practice in pharmaceutical research to adopt 24 h for the equilibration time ("one-shoe-size-fits-all" approach). In most cases, 24 h is enough, but certain practically-insoluble compounds, which consequently have very slow dissolution rates may require much longer times [34]. Loftsson *et al.* [60] allowed 3-7 days for equilibration to be reached for a variety of low soluble compounds. Other researchers used 2-10 days equilibration times for practically-insoluble anticancer drugs (Venkatesh *et al.* [61]; He *et al*. [62]). Fini *et al.* [63] allowed diclofenac suspensions to incubate for 30 days. Undeniably, the stability of the compound needs to be verified when such long times are used.

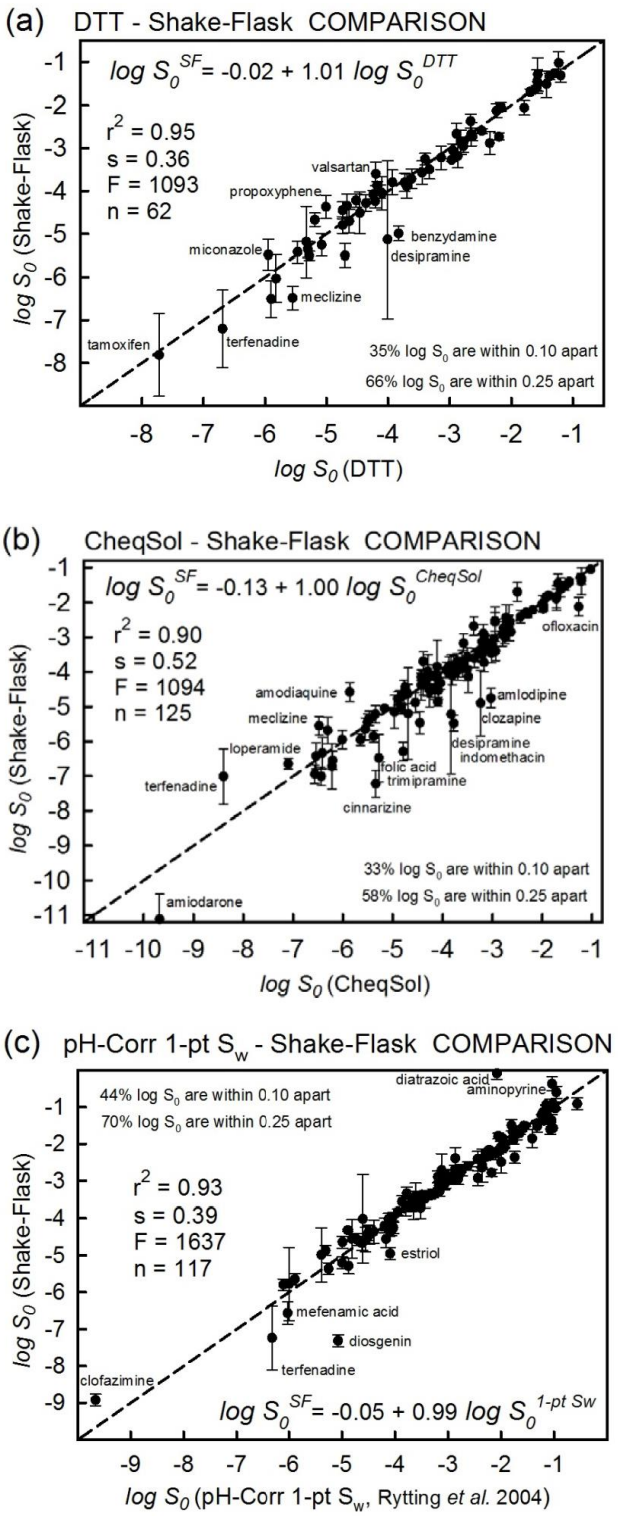

Figure 3. Comparison of traditional shake-flask measurements to (a) the Dissolution Template Titration (DTT) potentiometric method, (b) the CheqSol potentiometric method, and (c) the shake-flask method done in one laboratory using the same protocol.

All too often, at the end of equilibration, the actual form of the solid is not characterized in reported studies. Often the mono- or dihydrate is more stable (i.e., less soluble) than the anhydrous form of the solid. Sometimes, multiple-hydrate forms of the solid precipitate in the crystalline form. But this is not always characterized in published solubility-pH studies.

Solvates aside, different polymorphs of the same stoichiometry may form, depending on the specific assay protocol. Pudipeddi and Serajuddin [64] examined the differences between the solubility values of polymorphs of various substances and found that 0.3 log unit was a typical spread. A few substances showed differences as high as a log unit.

DL-racemates of optically active molecules can have solubilities lower than those of pure enantiomers. For example, at 25 °C, DL-tartaric acid has log S_0 = +0.18, but the L-form has +0.58, an increase of 0.40 log unit. Fourteen amino acids were found with solubility values reported for both the DL- and L- forms. On the average the log S_0 of the DL- enantiomer is 0.15 lower (more insoluble) than that of the L- enantiomer. The least-soluble amino acids (tryptophan, tyrosine, and cystine) show the largest differences in the solubility between the DL- and the L-forms (i.e., DL-forms less soluble). Very few chiral drugs have both DL- and L-/D-form solubilities reported. As an example, DL-atropine has log S_0 = -2.20 ± 0.03 (n=3); L-atropine has -1.84 ± 0.10 (n=2), an increase of 0.36 log unit.

When solubility of chiral molecules is reported without mention of the enantiomeric state, racemic mixture is probably implied.

As mentioned above, in many solubility studies, the solid added to solution is in the salt form (chloride, maleate, tartrate, sodium, etc.). The solid that is isolated at the end of equilibration may not be that of the original salt form, but rather of the free-acid/-base. This can lead to ambiguity in ascribing the reported solubility (Anderson and Flora [65]).

Consequently, crystal-form uncertainty translates to higher variances in prediction studies, where many literature values are pooled into the same training set. Molecular descriptors based on 2D considerations may be difficult to assign to test compounds which may exhibit significant polymorphism/solvatomorphism.

Reproducibility of Experimental Solubility Data

Interlaboratory reproducibility can be assessed for a few model compounds which are repeatedly used in solubility studies. For example, reported solubility of anthracene from 17 different laboratories indicate standard deviation, SD = 0.17 log unit (Kishi and Hashimoto [66]). Additional examples for phenytoin, SD = 0.15 (n=16), flurbiprofen, SD = 0.19 (n=16), and diclofenac, SD = 0.20 (n=20) show similar reproducibility (this work). Perhaps, these can be the expected interlaboratory reproducibility values for such relatively simple molecules. However, ionizable molecules with very low solubility might be expected to have poorer reproducibility. Particularly poignant examples are indicated (this work) by indomethacin (which can be unstable at elevated pH), SD = 0.79 (n=12), and terfenadine (whose unbuffered S_w is strongly affected by ambient CO_2), SD = 1.48 (n=12). The *Handbook of Aqueous Solubility Data* [19] is a massive source of values to estimate interlaboratory reproducibility, although a systematic analysis of the compiled data has not been published, as far as we are aware. For 411 compounds with reported replicate measurements, Katritzky *et al.* [67] found average SD = 0.58. Comparable values have been reported for research compounds. According to Taskinen and Norinder [11], an AstraZeneca in-house database of solubility measurements of different batches of the same compound typically showed reproducibility of 0.49 log units. Higher uncertainties had been suggested (Jorgensen and Duffy [5]; Palmer and Mitchell [17]).

However, there are many compounds determined in different laboratories that indicate reproducibility much less than the above SD values. For example, barbital, hydrochlorothiazide, and lidocaine show SD = 0.08 log unit for n = 10-14 each; for hydrocortisone SD = 0.07 (n=11); for testosterone SD = 0.06 (n = 10), and for acetanilide, aminopyrine, threonine, alanine, 5-fluorouracil, budesonide, minoxidil, fluconazole,

corticosterone, phenobarbital, lidocaine, hydroflumethiazide, acetaminophen, serine, glycine, atenolol, SD = 0.01 – 0.05 (n= 4-11).

In this review of shake-flask solubility measurement, it was decided to include data from two potentiometric methods (DTT and CheqSol – c.f., Appendix). It was thus of interest to estimate how concordant the DTT and CheqSol data are with shake-flask (SF) results for molecules evaluated by the different approaches from different laboratories.

Figure 3a shows a correlation plot of log S_0 (SF) vs. log S_0 (DTT). Although some replicate SF measurements possess large variances, the two sets of log S_0 values correlated well: log S_0^{SF} = -0.02 + 1.01 log S_0^{DTT}, r^2 = 0.95, s = 0.36, F = 1093, n = 62. Based on SF replicates, the average SD = 0.27, which is not very different for the correlation standard deviation, s = 0.36. The differences between the two log S_0 sets were ≤ 0.25 for 66 % of the molecules. A *weighted* linear regression, using the individual inverse variances of the SF data as weights did not appreciable change the statistics of the SF - DTT comparison.

Figure 3b shows a plot comparing SF to CheqSol measurements, indicating that the two sets appear comparable: log S_0^{SF} = -0.13 + 1.00 log $S_0^{CheqSol}$, r^2 = 0.90, s = 0.52, F = 1094, n = 125. There is a slight bias: $S_0^{CheqSol}/S_0^{SF}$ = 1.35 (based on the intercept in the fit). The average SD = 0.25, based on SF replicates, which is notably less than the s = 0.52 from the correlation plot. The differences between the two sets of log S_0 values were ≤ 0.25 for 61 % of the molecules. Using SF-based variances in a *weighted* linear regression analysis produced: log S_0^{SF} = -0.08 + 0.99 log $S_0^{CheqSol}$, with goodness-of-fit = 2.4. It appears that the bias in the unweighted regression is influenced, in part, by the high variance of some of the SF values.

To put the above two comparisons into perspective, we selected a one-source set of SF distilled-water S_w measurements (with pH_{sat} and S_0 calculated with *p*DISOL-X), performed in the same laboratory using the same assay protocol for 122 free-acids/-bases (Rytting *et al.* [24]). For 117 of the molecules, there were reported SF values from other laboratories. Figure 3c shows the SF-to-SF correlation plot. As expected, the two sets of data compared reasonably well: log S_0^{SF} = -0.02 + 1.01 log $S_0^{Rytting\text{-}SF}$, r^2 = 0.93, s = 0.39, F = 1637, n = 117. The average SD = 0.17, based on non-Rytting SF replicates. (It is noteworthy that – with the exception of clofazimine – the Rytting set of molecules were relatively simple.) The differences between log S_0 values from the two SF sources were ≤ 0.25 log unit for 70 % of the molecules.

From the correlation plots in Figure 3, one may conclude that diverse sets of molecules show average reproducibility in the range 0.17 to 0.39 log unit by the "gold standard" shake-flask method. The high-solubility end of the range may be better determined, whereas the low-solubility end may have higher measurement errors. The expected reproducibility of the DTT method, s = 0.36, appears to match that of the SF method, while the CheqSol method shows a slightly higher value, s = 0.52 (similar to the Katritzky *et al.* [67] estimate).

In the entire 4557-set, there were 786 replicate log S_0 values from different laboratories, where two or more values could be averaged. The mean SD of all the averaged values is 0.19 log unit. It is expected that the mean value of 0.19 could be further reduced, to near 0.15 log unit, if corrections were applied to normalize log S_0 values to a common temperature (e.g., 25 °C).

Summary of the Factors Affecting Reproducibility

Some of the factors affecting reproducibility of equilibrium solubility measurement discussed above may be summarized in the list:

- incomplete dissolution over the equilibration time (e.g., latent supersatured conditions)

- inappropriate phase separation (e.g., first centrifuging a saturated solution, then filtering the supernatant)
- adsorption to the filter or assay vial surfaces
- poor wettability
- formations of drug aggregates/oligomers (dimers, trimers, …), micelles, and drug-buffer complexes
- formation of ion-pair between ionic strength adjustor (e.g., NaCl, KCl, etc.) and charged form of drug
- polymorphs, hydrates, solvates, amorphous forms
- stereoisomers (DL-, D-, L-)
- cis-/trans- isomers
- not using buffers with low-soluble ionizable drugs
- not taking into account the effect of ambient CO_2 on the water solubility of low-soluble bases
- using unnecessarily high buffer concentrations, possibly effecting drug-buffer complexation
- not measuring the final pH of the equilibrated saturated solution of ionizable drugs
- inadequate pH electrode calibration procedure at low and high pH and in salt solubility studies
- effect of impurities, especially those which are ionizable
- "promiscuous inhibitor" particles passing through filter
- compound instability at the extremes of pH or over long saturation times
- not sufficiently sensitive analytical methods used to determine very low drug concentration

From the perspective of predicting solubility, the impact of many of the above factors can be minimized by employing good experimental and data analysis practices. However, some of the factors leading to variability in measured solubility, such as the formation of polymorphs, hydrates, solvates, amorphous solids, and the impact of stereoisomers will be harder to deal with. Further *in silico* insights will be needed to address these challenges.

Recommended Procedures for More Accurate Solubility-pH Measurements

Solubility Units, Tabulation of Results and the Use of Logarithmic Plots

Following the format used in the *Handbook of Aqueous Solubility Data* (Yalkowsky *et al.* [19]), it is recommended that solubility be *tabulated* both in molarity and in practical (mg/mL) units. Standard deviations in the measured solubility (based on averaging three or more values) should be included in the table of values. Additionally, a graphical display of log *S* vs. pH (but not *S* vs. pH) would be visually helpful. In the logarithmic forms, the plots can serve as templates [52,57], to recognize the presence of aggregates, incomplete equilibration, corrections for the presence of small quantities of DMSO, etc.

Solubility Methodology and the Benefits of Knowing the Accurate pKa

The "gold standard" multiple-pH buffer shake-flask measurement is recommended for challenging ionizable molecules. Other methods may also be satisfactory, provided that the Henderson-Hasselbalch relationship is independently validated. Miniaturization can be recommended, as long as the protocols are rigorous and well validated.

For ionizable molecules, the measurement of S_w without stating the pH is not recommended. It is far better to measure S_{pH} values in well-qualified buffers (see below), at three or more pH values, bracketing the pK_a.

Ionization Constant

The log *S* – pH data should be evaluated to estimate the value of the intrinsic solubility, S_0. To do this, the independently-determined pK_a is needed. It is sometimes very inaccurate to use the solubility-pH data to determine the pK_a, because usually the required assumption is that the Henderson-Hasselbalch equation is valid, but it may not be so for some particular low soluble molecule, or when high concentrations are used to characterize salt solubility. It is recommended that methods specifically designed to determine the ionization constants of very poorly soluble molecules are used (e.g., state-of-the-art UV spectrophotometry, capillary electrophoresis, potentiometry). These methods are widely available and have been fine-tuned for the challenge. Commercial pK_a instruments based on the above three technologies are generally well-supported by their manufacturers.

Temperature

Solubility is a function of temperature, so the assay temperature always needs to be reported. It is clear that "room temperature" can be different from laboratory to laboratory, and in some cases, seasonally variable. It is advisable to measure and record the actual temperature in the sample vessel during the equilibration period. Or better yet, the measurement is performed in a thermostated vessel kept at 25/37 °C.

Ionic Strength

Measured solubility can be affected by ionic strength (particularly when salt solubility is measured), so the ionic strength usually needs to be reported. Not only do the ionic strength adjustor (NaCl, KCl, etc.) and buffers contribute to the ionic strength, but so does the sample, especially when introduced in salt form.

Equilibration Time and Stirring Protocol

Finely granulated crystals dissolve more quickly than large crystals, illustrating the surface area effect in the classical Noyce-Whitney equation (Eq. 5 in the Appendix). Amorphous solids, which are generally more soluble than their crystalline counterparts, dissolve more quickly than crystals, illustrating the solubility effect – the rate of dissolution is proportional to the solubility (cf., Noyce-Whitney equation, Eq. 5 in the Appendix). Other examples of this are: anhydrous crystals dissolve more quickly than hydrates; usually pure enantiomers dissolve more quickly than racemic mixtures.

Vigorous stirring during the equilibration period can hasten the rate of dissolution, allowing for equilibrium to be reached more quickly (cf., simple Noyce-Whitney equation). However, for particle less than 1-5 µm in diameter, stirring speed has little influence on the rate of dissolution.

Towards the end of the equilibration period, stopping (or slowing) the stirring and allowing the solid to sediment probably contributes to formation of better quality crystals. The 80 % stirring – 20 % sedimentation timing protocol describe by Baka *et al.* [68] can be recommended.

In regions of pH where the sample molecule is largely ionized, equilibration times as short as 1-6 hours may be adequate. But for practically insoluble molecules, in pH regions where the molecule is largely in its uncharged form, equilibration times of 24-72 h may be required to reach equilibrium. Often, the conversion from less stable amorphous or anhydrous solids to more stable crystalline (often hydrate/solvate form) takes 12-24 h, and sometimes longer.

In rigorous applications, the shake-flask method usually determines the equilibrium solubility of the *most stable* solid state form of the compound. Equilibration times as long as several days have been used.

For example, terfenadine studies have used 3-4 d equilibration times. Steroids have been allowed to incubate for 2 -12 d. Probably 24 hours is a good average time, but it is a good idea to test longer times when measuring the equilibrium solubility of very poorly-soluble drugs: 48 h, or even 3 - 7 d. Perhaps special cases like amiodarone (30 d for full therapeutic effect of oral dose) may warrant long equilibration times. Ordinarily, such long times are not recommended.

Shortening the Equilibration Time

The CheqSol method uses cycles of dissolution and re-precipitation by pH adjustment to shorten the time to reach equilibrium. Presumably, the solid which re-precipitates in subsequent cycles is more active than the original crystalline material, perhaps being of small particle size (high surface area) and possibly amorphous.

Loftsson *et al.* [60] describes using temperature spiking cycles and seed crystal to hasten the equilibration period.

The Facilitated Dissolution Method of Higuchi, described in the Appendix can be used to speed up the rate of equilibration. The method is fundamentally rigorous and deserved much more attention than it has received. A comprehensive validation study would be welcomed.

Solution Composition

It is a good idea to keep the assay solutions simple and to define all components precisely!

When studying salt solubility, it is particularly helpful (and perhaps necessary) to specify the actual weight of sample in each vial. It is unhelpful to see statements such as “excess solid was added.”

It is not enough to state that “water was used” as the solvent. Was there any added ionic strength adjustor (e.g., 0.15 M NaCl, etc.)? Was carbon dioxide purged out? Low soluble bases such as terfenadine can indicate solubility over several magnitudes due to the effect of ambient CO_2 in unbuffered solutions.

pH Measurement using Glass Electrodes

It is not a good practice to assume that the assay buffer pH is not altered by the addition of sample. It is highly recommended that the final pH of the saturated solution be carefully measured, using a properly standardized electrodes.

Particular attention should be given to the pH electrode calibration, especially when extreme pH is measured (pH < 1 or pH > 12) or when the ionic strength reaches high values, as in the case of salt solubility measurement.

It is recommended that research-grade combination pH electrodes be used, with adequately described calibration procedure. Ideally, the four-parameter electrode calibration is recommended [55]. Those using the Sirius titrators will recognize the procedure as “Four-Plus” method, and those using the Pion titrators will recognize it as the “ABC” method. Simpler procedures may be satisfactory, as long as they are described precisely. The reported pH should be evidently either on the “operational” or the “concentration” scale; the “mixed” scale is not recommended [52].

A strong case can be made that general solubility equilibrium quotients are best formulated in the concentration scale (rather than activity), with pH electrodes standardized in a *constant ionic medium*, e.g., 0.15 M NaCl [52]. This may be especially important when salt solubility measurements are performed.

How was pH adjusted (e.g., 0.1 M HCl, 1 M NaOH)? Statements like: "concentrated H_3PO_4 was added drop-wise to adjust the pH" are not clear enough. It is far better to state the precise concentration of the acid used. Record the precise volume of pH adjustor solution added. If one wishes to use ionizable titrants, such as phosphoric acid, acetic acid, maleic acid, tartaric acid, etc., it may be useful to add enough of the titrant so the starting pH is as low as desired in the assay. That way, the pre-acidified solution pH can be solely regulated by the amount of base titrant added. There is no real need to have a separate burette dedicated to the ionizable acid titrant. Result computation will be greatly simplified.

Buffers

Sufficient buffering is needed so that reliable pH can be measured and that the titrant can be dispensed precisely enough to adjust the solution to the desired pH. Ordinarily, relatively low buffer concentrations (e.g., 5-10 mM) can be recommended. The sample itself may be an adequate buffer. It is not beneficial to overload the assay protocol with added complications arising from possible buffer-drug interactions. The use of universal buffers (e.g., Britton-Robinson, Sorensen, McIlvaine) can be very useful, but it may also be a source of unintended/unnecessary complications when it comes to processing log *S* – pH data, due to drug-buffer interactions, particularly when salt solubility is measured. Some buffer components may cause difficulties in UV measurement. High concentration phosphate buffer is not recommended; however drug regulatory agencies may prescribe it.

It is inadequate to state that "buffers were used". Precisely which buffer compounds were used? Which salt forms of buffers were used? At what concentrations were they prepared? This information will be needed to calculate the total ionic strength. It may be a good idea to use zwitterionic buffers generally, or acid buffers with weak acid samples and basic buffers with base samples. Phosphate buffers interact with weak base drugs. So, caution is needed when phosphate buffers are used with low-soluble basic drugs. Purpose designed assays can better address the effect of phosphate anions on positively-charged sample molecules.

Separating Solid from Saturated Solution

Filtration can be recommended, using hydrophilic filters, typically 0.2-0.45 µm pore sizes. Chen *et al.* [58] found the *hydrophilic* PVDF (polyvinylidene fluoride) and PES (polyether sulfone) filters performing the best, and nylon the worst, in terms of excessive compound adsorption. It is useful to discard the first 10-25 % filtered solution, to allow filters and surfaces to be saturated with adsorbed compound. The filtration step should not be rushed.

If "promiscuous inhibitors" (McGovern *et al.* [59]) are suspected, then the filtered solutions may be further subjected to ultracentrifugation. It is highly inadvisable to centrifuge first and then filter, for reasons discussed in the review.

Conclusions

Over 800 publications describing equilibrium solubility measurement of sparingly-soluble ionizable drug-like molecules by the shake-flask and related methods were examined. Many factors affecting the quality of the measurement were identified, and a number of suggestions were offered to improve the methodology. Some of the suggestions focused on improving methods for future measurements, and some referred to ways of extracting more reliable information from existing measurements. By normalizing data for pH (by using intrinsic solubility, S_0, derived from water solubility, S_w) and temperature effects (by

transforming measurements performed in the range 20 – 40 °C to the standard value of 25 °C), it can be demonstrated that the expected interlaboratory reproducibility of 0.7 log unit can be reduced to near 0.15.

Glossary

DTT Dissolution Titration Template potentiometric method used to determine intrinsic solubility, S_0

SF shake-flask method, the "gold standard" solubility measurement method

HH Henderson-Hasselbalch equation (e.g., Eq. 1)

K_n aggregation constant, where n is the degree of aggregation

pK_a negative logarithm of the ionization constant

pH_{sat} the equilibrium pH of a saturated solution

S solubility, ideally expressed in units of mol/L (M), µg/mL, or mg/mL

S_0 "intrinsic" solubility (i.e., the solubility of the *uncharged* form of the compound)

S_w "water" solubility, defined by dissolving enough pure free acid/base in distilled water (or water containing an inert salt - as ionic strength adjustor) to form a saturated solution. The final pH of the suspension, pH_{sat}, and S_0 can be calculated by the HH equation (when valid), provided the true pK_a is known. Compound added as a salt form may disproportionate into free acid/base, depending on how much solid had been added. It is not generally possible to calculate the pH and S_0 of such a drug salt suspension.

S_{pH} "pH buffer" solubility (i.e., the total solubility of the compound at a well-defined pH_{sat})

Acknowledgements

Helpful discussions with Antonio Llinàs (AstraZeneca), Krisztina Takács-Novák and Gergely Völgyi (Semmelweis University, Budapest), Christel Bergström (Uppsala University), and Tatjana Verbić (University of Belgrade) are much appreciated. We are greatly indebted to Agustin G. Asuero (Univ. of Seville), Michael H. Abraham (University of London), and William E. Acree, Jr. (Univ. of N. Texas) for pointing out many important log *S*-pH publications. Uko Maran (Univ. of Tartu), Manfred Kansy, Holger Fischer, and Stefanie Bendels (Hoffman-La Roche) have provided valuable insights and leads into the literature of chemoinformatics, for which we are grateful.

References

[1] J. Huuskonen, M. Salo, J. Taskinen, *J. Chem. Inf. Sci.* **38** (1998) 450-456.

[2] J. Huuskonen, J. Rantanen, D. Livingstone, *Eur. J. Med. Chem*. **35** (2000) 1081-1088.

[3] M.H. Abraham, J. Le, *J. Pharm. Sci.* **88** (1999) 868-880.

[4] W.L. Jorgensen, E.M. Duffy, *Bioorg. Med. Chem. Lett*. **10** (2000) 1155-1158.

[5] W.L. Jorgensen, E.M. Duffy, *Adv. Drug Deliv. Rev*. **54** (2002) 355–366.

[6] C.A.S. Bergström, U. Norinder, K. Luthman, P. Artursson, *Pharm. Res*. **19** (2002) 182-188.

[7] T.J. Hou, K. Xia, W. Zhang, X.J. Xu, *J. Chem. Inf. Comput. Sci*. **44** (2004) 266-275.

[8] J.S. Delaney, *Drug Discov. Today* **10** (2005) 289-295.

[9] J.C. Dearden, *Expert Opin. Drug Discov*. **1** (2006) 31-52.

[10] K.V. Balakin, N.P. Savchuk, I.V. Tetko, *Curr. Med. Chem*. **13** (2006) 223-241.

[11] J. Taskinen, U. Norinder, In silico predictions of solubility. In: B. Testa, H. van de Waterbeemd (eds.). *Comprehensive Medicinal Chemistry II*, Elsevier: Oxford, UK, 2007, pp. 627-648.

[12] P. Jain, S.H. Yalkowsky, *Int. J. Pharm*. **385** (2010) 1–5.

[13] A. Shayanfar, A. Jouyban, *Lat. Am. J. Pharm*. **30** (2011) 1525-1530.

[14] J. Wang, T. Hou, *Combi. Chem. High Throughput Scr*. **14** (2011) 1-11.

[15] D. Elder, R. Holm, *Int. J. Pharm.* **453** (2013) 3-11.

[16] J.L. McDonagh, N. Nath, L. De Ferrari, T. Van Mourik, J.B. Mitchell, *J. Chem. Inf. Model*. **54** (2014) 844-856.

[17] D.S. Palmer, J.B.O. Mitchell, *Mol. Pharmaceutics* **11** (2014) 2962-2972.

[18] B. Faller, P. Ertl, *Adv. Drug Deliv. Rev*. **59** (2007) 533–545.

[19] S.H. Yalkowsky, Y. He, P. Jain, *Handbook of Aqueous Solubility Data, Second Edition*. CRC Press, Boca Raton, FL, 2010.

[20] P. Howard, W. Meylan, *PHYSPROP DATABASE*. Syracuse Research Corp., Environmental Science Center, N. Syracuse, NY, Sept. 1999.

[21] Syracuse Research Corporation. The Physical Properties Database (PHYSPROP) http://www.srcinc.com/what-we-do/environmental/scientific-databases.html (accessed 18 Feb 2015).

[22] Analytical Profiles of Drug Substances (*Analytical Profiles of Drug Substances and Excipients; Profiles of Drug Substances, Excipients and Related Methodology*). K. Florey (ed., vols. 1-20),H.G. Brittain (ed., vols. 21-39). Academic Press, San Diego, 1972-2014.

[23] J.W. McFarland, A. Avdeef, C.M. Berger, O.A. Raevsky, *J. Chem. Inf. Comput. Sci*. **41** (2001) 1355-1359.

[24] E. Rytting, K.A. Lentz, X.Q. Chen, F. Qian, S. Venkatesh, *Pharm Res*. **21** (2004) 237-244.

[25] C.A.S. Bergström, C.M. Wassvik, U. Norinder, K. Luthman, P. Artursson, *J. Chem. Inf. Comput. Sci*. **44** (2004) 1477-1488.

[26] A. Llinàs, R.C. Glen, J.M. Goodman, *J. Chem. Inf. Model*. **48** (2008) 1289–1303.

[27] A.J. Hopfinger, E.X. Esposito, A. Llinàs, R.C. Glen, J.M. Goodman, *J. Chem. Inf. Model*. **49** (2009) 1-5.

[28] W.P. Walters, What are our models really telling us? A practical tutorial on avoiding common mistakes when building predictive models. In: J. Bajorath (Ed.). *Chemoinformatics for Drug Discovery*. John Wiley & Sons, Hoboken, NJ, 2014, pp. 1-31.

[29] G. Landrum, R. Lewis, A. Palmer, N. Stiefl, A. Vulpetti, *J. Cheminform*. **3** (2011) 1-1.

[30] M.H. Abraham, A. Ibrahim, A.M. Zissimos, Y.H. Zhao, J. Comer, D.P. Reynolds, *Drug Disc. Today* **7** (2002) 1056-1063.

[31] A. SID Lang, J-C Bradley, *ONS Melting Point Model 010. QDB archive,* DOI: 10.15152/QDB.104. QsarDB content. Property mpC.

[32] G. Völgyi, A. Marosi, K. Takács-Novák, A. Avdeef, *ADMET & DMPK* **1(4)** (2013) 48-62.

[33] A. Avdeef, *ADMET & DMPK* **2(1)** (2014) 33-42.

[34] A. Avdeef, *ADMET & DMPK* **2(1)** (2014) 43-55.

[35] M. Stuart, K. Box, *Anal. Chem.* **77** (2005) 983–990.

[36] C. Sköld, S. Winiwarter, J. Wernevik, F. Bergström, L. Engström, R. Allen, L. Box, J. Comer, J. Mole, A. Hallberg, H. Lennernäs, T. Lundstedt, A-L. Ungell, A. Karlén, *J. Med. Chem*. **49** (2006) 6660-6671.

[37] A. Llinàs, J.C. Burley, K.J. Box, R.C. Glen, J.M. Goodman, *J. Med. Chem*. **50** (2007) 979-983.

[38] K.J. Box, J.E.A. Comer, *Curr. Drug Metab*. **9** (2008) 869-878.

[39] L.Y.S. Narasimham, V.D. Barhate, *J. Pharmacy Res*. **4(2)** (2011) 532-536.

[40] Y.-L. Hsieh, G.A. Ilevbare, B. van Eerdenbrugh, K.J. Box, M.V. Sanchez-Felix, L.S. Taylor, *Pharm. Res*. **29** (2012) 2738-2753.

[41] J. Comer, S. Judge, D. Matthews, L. Towes, B. Falcone, J. Goodman, J. Dearden, *ADMET & DMPK* **2(1)** (2014) 18-32.

[42] D. Schönherr, U. Wollatz, D. Haznar-Garbacz, U. Hanke, K.J. Box, R. Taylor, R. Ruiz, S. Beato, D.

Becker, W. Weitschies, *Eur. J. Pharm. Biopharm*. **92** (2015) 155–170.

[43] A. Avdeef, *Pharm. Pharmacol. Commun*. **4** (1998) 165-178.

[44] A. Avdeef, C.M. Berger, C. Brownell, *Pharm. Res*. **17** (2000) 85-89.

[45] A. Avdeef, C.M. Berger, *Eur. J. Pharm. Sci*. **14** (2001) 281-291.

[46] A. Avdeef, *Curr. Topics Med. Chem*. **1** (2001) 277-351.

[47] B. Faller, F. Wohnsland, Physicochemical parameters as tools in drug discovery and lead optimization. In: B. Testa, H. van de Waterbeemd, G. Folkers, R. Guy, R. (Eds.). *Pharmacokinetic Optimization in Drug Research*. Verlag Helvetica Chimica Acta: Zürich and Wiley - VCH: Weinheim, pp. 257-274 (2001).

[48] C.A.S. Bergström, M. Strafford, L. Lazarova, A. Avdeef, K. Luthman, P. Artursson, *J. Med. Chem.* **46** (2003) 558-570.

[49] A.F. Fioritto, S.N. Bhattachar, J.A. Wesley, *Int. J. Pharm*. **330** (2007) 105-113.

[50] G. Ottaviani, D.J. Gosling, C. Patissier, S. Rodde, L. Zhou, B. Faller, *Eur. J. Pharm. Sci*. **41** (2010) 452-457.

[51] Y. Surakitbanharn, R. McCandless, J.P. Krzyzaniak, R-M. Dannenfelser, S.H. Yalkowsky, *J. Pharm. Sci.* **84** (1995) 720-723.

[52] A. Avdeef, *Absorption and Drug Development Second Edition*. Wiley-Interscience: New York. 2012.

[53] E. Shoghi, E. Fuguet, E. Bosch, C. Rafols, *Eur. J. Pharm. Sci*. **48** (2012) 290-300.

[54] I. Zimmermann, *Int. J. Pharm*. **31** (1986) 69-74.

[55] A. Avdeef, J.J. Bucher, *Anal. Chem*. **50** (1978) 2137-2142.

[56] Z. Wang, L.S. Burrell, W.J. Lambert, *J. Pharm. Sci*. 91 (2002) 1445-1455.

[57] A. Avdeef, *Adv. Drug Deliv. Rev*. **59** (2007) 568-590

[58] T-M. Chen, H. Shen, C. Zhu, *Combi. Chem. HTS* **5** (2002) 575-581.

[59] S.L. McGovern, E. Caselli, N. Grigorieff, B.K. Shoichet, *J. Med. Chem*. 45 (2002) 1712-1722.

[60] T. Loftsson, D. Hreinsdóttir, M. Másson, *Int. J. Pharm*. **302** (2005) 18–28.

[61] S. Venkatesh, J. Li, Y. Xu,R. Vishnuvajjala, *Pharm. Res*. **13** (1996) 1453-1459.

[62] Y. He, S.E. Tabibi, S.H. Yalkowsky, *J. Pharm. Sci*. **95** (2006) 97-107.

[63] A. Fini, G. Fazio, G. Feroci, *Int. J. Pharm.* **126** (1995) 95-102.

[64] M. Pudipeddi, A.T.M. Serajuddin, *J. Pharm. Sci*. **94** (2005) 929-939.

[65] B.D. Anderson, K.P. Flora, Preparation of water-soluble compounds through salt formation. In: C.G. Wermuth, (Ed.). *The Practice of Medicinal Chemistry*. Academic Press, London, pp. 739-754 (1996).

[66] H. Kishi, Y. Hashimoto, *Chemosphere* **18** (1989) 1749-1759.

[67] A.R. Katritzky, Y. Wang, S. Sild, T. Tamm, M. Karelson, *J. Chem. Inf. Comput. Sci.* **38** (1998) 720-725.

[68] E. Baka, J.E.A. Comer, K. Takács-Novák, *J. Pharm. Biomed. Anal*. **46** (2008) 335-341.

[69] A. Avdeef, D. Voloboy, A. Foreman, Dissolution and solubility. In: B. Testa, H. van de Waterbeemd (eds.). *Comprehensive Medicinal Chemistry II*, Elsevier: Oxford, UK, 2007, pp. 399-423.

[70] A. Avdeef, O. Tsinman, *Pharm. Res*. **25** (2008) 2613-2627.

[71] K. Tsinman, A. Avdeef, O. Tsinman, D. Voloboy, *Pharm. Res*. **26** (2009) 2093-2100.

[72] A. Avdeef, K. Tsinman, O. Tsinman, N. Sun, D. Voloboy, *Chem. Biodiv*. **11** (2009) 1796-1811.

[73] J.H. Fagerberg, O. Tsinman, K. Tsinman, N. Sun, A. Avdeef, C.A.S. Bergström, *Mol. Pharmaceutics* **7** (2010) 1419-1430.

[74] A. Avdeef, S. Bendels, O. Tsinman, M. Kansy, *Pharm. Res.* **24** (2007) 530-545.

[75] J. Alsenz, M. Kansy, *Adv. Drug Deliv. Rev*. **59** (2007) 546–567.

[76] J. Alsenz, E. Meister, E. Haenel, *J. Pharm. Sci*. **96** (2007) 1748-1762.

[77] C.A.S. Bergström, K. Luthman, P. Artursson, *Eur. J. Pharm. Sci*. **22** (2004) 387-398.

[78] A. Glomme, J. März, J.B. Dressman, *J. Pharm. Sci.*, **94** (2005) 1-16.
[79] T. Higuchi, F.M. Shih, T. Kimura, J.H. Rytting, *J. Pharm. Sci*. **68** (1979) 1267-1272.
[80] W.H. Streng, S.K. His, P.E. Helms, H.G.H. Tan, *J. Pharm. Sci.* **73** (1984) 1679-1684.
[81] F. Wohnsland, B. Faller, *J. Med. Chem*. **44** (2001) 923-930.
[82] G. Völgyi, E. Baka, K.J. Box, J.E.A. Comer, K. Takács-Novák, *Anal.Chim. Acta* **673** (2010) 40–46.

Appendix - Brief Summary of Methods Suitable for Measuring Equilibrium Solubility

The Appendix briefly describes methods which can potentially be used to generate intrinsic solubility of sparingly-soluble drugs. Kinetic methods are not suitable for such application, and are thus not considered.

Classical Saturation Shake-Flask (SF) – Still the 'Gold Standard'

Solubility measurement as a function of pH under equilibrium conditions usually requires long equilibration times (12 h - 7 d). The shake-flask (SF) method is a relatively simple procedure: the drug is added as solid to a standard buffer solution (in a flask) until saturation occurs, indicated by excess undissolved drug. The thermostated saturated solution is shaken as the suspension equilibrates. After filtration or centrifugation, the concentration of the substance in the supernatant solution is then determined using HPLC, usually with UV detection. For a solubility-pH profile, parallel measurements need to be performed in several different pH buffers. Baka *et al.* [68] suggested concrete ways to improve the quality of the SF measurements. In high-quality SF measurements, (a) it is actually verified that the dissolution/precipitation of the solid has reached equilibrium, (b) the final (equilibrium) pH is measured, and (c) the equilibrated solid is isolated and identified (or characterized). The measured solubility is expected to be that of the most stable polymorph, which can often be a hydrate.

Micro-Dissolution for Polymorph/Solvate Solubility Measurement (µDISS Profiler)

By USP standards, dissolution equipment typically uses 900-mL solution volumes, so it would be impractical to determine solubility of drugs, since an excessive amount of compound would be required to form a saturated solution. However, the miniaturized-volume dissolution apparatus, µDISS Profiler (Pion Inc.), uses 1.0 mL working volumes and makes it practical to determine solubility (Avdeef *et al.* [69]; Avdeef and Tsinman [70]; Tsinman *et al.* [71]; Avdeef *et al.* [72]; Fagerberg *et al.* [73]). Since the instrument uses *in situ* fiber optic UV (diode array) detection, the progression of dissolution may be followed in real time, making it possible to characterize the (transient) solubility of different polymorphs/solvates, over periods of days, if necessary. It has been demonstrated that 10-100 µg of powder can be characterized for both the transient polymorph solubility and equilibrium solubility of the most stable form of the solid. A hydroxypropyl-*β*-cyclodextrin phase-solubility study of itraconazole using the micro-dissolution apparatus estimated the active polymorph solubility of itraconazole to be < 9 ng/mL.

Equilibrium 96-Well Microtitre Plate Methods

To increase throughput and decrease sample consumption, several pharmaceutical companies have transferred the larger-volume classical SF method to smaller-volume 96-well microtitre plate format linked to robotic liquid handling systems, using several distinct approaches (including those which avoid use of DMSO). There are trade-offs in the high-throughput methods, and usually, the quality of the data are not expected to be as accurate as those obtained by the SF method.

Since the compound is usually introduced as aliquots of a 10 mM DMSO stock solution, such methods have an upper solubility limit, typically less than 150 µM. Also, the presence of the small amount of DMSO in the final buffer solution increases the measured solubility values of the most insoluble compounds by up to 100-fold (e.g., glibenclamide, clotrimazole), compared to DMSO-free conditions. This appears to be highly compound-dependent (Chen *et al*. [58]). Quality results require this DMSO-effect to be factored out (e.g., *p*DISOL-X method).

For acceptably reliable aqueous intrinsic solubility (S_0) to be determined from *single*-pH determination (of compounds introduced from DMSO stock solutions), it is necessary (a) to *remove the DMSO* originating from the stock solution, (b) to know the pK_a value of the test compound, and (c) to assume that the Henderson-Hasselbalch (HH) equation is a valid description of the log *S* - pH relationship.

If the measurement is performed in the presence of ≤1 % v/v DMSO, there is a way to determine S_0, provided that the solubility measurement is done at *several* values of pH over a wide enough a pH range ([52]; *p*DISOL-X method). If the pK_a is known, then it is *not* necessary to assume that the HH equation holds, as has been demonstrated by Avdeef *et al.* [74].

Lyophilization Method ('GeneVac' 96-Well DMSO-removing Device)

Some pharmaceutical companies have implemented methods where aliquots of 10 mM DMSO stock solutions of test compounds are added to a microtitre plate. Then, the DMSO is removed by lyophilization (e.g., using GeneVac vacuum centrifugation apparatus), after which, a pH 6.5 or 7.4 buffer is added to the compound residues in the microtitre plate. The plate is sealed and allowed to incubate up to 24 h, usually at room temperature, during which time the plate may be shaken. For best results, the final (equilibrium) pH needs to be measured. With the initial DMSO removed, measured solubility values can match those obtained by the traditional SF method. Alsenz and Kansy [75] described the Lyophilization Solubility Assay (LYSA) based on UV plate reader and HPLC methods.

Equilibrium Solubility Assay (DMSO-free 'THESA' Method)

Alsenz and Kansy [75] also described the Thermodynamic Solubility Assay (THESA) microtitre plate method where < 1 mg of weighed solid sample is added a pH 6.5 buffer solution. The suspensions are stirred for 2 h and allowed to sit for another 20 h, before filtration. A provision for reading the final pH is included in the protocol. The advantage of the THESA method is that the compound is introduced in the original solid state.

Partially-Automated Solubility Screening (DMSO-free 'PASS' Method)

Alsenz *et al*. [76] described the microtitre plate Partially-Automated Solubility Screening (PASS), where solid compounds (DMSO-free) are suspended as slurries in volatile heptane, sonicated to increase fractionation and dispersion, and then dispensed quickly in small aliquots into microtitre plate wells. From the dispensed volume, the weight of drug is calculated. The advantage of the PASS method is that the compound is introduced in the original solid state. The heptane is allowed to evaporate, before buffer is added. This procedure inherently can be used to assess relatively high solubility (several mg/mL) by a microtitre plate method. The comparison of PASS solubility values to those from standard SF values was good.

Small-Scale Shake-Flask Method (SSF)

Bergström *et al.* [6] described the Small-Scale Shake-Flask (SSF) method, where crystalline compounds were added to 50-200 µL solutions whose pH was then adjusted to at least one pH below the pK_a for acids and one pH above the pK_a for bases, in an effort to measure S_0 directly. After 24-72 h of stirring, the solutions were centrifuged to separate the phases. Seventeen compounds were measured (0.7 ng/mL to 6 mg/mL). Expanding the SSF approach, Bergström *et al*. [77] further studied 25 bases over a wide pH range, using 0.15 M phosphate buffer adjusted with KOH or H_3PO_4. The suspensions were incubated for 24 h. Phases were separated by centrifugation. An in-depth analysis of the 25 bases has been recently reported

[34], where it was tentatively suggested that 24 h may not be sufficient equilibration time for several of the sparingly-soluble bases studied. Other concerns about pH control were raised by Völgyi et al. [82].

Miniaturized Shake-Flask (MSF)

Glomme *et al.* [78] described the Miniaturized Shake-Flask (MSF) method. The sample is weighed (twice the *in silico*-estimated solubility) directly into the Whatman UniPrep filter chamber, which is then filled with 2 mL of buffer solution. The capped chambers are agitated for 24 h at 37 °C. Afterwards, a chamber-mated plunger equipped with a filter is pushed over the sample solution to separate the solid. The final pH is read at the end of the equilibration period.

Dual-Phase Potentiometric Methods

The two related potentiometric methods described below are only suitable for ionizable compounds, whose pK_a can be determined *in situ* (DTT) or need to be determined in a separate method (CheqSol).

Dissolution Template Titration Method (DTT)

The Dissolution Template Titration (DTT) semi-automated intrinsic solubility potentiometric method [43] takes the estimated pK_a and the octanol/water partition coefficient, log P_{OCT}, as input parameters to predict S_0. The DTT procedure then simulates the entire titration curve *before* the assay starts. This defines a "template" for data collection. Titration is done in the direction of increasing dissolution, taking the saturated solutions past the point of complete dissolution. The Noyes-Whitney equation (Eq. 5 below) is used to set the pace of data collection. The more insoluble the compound is (based on the template), the longer is the assay time (typically, 3 - 10 h).

Data are analyzed using Bjerrum plots: $\overline{n}_H$, the average number of *bound* protons, versus pH. Since it is known how much strong acid, [HCl], and strong base, [KOH], have been added to the solution at any point and since it is known how many dissociable protons, n, the sample adds to the solution, the total hydrogen ion concentration is defined. The difference between the latter and the free hydrogen (pH electrode reading) concentrations is equal to the concentration of the bound hydrogen, which, when divided by the sample concentration, *C*, results in $\overline{n}_H = \{ [HCl] - [KOH] + n\,C - [H^+] + [OH^-] \} / C$.

The pH at half-integral value of $\overline{n}_H$ equals pK_a^{APP}, the apparent pK_a. The presence of precipitate is indicated by the pK_a^{APP} shifting to a higher value for acids and to a lower value for bases, compared to the true pK_a. The intrinsic solubility can be deduced by inspection of the curves and applying the relationship $\log S_0 = \log(C/2) - | pK_a^{APP} - pK_a |$. The graphically-estimated constant is subsequently refined by a mass-balance weighted nonlinear regression procedure, which does *not* require that the Henderson-Hasselbalch relationship be valid (although, often, it is assumed to be so). The pK_a can be determined alongside intrinsic solubility.

CheqSol Potentiometric Method

Stuart and Box [35] embraced the $\overline{n}_H$ part of the DTT method and developed a valuable novel way to speed up the time to reach equilibration. Focusing on the pH region where $\overline{n}_H$ is expected to be near half-integral, the CheqSol instrument quickly dissolves the solid by raising the pH for acids (e.g., to 10-12) or lowering the pH for bases (e.g., to 1-2). Afterwards, the original pH is re-established by adding acid/base titrant, whereupon the solid re-precipitates, possibly as an amorphous phase. The procedure of dissolution-precipitation is cycled several times, from which the pH corresponding to a known value of n_H (near 0.5) is determined by interpolation. Using the DDT n_H relationships, the intrinsic S_0 is calculated from

a *single* pH point, suggested by the value of the pK_a. Since the method is not based on the general mass-balance base regression analysis, CheqSol implicitly assumes that the Henderson-Hasselbalch equation is always valid, which in some cases could potentially be a source of systematic error.

Facilitated Dissolution Method (FDM)

The Facilitated Dissolution Method (FDM), devised by Higuchi *et al.* [79], can be used to overcome extremely low equilibration rates during solubility measurement of practically-insoluble compounds. Since it is rarely practiced, the method is described here in some detail (cf., [52]). In the FDM approach, a two-fold excess of solid over that needed to saturate the solution is recommended. To overcome the expected slow dissolution, a small volume of an immiscible organic solvent (e.g., 2 % v/v hexadecane) is added to the aqueous solution of the sparingly-soluble compound.

As long as the saturated system contains three distinct phases (solid, oil, and water), the solubility value is not altered by the presence of the water-immiscible oil. (This is sufficiently but not precisely true for ionizable compounds, as commented below.) To show this, consider a weak base example (e.g., terfenadine), for which the FDM equilibrium reactions are:

$$B(s) \leftrightarrow B(org) \qquad S_{ORG} = [B(org)] \tag{2}$$

$$B \leftrightarrow B(org) \qquad P_{O/W} = [B(org)] / [B] \tag{3}$$

where Eq. 2 denotes the solubility of the compound in oil, S_{ORG}, and Eq. 3 denotes the oil-water partition coefficient of the compound, $P_{O/W}$. By subtracting the partition reaction from the solubility-in-oil reaction, one gets the expected solubility-in-water equation,

$$B(s) \leftrightarrow B \qquad S_W = S_{ORG} / P_{O/W} \tag{4}$$

So, the presence of a small quantity of oil, into which the water sparingly-soluble compound can appreciably dissolve, does not affect the aqueous solubility value. According to the Noyes-Whitney dissolution rate (μg/cm^3·s) equation under sink condition,

$$d[B]/dt = (A / V)\, P_{ABL}\, S_W \tag{5}$$

where A = powder surface area (cm^2), V = volume of aqueous solution (cm^3), P_{ABL} = permeability of the aqueous boundary layer adjacent to the surface of the solid particles (cm/s), and S_W = aqueous solubility (μg/cm^3).

The addition of the oil may also alleviate problems due to poor wettability of the original crystalline solid.

Let's consider the terfenadine FDM example explicitly. The intrinsic solubility of the weak base in water is 5.6x10^{-8} M (log S_0 = -7.25) at 25 °C (Streng *et al.* [80]; data analyzed using *p*DISOL-X). The measured pK_a is 9.91 at 25 °C, I = 0.15 M (Origin-shifted Yasuda-Shedlovsky method [52]). Consider 20 μg of terfenadine free base added to 1 mL of 10 mM taurine buffer in 0.15 M NaCl at pH 9.0, containing 20 μL of hexadecane. Let's assume 100 μm (diam.) spherical particles of solid in the suspension at the start. The hexadecane-water partition coefficient of terfenadine is estimated to be log $P_{HXD/W}$ = 2.3 (using calibration standards from Wohnsland and Faller [81]). The dissolution simulation feature of *p*DISOL-X was used to calculate the precise concentrations and quantities in each of the three phases. At pH 9.0, 0.302 μg of the drug is predicted to dissolve in the buffer in the absence of added hexadecane, with 19.70 μg remaining in the solid state. The calculated total surface area of the solid is initially 0.0086 cm^2 and decreases by 1.3 % at saturation. The (A/V) factor for Eq. 5 is 0.0086 cm^{-1}. After the addition of 20 μL of hexadecane, the

calculated amount of the drug in water is still exactly 0.302 µg; the amount in the solid is now 19.59 µg, as 0.20 µg of the drug partitions into the 20 µL of hexadecane. The solubility in hexadecane is calculated to be $2.2x10^{-7}$ M, compared to $6.4x10^{-7}$ M in the buffer. The smaller volume of the oil phase increase the rate of dissolution into the oil phase: the (A/V) factor between the solid surface *and the oil volume* is 0.0086 cm^2/0.02 cm^3 = 0.43 cm^{-1}. This suggests that the rate of dissolution into the oil phase will be about 50 times greater than the rate of dissolution into the water phase. What about the rate of transfer from the oil phase into water (to complete the transfer cycle in Eq. 4)? This is expected to be high in a well stirred (e.g., 500 RPM) solution, dispersing the oil into tiny droplets, thus increasing oil-water interfacial surface area. Hence, a small amount of adjunct oil substantially increases the overall dissolution rate associated with Eq. 5, without affecting the equilibrium solubility of terfenadine.

It is important to point out that the FDM approach does not work for ionizable compounds in *poorly-buffered* solutions, and generally cannot be applied in the DTT or the CheqSol method (since buffers are not generally used). Using the *p*DISOL-X program, simulations in buffer-free solutions show that the addition of 10 µL hexadecane can affect the water solubility of terfenadine, through a subtle interplay of the various pH-dependent equilibria. The effect is lessened if a very large excess of terfenadine were added. But this is not recommended, as discussed by Higuchi *et al.* [79].

As far as we are aware, the FDM approach was last applied by Venkatesh *et al.* [61] in the solubility determination of cosalane, a steroid derivative with aqueous intrinsic solubility of *much less* than 1 ng/mL. The pK_a values of cosalane have not been reported to date, so there may be some uncertainty in the intrinsic solubility of cosalane. Since the FDM method is best applied to practically-insoluble molecules, probably LC/MS-MS resources would be required to measure the extremely low sample concentrations in saturated solutions.