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Study of equilibrium solubility measurement by saturation shake-flask method using hydrochlorothiazide as model compound

Edit Baka [a], John E.A. Comer [b], Krisztina Takács-Novák [a,*]

[a] *Semmelweis University, Department of Pharmaceutical Chemistry, H-1092 Budapest Hőgyes, Endre u. 9, Hungary*
[b] *Sirius Analytical Ltd., Riverside, Forest Row Business Park, Forest Row, East Sussex, RH18 5DW, United Kingdom*

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Abstract

The experimental conditions that affect equilibrium solubility values measured by the classical saturation shake-flask method have been examined, using hydrochlorothiazide as a model compound. Modifications in temperature, sedimentation time, composition of aqueous buffer and the technique of separation of solid and liquid phases were all found to influence the equilibrium solubility results strongly. However, variations in the amount of solid excess and stirring time were found to have less influence. In the light of these observations, a new, shorter protocol has been developed for measurements of equilibrium solubility, together with recommendations for good analytical practice. The equilibrium solubilities of five other drugs were measured to verify the new protocol.

Keywords: Solubility; Saturation shake-flask method; Hydrochlorothiazide

1. Introduction

Determination of solubility of drug candidates is important in drug research, both in discovery and development phases. In early stages of drug research, solubility together with other physicochemical parameters (lipophilicity, ionization, permeability) is used to screen out drug-like candidates, while in the development phase it is needed for biopharmaceutical classification and bioequivalence issues. Solubility is also required for formulation optimization and salt selection [1–3].

In the pharmaceutical literature two commonly used solubility terms are *kinetic* solubility (the concentration of a compound at the time when an induced precipitate first appears in the solution) and *equilibrium* (or *thermodynamic*) solubility (the concentration of a compound in saturated solution when excess of solid is present, and solution and solid are at equilibrium). The term *intrinsic solubility* refers to the equilibrium solubility of the free acid or base form of an ionizable compound at a pH where it is fully un-ionized [1,3].

Various methods have been developed for the measurement of solubility in the last two decades. In the early phase of discovery, kinetic solubility is often measured by turbidity-based [4] and UV plate scanner-based detection systems [4,5]. Most of these methods are ranking assays for high throughput screening where the buffered sample solutions are prepared by adding aliquots of 10 mM DMSO stock solutions [3]. In the development phase of drug research, equilibrium solubility of drug-like molecules is measured by different methods, among them new potentiometric methods like DTT or CheqSol [5–9]. However, the basic method (against which new solubility methods are generally validated) has remained the classical *saturation shake-flask* method.

Despite the numerous experimental methods developed precise equilibrium solubility values are difficult to obtain, because they are affected by many known and unknown factors [10]. Table 1 shows some examples for the wide variation of published aqueous solubility values [8,11,12b–19,29]. The differences of the solubility data among reports sometimes are very large, for example in case of cholesterol it exceeds the five orders of magnitude. The most likely explanation for this discrepancy can be the effect of particle size, crystallinity and other molecular features of the sample [10,20] or the great differences in the applied experimental conditions.

* Corresponding author. Tel.: +36 1 215 5241; fax: +36 1 217 0891.
E-mail address: NOVKRI@HOGYES.SOTE.HU (K. Takács-Novák).

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Table 1
Variation of aqueous solubility in literature (25 °C)

Compound	Solubility range (g/ml)	Solubility range (μg/ml)
Cholesterol [12b,30]		0.025–2600
Dexamethasone [11,29]	89.1–121.0	
Diclofenac [8,12b,13]		0.6–2.4
Digoxin [11,29]	28.0–97.9	
Estradiol [11,29]	0.16–5.00	
Hydrocortisone [11,29]	280–359	
Ibuprofen [8,13–15]		20–80
Indomethacin [11,19]	4.00–14.0	
Lidocaine [16–19]	2.30–4.60	
Progesterone [11,29]	7.90–200	
Riboflavine [11,29]	66.0–99.9	

Shake-flask technique is based on simple procedures, but it is time-consuming, and requires lots of manual work. Moreover, there is no accepted standard way to carry out this method [1], and published solubility studies show great differences in the experimental conditions used, in particular the stirring and the sedimentation times, and in separation techniques. For example, it is possible to find reported stirring times from 48 h to 2 weeks, and sedimentation times from 24 h to 3 days [3,22–25]. In addition, different techniques (sedimentation, centrifugation or filtration) are used for separation of solid and liquid phases [21]. A comprehensive, systematic study of the experimental conditions of the shake-flask method, and their influence on solubility measurement – similar to the paper of Dearden and Bresnen about log *P* measurement [26] – has not been yet published.

The aim of the present study was to examine the factors that influence the measurement of equilibrium solubility and to reveal the most critical steps in the application of the traditional shake-flask solubility method. As a physicochemical property, solubility is influenced by temperature (and pressure), purity of materials, composition of buffer solutions and properties of the compound such as polymorphism, aggregation and the formation of supersaturated solutions [5,21]. The effect of pH on the solubility of ionizable compounds is well known and extensively examined phenomenon. Recently an excellent review has summarized this important aspect of solubility [27]. Here we focus only on the measurement of *intrinsic* solubility of compounds thus the effect of pH is out of the scope of this study. As a measurement, solubility is also influenced by several experimental factors, including stirring time, sedimentation time, composition of the aqueous buffer, temperature, amount of solid excess, and the technique of phase-separation. In this study, these experimental factors were systematically varied to check their effect on measured solubility values measured by a "standard" protocol, described in Section 3. Based on these results, a new, faster protocol is proposed for the measurement of equilibrium solubility, which was verified by measuring the solubility of five drugs by both protocols.

2. Materials and methods

2.1. Samples and chemicals

Hydrochlorothiazide, furosemide, nitrofurantoin, piroxicam, and quinine hydrochloride were of pharmacopoeial grade (Ph. Eur. 5.8) and supplied by Reanal (Budapest, Hungary) or Hungaropharma (Budapest, Hungary). Trazodone was purchased from Sigma (Poole, Dorset, UK) and used without further purification. All samples were supplied as small crystals or powders. Distilled water of pharmacopoeial grade was used to prepare all solutions.

Three buffer solutions were used in the solubility experiments:

A) A Britton-Robinson (BR) buffer solution (a mixture of acetic, phosphoric, and boric acids, each at 0.04 M) was prepared, and various amounts of 0.2 M NaOH were added to give the pH required for each shake-flask experiment. The ionic strength of Britton–Robinson buffer is 0.089.
B) Sörensen I (SöI) buffer solutions were prepared by mixing various amounts of 1/15 M Na_2HPO_4 and 1/15 M KH_2PO_4 solutions to reach the required pH. The ionic strength of Sörensen I buffer solution is 0.076.
C) Sörensen II (SöII) buffer solutions were prepared by mixing various amounts of 0.1 M sodium citrate solution and 0.1 M NaOH. The ionic strength of Sörensen II buffer solution is 0.318.

2.2. Apparatus

The pH of the buffer solutions was measured by a Radiometer PHM 220 pH meter with combined Ag/AgCl glass electrode (PHC 3359-9). The temperature of the samples was maintained at 25 ± 0.1 °C during the solubility measurements using a Lauda thermostat. A Heidolph MR 1000 magnetic stirrer was used to mix the two phases. Samples were filtered using a Whatman PVDF membrane (0.45 μm pore size) syringe filter, as the most suggested one by the manufacturer for quantitative analysis and dissolution testing. The concentration in the supernatant of the samples was measured spectrophotometrically using a Jasco V-550 UV–vis spectrophotometer. The dissociation constants (pK_a) of the compounds have been determined in previous works [1,6] using a GLpKa automated pK_a and log *P* analyser (Sirius Analytical Instruments Ltd., UK), at 25 °C and 0.15 M ionic strength.

2.3. Saturation shake-flask solubility method

Knowing the pK_a values and class (acid, base, ampholyte) of the compounds examined, the pH of the aqueous solution could be selected to assure the predominant presence of the unionized form. Low pH was selected for acids and high pH was selected for bases. The pH value of the isoelectric point (the pH at which the substance has an equal number of positive and negative charges) was used for ampholytes (Table 2).

Table 2
pK_a values, pH of solubility and $A^{1\%}_{1\,cm}$ experiments and spectroscopic data of examined compounds

Compound	pK_{a_1}	pK_{a_2}	pH	$A^{1\%}_{1\,cm}$ ± S.D. (r)	λ_{max} (nm)
Hydrochlorothiazide (bivalent acid)	8.75	9.88	6.00	696 ± 12 (0.9996)	271
Furosemide (bivalent acid)	3.60	10.15	2.00	1225 ± 20 (0.9990)	234
Nitrofurantoin (acid)	7.05		2.50	510 ± 35 (0.9979)	265
Piroxicam (ampholyte)	1.87	5.29	3.73	640 ± 3 (0.9999)	361
Quinine-hydrochloride (bivalent base)	4.24	8.55	11.50	122 ± 3 (0.9996)	329
Trazodone (base)	6.81		11.50	278 ± 10 (0.9981)	246

To facilitate the measure of concentration by UV spectrophotometry, the specific absorptivity ($A^{1\%}_{1\,cm}$, the absorbance of 1 g/100 ml solution measured with 1 cm path length at a given wavelength) of each sample at a pH where it was un-ionized was determined separately at a selected wavelength using 12–18 points of a minimum of two parallel dilution series, from the linear regression equation (Table 2).

The equilibrium solubility of the un-ionized form of the samples was determined by the shake-flask method. For each reported solubility result, three (occasionally six) independent shake-flask experiments were carried out in parallel. For each experiment, the solid sample was added carefully using a spatula to 5–10 ml of the aqueous buffer in a glass vial, while stirring until a heterogeneous system (solid sample and liquid) was obtained. The solution containing solid excess of the sample was then capped, and stirred at the chosen temperature for a specified time before separating saturated solution and precipitate by sedimentation, filtration or centrifugation. Three (occasionally four) aliquots of supernatant were then taken out with a fine pipette from the saturated solution and diluted with solvent if necessary, and the concentration of sample in each aliquot was measured by UV spectrophotometry. As mentioned above, three (or six) independent shake-flask experiments were made for each reported result. The reported results were thus the mean of at least 9 (or 18) measured concentrations. The standard deviation of the mean result was also calculated.

2.4. Statistical evaluation

The two-sample *t*-test statistical technique was applied to investigate the significance of the results obtained under different experimental conditions [28].

3. Results and discussions

Hydrochlorothiazide was chosen as the model compound for this standardization study of the saturation shake-flask solubility method. This substance seems to be ideal for investigation, being ionizable, sparingly soluble in water and chemically stable, with intensive UV absorbance and no polymorphism. Hydrochlorothiazide is an acid with pK_as of 8.75 and 9.88; from consideration of this information, it could be deduced that hydrochlorothiazide would be un-ionized at pH 6.

First, the equilibrium solubility of the un-ionized form of hydrochlorothiazide (i.e. the intrinsic solubility) was measured according to a "*standard protocol*". In this protocol, a Britton–Robinson buffer solution at pH 6.0 was prepared, and enough solid was added to a 5 ml aliquot of the buffer to cause a small excess of solid. This was followed by 48 h stirring in a glass vial immersed into a thermostat (the so-called saturation time) at 25 ± 0.1 °C, allowing time for the system to achieve thermodynamic equilibrium. The sample was left without stirring for a further 24 h of sedimentation for phase-separation. The equilibrium solubility of hydrochlorothiazide measured by this standard protocol was $\log S = -2.73 \pm 0.01$ (S in molarity, $n = 18$). This result expressed in μg/ml is indicated in Table 3. In case of hydrochlorothiazide the available literature data: $\log S = -2.67$ [6]; $\log S = -2.63$ [8,31] do not exhibit significant deviation and our result is in good agreement with them.

Next, the effect on measured solubility of varying one or other of the parameters in the standard protocol was examined. In subsequent tests, one of six parameters (buffer choice, amount of solid excess, temperature, time of stirring, time of sedimentation, phase separation technique) was varied while the other conditions were kept unchanged. The results are summarized in Table 3 and in Figs. 1–3.

Table 3
The effect of the phase-separation technique, temperature and buffer composition on the equilibrium solubility of hydrochlorothiazide

	Standard protocol	Different phase separations		Different temperatures		Different buffers	
Buffer	a: BR, pH 6	a	a	a	a	Sö I, pH 6	Sö II, pH 6
Solid excess	b: small excess	b	b	b	b	b	b
Temperature	c: 25 ± 0.1 °C	c	c	15 °C	37 °C	c	c
Stirring time	d: 48 h	d	d	d	d	d	d
Sedimentation time	e: 24 h	e	e	e	e	e	e
Phase separation	f: sedimentation	Centrifuge	Filtration	f	f	f	f
Result (μg/ml)	556	591	661	450	1036	565	779
±	13.2	15.4	3.5	20.3	36.3	10.4	41.3
n	18	12	12	12	12	12	22

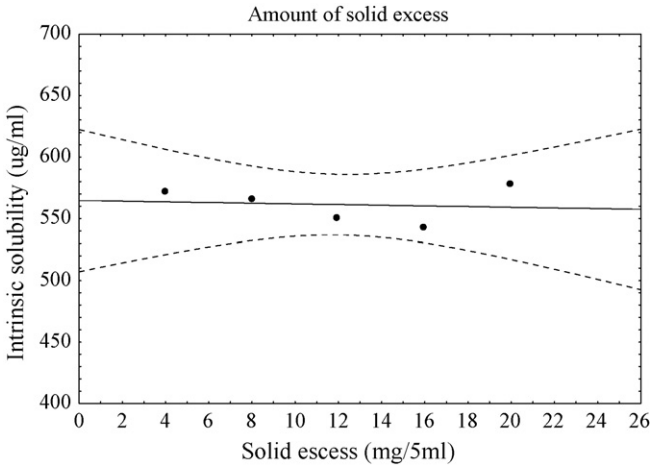

Fig. 1. The effect of the solid excess on the equilibrium solubility of hydrochlorothiazide.

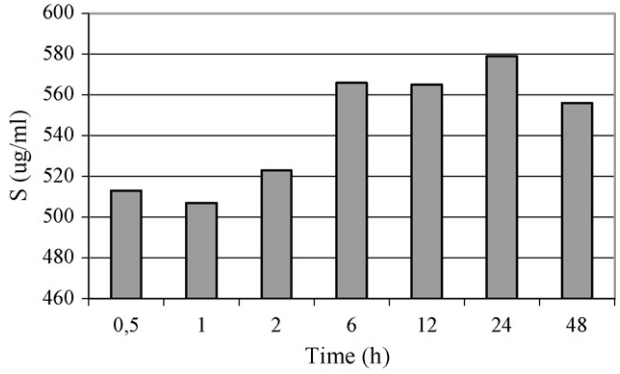

Fig. 2. The effect of stirring time on the equilibrium solubility of hydrochlorothiazide. Each result is the mean of three-independent shake-flask results, each based on the mean of four concentration measurements using different aliquots of supernatant.

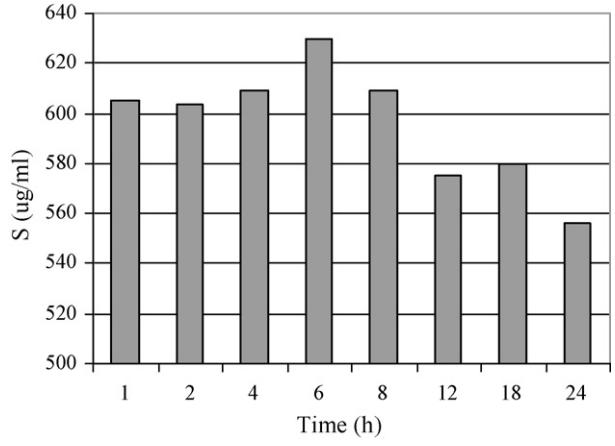

Fig. 3. The effect of sedimentation time on the equilibrium solubility of hydrochlorothiazide. Each result is the mean of three-independent shake-flask results, each based on the mean of four concentration measurements using different aliquots of supernatant.

3.1. Effect of buffer solution

Measurements of aqueous equilibrium solubility of ionizable drugs are made in buffered solution, because their solubility is a pH-dependent parameter. Weak acids and bases ionize in solutions to varying extent, depending on their pK_a values and the pH of the medium [7–9]. For measurement of the intrinsic solubility the experimental pH is chosen at which the sample is not ionized.

To measure hydrochlorothiazide, three buffer solutions were used at pH 6.0; the obtained results are shown in Table 3. The statistical analysis has indicated that the S (μg/ml) value of hydrochlorothiazide in Britton–Robinson and Sörensen I buffer are in accordance but the solubility in Sörensen II buffer deviates significantly from previous ones. The ionic strength of BR and Sö I buffers is almost identical, while Sö II buffer has four times higher ionic strength. This fact and the possible specific interaction between solvent and the citrate component of this buffer may be the reason of the higher solubility of the compounds in this medium.

3.2. Amount of solid excess

The second parameter studied was the amount of solid excess in the saturated solution. The presence of solid excess in the solution forming a heterogeneous system is necessary to reach equilibrium. This question has been nicely studied in a paper of Kawakami et al. [29], where the impact of solid excess on the apparent solubility was described. They concluded that the apparent solubility was affected by the amount of the solid excess most likely due to a competition between the crystallization and dissolution rates. However, in the literature, there do not appear to be unambiguous guidelines stating how much solid material has to be used in the shake-flask method in measuring equilibrium solubility thus we examined this aspect. In this study, the amount of solid weighed was from 4 to 20 mg by 4 mg steps. The results did not show significant differences (Fig. 1). Consequently the equilibrium solubility does not appear to depend on the amount of solid excess in the solution. This finding is not in contradiction with results of Higuchi et al. [12a] who reported that "the final rate of approach to saturation is approximately directly proportional to the excess of solid present—the larger the excess, the faster the saturation rate. A slightly soluble solid of equivalent surface area would require many times greater excess for saturation than would be needed for a moderately soluble solid". We recommend using only a small (but sufficient) excess (for example 5–10 mg/5 ml) to avoid difficulties in sampling.

3.3. Temperature

The dependence of solubility on temperature is well known, so the measurements have to be carried out at thoroughly controlled, constant temperature. In this section the solubility of hydrochlorothiazide is compared at three different temperature values: 15, 25 and 37 °C. The results are summarized in Table 2.

Most drugs have an endothermic dissolution process, so the solubility increases with temperature rise. Hydrochlorothiazide belongs to this type of compound since its *S* value increases significantly between 15 and 37 °C. In this study, the solubility at body temperature (37 °C) was found to be almost double the solubility at room temperature (25 °C).

The fact that most compounds have higher solubility at body temperature than at 25 °C may be an advantage in drug design, because better bioavailability can be expected. This shows the usefulness and supports the need for solubility measurement at biomimetic temperature as well as at room temperature.

3.4. Time of stirring

In the shake-flask solubility method, the achievement of equilibrium consists of two important but different parts: vigorous agitation of the phases (e.g. by stirring), and sedimentation. To discover which of these parts plays a higher role in formation of equilibrium, the time of stirring and the time of sedimentation were independently varied.

First, the time of stirring was varied between 30 min and 48 h. As shown in Fig. 2, the measured solubility of hydrochlorothiazide increases with increasing stirring-time. Values obtained after stirring for 2 h or less were lower than values measured by the standard protocol. However, there are no significant differences in the solubility results obtained after stirring for 6 h or more. This suggests that 48 h of stirring time is not required for the measurement of solubility of hydrochlorothiazide, and that a shorter stirring time may considerably reduce the whole time of the shake-flask method. We have to note however, there may be compounds particularly those are sparingly soluble in water for which longer stirring time is necessary for equilibrium. So in the most rigorous application of the shake-flask method, solubility would be measured after checking the required equilibration time from compound to compound. However, the results of this study suggest that it is reasonable to start with 6 h of stirring time.

3.5. Time of sedimentation

The time of sedimentation was varied between 1 and 24 h. The results are illustrated in Fig. 3. The outcome of this test was somewhat surprising. The solubility values of hydrochlorothiazide were higher in the 1–8 h interval, with a maximum at 6 h of sedimentation. Despite the 48 h stirring, the time of sedimentation significantly affects the *S* values, and longer sedimentation times appear to be required for accurate measurement. This suggests that the time of sedimentation plays a greater role in development of thermodynamic equilibrium than the time of stirring.

3.6. Alternative techniques for separation of solid and liquid phases

The technique of phase-separation is a key part of the shake-flask method. After stirring, the two phases (solution and solid material) of the saturated solution have to be separated before aliquots of supernatant can be taken out for concentration measurement by UV spectrophotometry. The aliquots taken out must be completely transparent, and free of any solid particles.

Rather than waiting for sedimentation to occur naturally, samples can be separated immediately after stirring by centrifugation or filtration. In this study, 12 samples were centrifuged at 2000 rpm for 10 min at 25 ± 0.1 °C, while 12 samples were filtered through 0.45 μm membrane filters. This pore size of filter widely used in the literature for analytical purposes was found suitable by us to obtain clear filtrate without any retention of the solute.

According to the two-sample *t*-test the obtained solubility results (columns 3–4 in Table 2) are significantly different. The highest deviation is caused by filtration. Presumably, this is because the filtration was executed directly after stirring, followed by concentration measurement directly after separation. During the stirring time a supersaturated solution may be formed. Consequently, if samples are not allowed to

Table 4
The solubility of compounds measured by the standard and the new protocols

Compound	MW	μg/ml	Solubility, S (M)	log *S* (M)	Solubility, S (μM)	log *S* (μM)	*n*
Standard protocol							
Hydrochlorothiazide	297.7	556 ± 13.2	0.001868	−2.73	1867	3.27	18
Furosemide	330.8	20.4 ± 2	0.000062	−4.21	61.7	1.79	8
Nitrofurantoin	238.2	109.5 ± 3	0.000460	−3.34	460	2.66	8
Piroxicam	331.4	5.95 ± 0.4	0.000018	−4.75	17.9	1.25	2
Quinine-HCl	360.4	201 ± 10	0.000558	−3.25	558	2.75	6
Trazodone	371.4	138 ± 10	0.000372	−3.43	370	2.57	6
New protocol							
Hydrochlorothiazide	297.7	571 ± 8.6	0.001918	−2.72	1918	3.28	12
Furosemide	330.8	18.7 ± 1.2	0.000057	−4.25	56.4	1.75	8
Nitrofurantoin	238.2	99 ± 4.1	0.000416	−3.38	416	2.62	8
Piroxicam	331.4	6.36 ± 0.04	0.000019	−4.72	19.2	1.28	3
Quinine-HCl	360.4	285 ± 30	0.000791	−3.10	717	2.86	5
Trazodone	371.4	176 ± 1.8	0.000474	−3.32	473	2.67	12

BR buffer used for all samples except furosemide, which was measured in 0.01 M HCl solution. The pH values of the measurements are shown in Table 2.

stand after stirring (as they are in the sedimentation technique), solubility will be higher than at the real thermodynamic equilibrium. Moreover, it is possible that suspended particles smaller than the filter pore size will not be separated, leading to falsely high concentrations measured in the filtrate. We do not recommend the usage of filtration since it strongly perturbs the heterogeneous system and finally eliminates it. Sedimentation is considered to be the safest technique for separation of phases. However, solutions sometimes fail to clarify, for example with compounds that form micelles or aggregates and produce opalescent solutions; for such samples, the separation of phases can only be carried out by centrifugation.

3.7. New shake-flask protocol

Based on the results above we developed a new protocol for measurements of equilibrium solubility taking less than 36 h. The standard 48 h of stirring time was reduced to 6 h and the sedimentation time from 24 to 18 h. Other parameters were kept as in the standard protocol.

The equilibrium solubilities of hydrochlorothiazide and five other drugs were measured according to both protocols. Table 4 shows that results are in excellent agreement for four of the drugs, while two compounds (quinine-hydrochloride and trazodone) exhibit slightly greater (but still acceptable) differences.

4. Conclusions

This paper describes a comprehensive and systematic study on equilibrium solubility measurement by the saturation shake-flask method using hydrochlorothiazide as model compound and five drugs as test set. Based on 135 separate measurements, the main factors influencing the solubility results have been revealed.

Parameters like temperature, sedimentation time, type of aqueous buffer, and technique of phase separation strongly influenced the equilibrium solubility results. On the other hand, the amount of solid excess did not appear to influence the results significantly.

We recommend – according to our experiences – to apply the following experimental conditions in a new protocol for the saturation shake-flask measurement of aqueous solubility:

- the measurements must be carried out at controlled, standard temperature
- Sörensen phosphate buffer can be used between pH 3–7; Britton–Robinson buffer solution can be used between pH 2.5–11.5; HCl of appropriate concentration can be used below pH 2.5
- to avoid difficulties in sampling, only a small excess (∼5–10 mg/5 ml) of solid should be present
- a minimum of 24 h is necessary to reach the thermodynamic equilibrium; this time should consist of 6 h of stirring plus 18 h of sedimentation; but in case of very sparingly soluble compounds longer stirring time may be necessary for equilibrium, so in the most rigorous application of the shake-flask method, solubility would be measured after checking the required equilibration time from compound to compound
- the safest technique of phase separation is sedimentation, which assures the existence of a heterogeneous system until equilibrium has been achieved.

If the parameters above are strictly kept, the experimental error of the solubility measurements can be reduced to about 4%.

With this new protocol for the saturation shake-flask method, the log S value of a compound can be measured in less than one and a half days.

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