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# Equilibrium drug solubility measurements in 96-well plates reveal similar drug solubilities in phosphate buffer pH 6.8 and human intestinal fluid

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ABSTRACT

This study was conducted to develop a high throughput screening (HTS) method for the assessment of equilibrium solubility of drugs. Solid-state compounds were precipitated from methanol in 96-well plates, in order to eliminate the effect of co-solvent. Solubility of twenty model drugs was analyzed in water and aqueous solutions (pH 1.2 and 6.8) in 96-well plates and in shake-flasks (UV detection). The results obtained with the 96-well plate method correlated well ($R^2$ = 0.93) between the shake-flask and 96-well plates over the wide concentration scale of 0.002–169.2 mg/ml. Thereafter, the solubility tests in 96-well plates were performed using fasted state human intestinal fluid (HIF) from duodenum of healthy volunteers. The values of solubility were similar in phosphate buffer solution (pH 6.8) and HIF over the solubility range of $10^2$–$10^5$ μg/ml. The new 96-well plate method is useful for the screening of equilibrium drug solubility during the drug discovery process and it also allows the use of human intestinal fluid in solubility screening.



## 1. Introduction

Inadequate drug solubility in intestinal fluids may limit the oral bioavailability of drugs, and even 40% of the newly developed lead compounds are poorly water-soluble (Lipinski, 2002). Drug solubility can be measured either as time dependent kinetic solubility or thermodynamic equilibrium solubility. In kinetic solubility measurements the compound is usually dissolved in a pre-solvent (e.g. dimethyl sulfoxide) and small volumes of the aqueous medium are added until solubility limit is reached. Drug precipitation is followed by turbidimetric titration, nephelometry or UV-absorbance (Lipinski et al., 1997; Bevan and Lloyd, 2000; Pan et al., 2001). The thermodynamic equilibrium solubility is usually studied using the shake-flask method, but these experiments may take days or even weeks (Lipinski, 2003).

High throughput screening (HTS) of kinetic solubility is used in drug discovery, but the use of co-solvents may lead to overestimation of solubility values (Pan et al., 2001; Chen et al., 2002b; Takano et al., 2006; Bard et al., 2008; Dai et al., 2008; Alelyunas et al., 2009). Recently, Alelyunas et al. (2009) determined the equilibrium solubility in 96-well plates using solid drug as a starting material. The drug concentrations were determined by HPLC, which is not optimal for HTS systems. Fast and reliable solubility methods based on plate reader assays are needed for drug solubility measurements.

Various media have been used for the dissolution determinations. Simple buffers (pH 1.2 and pH 6.8) are used most frequently, but also more complex simulated intestinal fluids, like FaSSIF (fasted-state simulated intestinal fluid) and FeSSIF (fed-state simulated intestinal fluid), have been introduced (Galia et al., 1998). Still, the human intestinal fluid (HIF) is the most realistic solubility medium. Aspirated HIF has been used in some dissolution and solubility studies (Pedersen et al., 2000a,b; Persson et al., 2006; Clarysse et al., 2009), but the use of HIF is limited by the sparse availability of the fluids. Therefore, use of HIF in drug discovery would require a miniaturized solubility platform. For these reasons, HIF and other media have not been compared in broad scale and, thus, the true predictive value of the substitute media has not been defined. The aims of this study were to develop 96-well plate method for the thermodynamic solubility measurements and use this method to compare drug solubility in simple buffers and aspirated human duodenal fluid.

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**Table 1**
Model drugs. Drug properties and experimental conditions are presented. p$K_a$-values were obtained with ACD LabSystem Software (Advanced Chemistry Development Inc., Canada).

| Model compound | BCS class | Drug concentration in the stock solution | Volume pipetted into the 96-well plate (μl) | Absorption maximum (nm) | p$K_a$ | Acidic/basic |
|---|---|---|---|---|---|---|
| Antipyrine | I | 177.5 mg/ml in MeOH | 250.0 | 266 | 0.7 | B |
| Atenolol | III | 40.0 mg/ml in MeOH | 187.5 | 275 | 9.16 | B |
| Caffeine | I | 15.0 mg/ml in water | 875.0 | 275 | 0.73 | B |
| Carbamazepine | II | 1.0 mg/ml in MeOH | 62.5 | 285 | 13.94 | A |
| Diclofenac sodium | II | 40.0 mg/ml in MeOH | 125.0 | 254 | 4.18 | A |
| Furosemide | IV | 9.0 mg/ml in MeOH | 62.5 | 271 | 3.04 | A |
| Hydrochlorothiazide | III | 4.0 mg/ml in MeOH | 62.5 | 254 | 8.95 | A |
| Ibuprofen | II | 100.0 mg/ml in MeOH | 62.5 | 254 | 4.41 | A |
| Indomethacin | II | 16.0 mg/ml in MeOH | 62.5 | 254 | 3.93 | A |
| Ketoprofen | II | 100.0 mg/ml in MeOH | 62.5 | 258 | 4.23 | A |
| Metformin HCl | III | 200.0 mg/ml in water | 500.0 | 250 | 13.86 | B |
| Metoprolol tartrate | I | 100.0 mg/ml in MeOH | 500.0 | 254 | 9.17 | B |
| Naproxen | II | 2.0 mg/ml in MeOH | 62.5 | 254 | 4.84 | A |
| Phenytoin sodium | II | 2.0 mg/ml in MeOH | 62.5 | 250 | 8.33 | A |
| Piroxicam | II | 1.0 mg/ml in MeOH | 62.5 | 254 | 3.80,4.50, 13.33 | B, A, A |
| Propranolol HCl | I | 200.0 mg/ml in MeOH | 250.0 | 290 | 9.14 | B |
| Ranitidine HCl | III | 267.5 mg/ml in MeOH | 125.0 | 322 | 8.40 | B |
| Rifampicin | II | 150.0 mg/ml in MeOH | 250.0 | 254 | 9.92 | A |
| Spironolactone | IV | 3.0 mg/ml in MeOH | 62.5 | 254 | N.A. | N.A. |
| Trimethoprim | II | 10.0 mg/ml in MeOH | 62.5 | 254 | 7.2 | B |

## 2. Materials and methods

### 2.1. Materials

The model compounds (Table 1) were from Orion Pharma (Finland) except for antipyrine (Aldrich Chemical Company Inc., USA), diclofenac sodium (Sigma–Aldrich Chemie GmbH, Germany), indomethacin (Fluka Biochemika, Italy) and piroxicam (Hawkins Inc., USA). Methanol (Riedel-de Haën, Germany) was used as a pre-solvent in the stock solutions before pipetting the drugs into the 96-well plates for the HTS method.

The media at pH 1.2 (0.2 M aqueous hydrochloric acid solution) and pH 6.8 (0.2 M phosphate buffer solution) were prepared with potassium chloride (Riedel-de Haën, Germany), hydrochloric acid (Riedel de-Haën, Germany), potassium dihydrogen phosphate (Sigma–Aldrich Laborchemikalien GmbH, Germany) and sodium hydroxide (Tamro, Finland) (USP, 2007). Ultrapure ion exchanged water was used in all studies (Millipore, France).

### 2.2. Collection of human intestinal fluid (HIF)

HIF was collected at the University Hospitals Leuven, Belgium, with approval by the Committee of Medical Ethics (ML3242). The HIF was collected from five healthy volunteers (three females and two males, aged 24–39 years, body mass index $21.6 \pm 0.8\ kg/m^2$) after an overnight fast by a double-lumen catheter (Salem Sump Tube 14 Ch, external diameter 4.7 mm, Sherwood Medical, Petit Rechain, Belgium) from the duodenum (D2/D3) (Fritsch and Kuehnel, 2008). The position of the catheter was confirmed with fluoroscopy. The double-lumen catheter allowed the collection of intestinal fluids by means of a syringe without generation of underpressure in the gastrointestinal tract. Upon positioning the catheters, the volunteers were given 250 ml of water. Sampling of HIF was initiated 15 min after water ingestion and then continued for 3 h. HIF was collected every 15 min and kept on ice in closed tubes.

After the sampling period, all 15-min fractions from one volunteer were pooled and centrifuged at 4000 rpm and 4 °C for 20 min. The pH ($6.24 \pm 0.54$) (Hamilton Slimtrode, Bonaduz, Switzerland) and osmolality ($205 \pm 15\ mOsm\ kg^{-1}$) (Advanced Osmometer 3250, Advanced Instruments, Norwood, MS) were measured from the individually pooled HIF before storing them at −30 °C or colder until further use. The total sample volume from each individual varied from 35 ml to 100 ml, resulting in a total volume of 345 ml. Prior to the solubility studies all the individual HIF samples were pooled together.

### 2.3. 96-Well plate method for equilibrium solubility

The model drugs were first dissolved in methanol or water to prepare stock solutions (Table 1). The stock solutions were pipetted into the 96-well plates (96F untreated 260895, Nunc, Denmark) (Table 1) and the solvent was evaporated. This ensured that the drug was in solid form in the beginning of the experiment. Thereafter, 250 μl of the dissolution medium (water, aqueous buffer solution at pH 1.2 or pH 6.8, HIF) was added to the wells and the 96-well plates were shaken horizontally at 600 rpm (Heidolph Titramax 100, Germany). The tests were carried out at room temperature ($23 \pm 1$ °C) and the plates were protected from light. The plates were centrifuged at 3000 rpm for 15 min (Eppendorf AG, Germany). Samples of 200 μl were withdrawn after 30 min, 1, 2, 5, 24 and 30 h and pipetted into UV 96-well plates (UV FB microtiter Plates 8404, Thermo Fisher, USA) for analyses with plate reader at λ = 230–400 nm (Varioskan, Thermo Electron Corporation, Finland). The analyses were performed in triplicate for each compound.

### 2.4. Shake-flask method for drug solubility testing

The shake-flask method was used as a reference assay (Lipinski, 2003). Excess of the model compounds was weighed into 40 ml glass vials, and the medium (water, aqueous buffer solution at pH 1.2 or pH 6.8) was added. The vial contents were mixed with a magnetic stirrer at 100 rpm. Also these tests were performed at room temperature. Samples were taken at 1, 2, 3, 4, 5, 24 and 30 h until the equilibrium plateau was reached. The samples were filtered (0.45 μm, Minisart, Sartorius, UK) before the analyses using UV-spectrophotometer (Pharmacia LKB, Ultrospec, Sweden). The wavelengths for detection are shown in Table 1. These determinations were performed in duplicate.

### 2.5. Analysis of bile salts and phospholipids

Bile salts of the HIF were measured by GC–MS-selected ion monitoring analysis (Lütjohann et al., 2004). After alkaline hydrolysis

and acidification, free bile acids were extracted with diethylether and, thereafter, trimethylsilylated for GC–MS-selected ion monitoring analysis. The chromatographic separation was performed with a DB-XLB capillary column at constant helium flow of 0.8 ml/min with a final temperature of 290 °C. Total phospholipid levels were determined enzymatically according to Gurantz et al. (1981), using phospholipase D and choline oxidase. The reagent kit was from Wako Chemicals (Neuss, Germany).

### 2.6. HPLC method

The accuracy of the UV spectrophotometric determinations was checked for selected compounds (ibuprofen, indomethacin, phenytoin sodium) using HPLC (Agilent 1100 series, Agilent Technologies, Germany). The HPLC analyses were performed using a reversed-phase C8 column for ibuprofen (Zorbax Eclipse XDB 150 mm × 4.6 mm, Agilent Technologies, Germany) with pre-column (LiChroCART 4-4 RP-8e, LiChrospher, Germany) and C18 column for indomethacin and phenytoin sodium (Luna 3 μ 100 Å 150 mm × 4.6 mm, Phenomex, USA) with pre-column (C18, Phenomex, USA). Aqueous trifluoroacetic acid (0.1%, pH 1.2) solution in acetonitrile was used as the mobile phase for ibuprofen. Aqueous phosphoric acid (0.2%, pH 2.0) solution in acetonitrile was the mobile phase for the other compounds. These studies were performed at room temperature using flow-rates of 1.0 ml/min or 1.5 ml/min. Multiple wavelength detector (Agilent 1100, Agilent Technologies, Germany) was used to analyze the samples and the wavelengths were taken from the USP 2007. Injection volumes were 10.0–20.0 μl and the determinations were done in triplicate.

### 2.7. XRPD

X-ray diffraction patterns were measured using an X-ray powder diffraction theta–theta diffractometer (Bruker AXS D8 advance, Bruker AXS GmbH, Germany), in order to study the solid state forms of the model compounds and to confirm possible polymorphic changes. The XRPD experiments were performed in symmetrical reflection mode with Cu$K_\alpha$ radiation (1.54 Å) using Göbel Mirror bent gradient multilayer optics. The scattered intensities were measured with a scintillation counter. The angular range was 5–30°, steps of 0.1° were used and each step was measured for 20 s. The reference codes to identify the experimental polymorphs were taken from CSD database (Cambridge Structural Database, The Cambridge Crystallographic Data Centre, UK) (Allen, 2002).

### 2.8. DSC

Differential scanning calorimetry (DSC) was used to confirm the XRPD results. The thermal analyses were performed with a Mettler DSC 823e (Mettler-Toledo AG, Switzerland) and analyzed by STAR$^e$ Software (Mettler-Toledo AG, Switzerland). Samples of 4–5 mg were heated at a rate of 10 °C/min from 25 °C to 10 °C above the melting point of each compound under nitrogen atmosphere. The calibration of the DSC was performed with indium.

## 3. Results and discussion

### 3.1. Compound selection

The 20 model compounds were selected to cover a broad range of physicochemical parameters: the range of computed (ACD LabSystem) log *P* values was −2.31 to 4.06 and the polar surface areas were 23.55–220.15 Å$^2$. The compounds also represented all four BCS classes (Table 1).

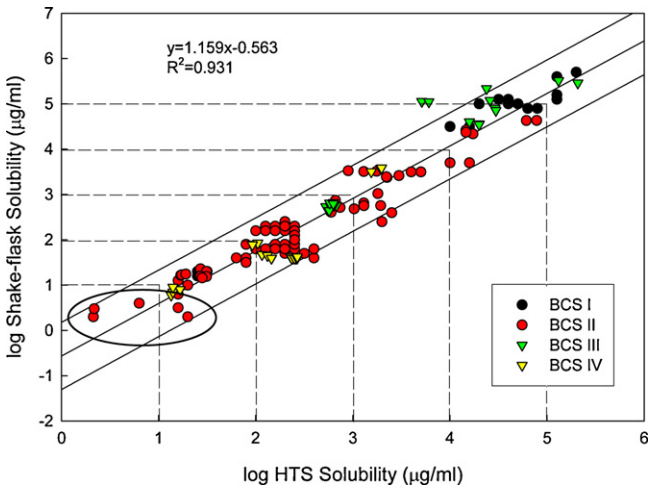


**Fig. 1.** The correlation between the shake-flask (*y*-axis) and the 96-well plate (*x*-axis) solubility results in aqueous media (pH 1.2 and pH 6.8 buffer solutions, purified water). The predictive confidence intervals in the figure are 95%.

### 3.2. Equilibrium solubility determinations

In the shake-flask and 96-well plate systems, equilibrium solubility was usually reached within 5 h (data not shown). Overall, the 96-well plate solubility results correlated well ($R^2$ = 0.931) with the data from the shake-flask method over a wide (five orders of magnitude) aqueous solubility range (Fig. 1). The relative deviation of the 96-well plate results was less than 2-fold for 7 model compounds, 2–5-fold for 10 model compounds and over 5-fold for 3 model compounds, but always less than 7-fold. Relative overestimation by the 96-well method was the most significant for compounds with solubility values lower than 10 μg/ml (for example BCS class II drugs in aqueous solution at pH 1.2; circled area in Fig. 1). Altogether, the 96-well plate method provides adequate estimates for the water solubility in drug development.

In the study by Alelyunas et al. (2009), the 96-well plate method gave higher values for some drugs compared to the reference method, but solubilities of more than 80% of the drugs were reliably estimated in the 96-well system. Previous kinetic HTS solubility methods have overestimated the water solubilities (Pan et al., 2001; Chen et al., 2002b; Bard et al., 2008; Heikkilä et al., 2008). In this equilibrium drug solubility study, the 96-well plate method gave reliable solubility profiling: 98% of the tests gave reliable results within the predictive confidence intervals of 95%.

The 96-well plate method gave similar values with the traditional shake-flask method when pH of the buffer was changed. The pH change caused even 10,000-fold changes in drug solubility, but the correlation between the HTS and shake-flask methods remained good. For example, the solubility of diclofenac sodium at pH 1.2 was 0.002 mg/ml and at pH 6.8 15.3 mg/ml using the 96-well plate method; the corresponding values obtained by the shake-flask method were 0.003 mg/ml and 24.0 mg/ml, respectively.

Accurate determination of drug solubility over a wide solubility scale (0.002–169.2 mg/ml) is a true advantage of the newly developed 96-well plate method: compounds with vastly different solubility properties can be determined with the same method using small drug quantities. This is important in the early drug discovery when only small quantities of compounds are available.

Methanol is suitable solvent for the 96-well method. Methanol can be easily evaporated and it is compatible with the 96-well plates (unlike chloroform and acetone). Only two drugs (caffeine, metformin) were not soluble in methanol, and they had to be dissolved in water. This is not a major problem, because such drugs have high water solubility, and their absorption may not be limited

**Table 2**
Results from the XRPD (polymorphic form) and DSC (melting temperatures) measurements ($n = 3$). Literature values for diclofenac sodium are by Llinas et al. (2007), for indomethacin by Chen et al. (2002a) and for piroxicam by Vrecer et al. (2003).

| Drug | Physical form according to XRPD | Melting points from DSC measurements | Literature |
|---|---|---|---|
| Diclofenac sodium | | | |
| Starting material | I, III | 288.3 °C | II = 180.5 °C |
| Starting material (HTS) | II, III | 285.5 °C | |
| Indomethacin | | | |
| Starting material | I (γ) | 160.0 °C | I (γ) = 160–161 °C |
| Starting material (HTS) | II (α), I (γ) | 154.0 °C, 160.5 °C | II (α) = 152–154 °C |
| Piroxicam | | | |
| Starting material | I (α), II (β) | 202.4 °C | I (α) = 202.6 °C |
| Starting material (HTS) | Monohydrate | 201.5 °C | II (β) = 199.7 °C monohydr. = 202.0 °C |

**Table 3**
Mean HIF composition from five volunteers.

| | Bile salts (mM) | Phospholipids (mM) | Cholesterol (mM) | Deoxycholate (%) | Cholate (%) | Chenodeoxycholate (%) | Ursodeoxycholate (%) |
|---|---|---|---|---|---|---|---|
| Mean | 3.64 | 1.81 | 1.80 | 14.92 | 57.21 | 24.11 | 3.74 |
| ± S.D. | 0.42 | 0.16 | 0.21 | 3.31 | 5.70 | 1.73 | 0.75 |

by the solubility. Incomplete evaporation of methanol disturbed the test in one case (metoprolol). Complete evaporation of the solvent is necessary in the solubility tests with solid drug materials.

### 3.3. Polymorphic changes

During evaporation the stock solvent may cause (pseudo) polymorphic changes in the physical structure of the drug precipitate. Among the 20 test compounds four cases of polymorphism were seen in 96-well plate method: piroxicam, indomethacin, diclofenac, and spironolactone. In shake flask method one case of polymorphism (piroxicam) was seen. The changes were confirmed by XRPD and DSC (Table 2).

Possible polymorphic changes of drug candidates may influence the results of equilibrium solubility screening. Later the polymorphic changes may have an impact in the drug development (e.g. formulation, stability, bioavailability) (Bauer et al., 2001).

Thus, the polymorphic changes could be detected early by combining the 96-well plate dissolution tests and analysis of the polymorphism.

### 3.4. Composition of human intestinal fluid (HIF)

The mean of the total bile salt concentration in HIF was 3.64 mM. This is in line with the earlier studies: 2.9 mM (Lindahl et al., 1997), 2.0 mM (Persson et al., 2005), 2.6 mM (Clarysse et al., 2009), 3.06 mM (Moreno de la cruz et al., 2006) and 3.5 mM (Brouwers et al., 2006). The phospholipid concentration (1.8 mM) was higher than in the earlier studies (0.2–0.6 mM) (Persson et al., 2005; Clarysse et al., 2009) because one sample had very high phospholipid content. These values vary between individuals and concentrations of up to 2.7 mM have been found in fasted upper small intestine. The mean cholesterol level of HIF was 1.8 mM, which is close to the level found by Mansbach et al. (1975) (1.5 mM).

The lipid composition of HIF varies between individuals (inter-individual variation) and in the same individuals at different times (intra-individual variation) (Lindahl et al., 1997; Clarysse et al., 2009). Inter-individual variation was also observed in this study (Table 3). The mean pH and osmolality were in line with previously published results. The mean pH was 6.24. Previously pH values of 6.5–7.5 have been reported (Lindahl et al., 1997; Persson et al., 2005; Moreno de la cruz et al., 2006; Clarysse et al., 2009). The mean osmolality was 205 mOsm $kg^{-1}$, which is in line with previous values of 137–224 mOsm $kg^{-1}$ (Moreno de la cruz et al., 2006; Clarysse et al., 2009).

### 3.5. Drug solubility in human intestinal fluid (HIF)

Equilibrium drug solubility tests in the 96-well plates were also performed using the fasted state HIF (pH 6.24) for 13 model drugs, mainly from the BCS class II, and the results were compared to the HTS solubility results in the aqueous buffer solution at pH 6.8 (Fig. 2). The results showed a fair correlation ($R^2 = 0.630$). More importantly, the absolute values of drug solubility differed only modestly (less than 6.9-fold in all cases) even though 1000-fold solubility range was studied. Slightly increased solubility values in HIF compared to the pH 6.8 buffer were seen for piroxicam, naproxen, rifampicin and diclofenac sodium. Vice versa, the solubility in HIF was lower than in buffer pH 6.8 for ibuprofen and ketoprofen.

No major differences were seen between the equilibrium solubility values in the buffer at pH 6.8 and HIF, which suggests that the aqueous buffer at pH 6.8 is an adequate solubility test medium for drug discovery at surprisingly wide range solubility (100 μg/ml to 100 mg/ml). However, we cannot rule out the possibility that more

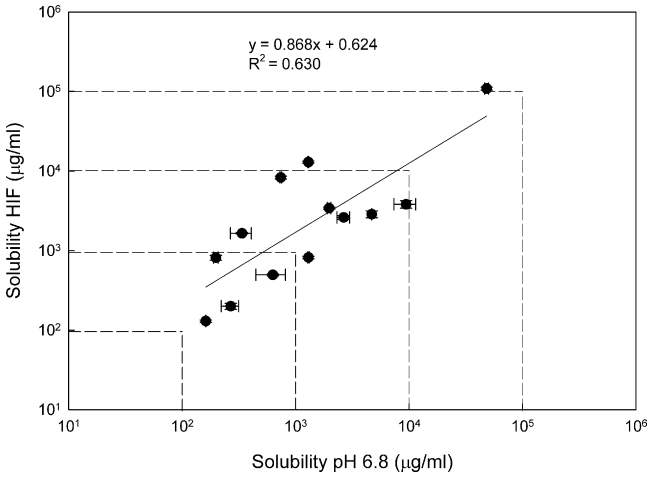


**Fig. 2.** The 96-well plate equilibrium solubility results in HIF and aqueous buffer at pH 6.8. The mean solubility values of 3 experiments are presented on a logarithmic scale.

significant solubility differences would be seen for compounds with aqueous solubility below 100 μg/ml. For example, preliminary solubility test of beclomethasone dipropionate, showed 150 times higher solubility in HIF (0.6 μg/ml) than in the buffer at pH 6.8 (0.004 μg/ml). Obviously, more detailed studies are needed to determine the limits and utilization potential of HIF in drug solubility and dissolution testing, especially in the case of the compounds with very low solubility. It is worth noting, however, that the simple buffer is useful medium over a wide solubility range that extends at least to $10^2$ μg/ml.

### 3.6. Dissolution testing

The 96-well plate method introduced here is applicable also to the dissolution rate testing with small quantities of compound. In that case, concentration determinations are carried out at different time points. Dissolution testing is done typically later in drug discovery and development than the solubility tests. Furthermore, particle size influences the dissolution rates, and thus it must be well-controlled.

## 4. Conclusions

In this study, 96-well plate method was developed for measuring the equilibrium solubility of compounds during drug discovery and development. The small volumes in this method enable utilization of the most relevant medium (human intestinal fluid) as a solubility medium. Buffer at pH 6.8 is a good substitute for HIF in the solubility range of 100 μg/ml to 100 mg/ml. This method is useful for the solubility tests with small quantity, but large number, of compounds.

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