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Use of ^{1}H NMR to facilitate solubility measurement for drug discovery compounds

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ABSTRACT

The use of ^{1}H NMR experiments to determine the solubility of potential drug candidates using a panel of solubilizing agents is proposed as an alternative to an HPLC-UV method. The advantages of using this approach will be discussed and results comparing the two methodologies will be presented. This effort highlights the importance of a simple method for determining a suitable formulation for discovery compounds for studies using a minimal amount of material in support of early *in vivo* studies.

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1. Introduction

The aqueous solubility of potential drug candidates is an important physicochemical property, which can significantly impact the bioavailability of a compound. Drugs that demonstrate good bioavailability typically have adequate aqueous solubility and permeability. It has been recognized that evaluation of drug solubility early in the drug discovery process is important for drug candidate assessment. In addition, early solubility evaluation can provide valuable information on the reliability of *in vitro* results of bioassays for poorly soluble compounds. Since the introduction of automated screening technologies into the drug discovery process, drug leads have been produced at an accelerating rate. As a result, new methods have emerged to accommodate the increased demand for physicochemical screening including the rapid measurement of compound solubility. Thus far, much attention has been focused on high-throughput solubility screens (Lipinski et al., 2001; Dehring et al., 2004; Chen and Venkatesh, 2004) to support *in vitro* biological assays, which are conducted at micromolar concentrations. The development of high-throughput solubility assays for samples already dissolved in DMSO utilizing UV plate readers for quantitation as well as nephelometric methods to determine the solubility of compounds have been shown to be quite valuable for measuring samples at relatively low concentrations, but suffer from dynamic range issues and interference from absorption of the vehicle components at higher concentrations (Pan et al., 2001). The solubility results obtained from these high-throughput assays may not be suitable for guiding the design of pharmacokinetic and pharmacologic studies in drug discovery.

Early *in vivo* dosing in drug discovery has many challenges. One of these is the low aqueous solubility of many small molecule drug candidates (Sun et al., 2004). *In vivo* dosing is typically done at much higher concentrations than *in vitro* bioassays, and some formulation efforts are usually required to achieve the desired outcome. Unfortunately, extensive formulation development for early discovery compounds is not practical due to resource and time limitations, as well as limited compound availability. The ability to choose a suitable formulation in a short time period with a minimal amount of compound is, therefore, highly desirable in the drug discovery setting. For this reason, a convenient method to rapidly evaluate a useful *in vivo* solubility range of drug discovery compounds rather than determining the aqueous solubility limit prior to dosing studies utilizing a panel of formulations is required.

Traditionally, solubility measurements from aqueous suspensions are performed using a shake flask method, which is conducted by introducing compounds into the desired media, equilibrating the compound for a sufficient time period, then separating the phases (Avdeef et al., 2000; Avdeef and Berger, 2001). The concentrations of the aqueous soluble solutions are usually evaluated by high performance liquid chromatography with UV detection (HPLC-UV). The

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Table 1
Solutions used for solubility measurement.

Formulation	Compound class
2% polysorbate 80	Acid, base, neutral
2% polysorbate 80, 10% Solutol HS15	Neutral
10% Solutol HS15	Neutral
2% polysorbate 80, 10 mM acetic acid	Basic
10 mM acetic acid	Basic
2% polysorbate 80, 10 mM sodium bicarbonate	Acidic
10 mM sodium bicarbonate	Acidic

HPLC measurement is relatively slow, often requiring long runs as well as the production of a calibration curve for quantitation. With an ever-increasing number of compounds generated for *in vivo* experiments, there is a need to develop a faster method for solubility measurement.

In this paper we describe an NMR-based method, which facilitates solubility determination in simple formulations. Twelve commercially available drug-like compounds and nine Wyeth compounds, including acids, bases and neutral compounds were tested using both the NMR method and the traditional HPLC-UV method to compare the two. The results from the comparison of these two methods are discussed.

2. Materials and methods

Chemicals: 3-(Trimethylsilyl)propionic-2,2,3,3-d_4 acid, sodium salt (TSP) was purchased from Sigma–Aldrich Co. (St. Louis, MO). Deuterium oxide (D_2O, 99% D, Cambridge Isotope Laboratories, Andover, MA), deuterated acetic acid (Aldrich), polysorbate 80 (Spectrum Chemicals), Solutol HS15 (BASF) and sodium bicarbonate (JT Baker), and de-ionized water (Millipore filtration, in-house) were used in the preparation of the formulation vehicles. The following chemicals were purchased from Sigma–Aldrich Co. (St. Louis, MO) for solubility measurement and were used as received: gemfibrozil, bendroflumethiazide, acetazolamide, ketoprofen, phenyl salicylate, 2-(4-hydroxyphenylazo)benzoic acid (HPBA), 4-(4-methylphenoxy)benzoic acid (MPBA), salicylic acid, benzocaine, tamoxifen, trimethoprim, carbamazepine, prednisolone, griseofulvin, and triamcinolone.

Internal Wyeth Research compounds (>98% purity) (Compounds 1–9) were also used for solubility measurement.

2.1. Formulation vehicle preparation

Seven aqueous formulations were used in the study: (1) 2% Tween 80, (2) 10% Solutol, (3) 2% Tween 80/10% Solutol, (4) 10 mM sodium bicarbonate, (5) 2% Tween 80/10 mM sodium bicarbonate, (6) 10 mM acetic acid, (7) 2% Tween 80/10 mM acetic acid. The vehicles were prepared by weighing the ingredients into a volumetric flask and completing the volume with deuterium oxide or de-ionized water.

2.2. Solubility sample preparation

Approximately 15 mg of each compound was weighed into 4 mL scintillation vials. Three milliliters of the appropriate formulation vehicle was then added and the sample vortexed. Samples were placed on an end-over-end rotator and equilibrated for 2 days. The pH was measured from the suspensions at the end of the equilibration period. For analysis, the suspensions were filtered using 0.22 μm Millipore PVDF syringe filters. After discarding the first 5 drops, each filtrate was collected and a portion appropriately diluted for HPLC analysis with an acetonitrile-buffer solution. The remaining portion was subjected to NMR analysis.

2.3. HPLC analysis

A PerkinElmer Series 200 HPLC equipped with a vacuum degasser, column oven, autosampler, and photodiode array detector was used for the analysis. The column used for the Wyeth research compounds was a Waters 100 mm × 4.6 mm, C18 SymmetryShield with 3.5 μm particle size maintained at 30 °C, while a Waters 100 mm × 4.6 mm, C18 Xterra with 3.5 um particle size, also maintained at 30 °C, was used for the other test compounds. A linear gradient was used for all compounds and run from 5% to 95% acetonitrile in 10 or 30 min with a 1 mL/min flow rate. An aqueous phase of 0.05% TFA was used for neutral and basic compounds, a 0.1% ammonium acetate solution was used for acidic compounds. The autosampler injection volume was 5 μL. Optimal detection

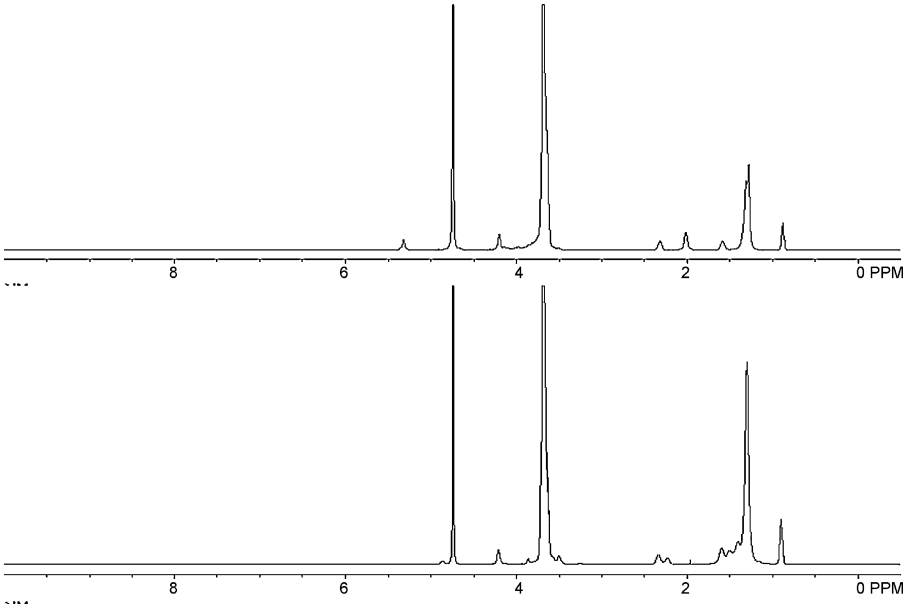

Fig. 1. NMR vs. HPLC solubility data for the commercial compounds listed in Table 2.

wavelengths were identified for each compound from their UV-spectra. Solution concentrations were determined by comparison with standards of known concentration.

2.4. NMR analysis

NMR experiments were conducted on a Bruker DPX400 MHz spectrometer equipped with a 5 mm broadband probe with *Z*-axis gradient. All experiments were carried out at 298 K. The 1D proton spectra were acquired using a 30° pulse with a relaxation delay of 6 s, 128 scans were acquired with acquisition time of 2.4 s. For H_2O samples, the H_2O signal was suppressed using a 1D NOESY sequence, with irradiation during the last 2 s of relaxation delay and the mixing time. All spectra were carefully baseline corrected before integration.

The quantitation was accomplished using an internal standard, TSP. A typical NMR sample was prepared by adding 60 μL of TSP standard solution (1 mg/mL in D_2O) to 540 μL of sample and the solution was transferred to an NMR tube after thorough mixing.

The concentration of the compound in each formulation vehicle was calculated based on the integration ratio of the drug signal to the internal standard TSP signal (δ 0 ppm, 9H).

3. Results

Acidic, basic, and neutral drugs or drug-like compounds were chosen to represent the types typically encountered in small molecule drug discovery. Several were selected from chemical test sets used in the literature in the development and comparison of high-throughput solubility methods, while the remaining samples were Wyeth Discovery compounds.

It was envisioned that the NMR method would enable the rapid evaluation of a panel of potential formulations for use in pharmacokinetic and pharmacologic studies. The optimal vehicle was to impart intermediary solubility (~0.5–3 mg/mL) to enable adequate absorption while limiting the potential for GI toxicity that might arise from a completely solubilized oral dose. The vehicles selected for the study were therefore variations on a standard suspension formulation containing 2% polysorbate 80 surfactant minus the standard viscosity-increasing agent, methylcellulose. For acidic and basic compounds, the potential formulations included 2% polysorbate 80 with and without a pH modifier, 10 mM sodium bicarbonate and 10 mM acetic acid, for acidic and basic compounds, respectively. For the neutral compounds, the formulations consisted of 2% polysorbate 80 alone and with a second surfactant, 10% Solutol HS 15. Although not specifically intended for use as preclinical animal formulations, solutions of 10 mM sodium bicarbonate, 10 mM acetic acid, and 10% Solutol HS 15, all without polysorbate 80, were included in the study to better demonstrate the range of the NMR solubility method. The vehicles are listed in Table 1. The solubility results obtained from HPLC and NMR measurements are summarized in Tables 2–4 and Figs. 1 and 2.

As seen in the tables, the solubilities of the compounds tested in the current study range from a low of 40 μg/mL to greater than 10 mg/mL. The solubility results measured using the NMR method agree well with those obtained from the HPLC-UV method, particularly in the target formulation solubility range of 0.5–3 mg/mL. This is readily apparent from the excellent fit of the data to the lines of identity in Figs. 3 and 4 (r^2 values for the best-fit regressions are 0.986 and 0.988 for the commercial and Wyeth research compound data sets, respectively). The solubility values followed an expected trend for the acidic compounds, with solubility generally increasing as the vehicle changed from 10 mM bicarbonate to 2% Tween 80 to

Table 2
Solubility of commercially available compounds in various solutions obtained from NMR and HPLC methods.

	Formulation	HPLC solubility (mg/mL)	NMR solubility (mg/mL)
Acids			
Gemfibrozil	ABN	2.31	2.18
	A2	0.91	0.91
	A3	3.08	2.98
Bendroflumehiazide	ABN	0.79	0.88
	A2	0.02	0.05
	A3	0.80	0.94
Acetazolamide	ABN	0.94	0.84
	A2	0.88	0.97
	A3	1.02	1.04
Ketoprofen	ABN	2.48	2.56
	A2	2.86	2.84
	A3	4.86	4.73
Phenyl salicylate	ABN	1.69	1.73
	A2	0.08	0.04
	A3	1.67	1.69
HPBA	ABN	0.93	0.91
	A2	2.22	2.13
	A3	2.98	2.98
7MPBA	ABN	0.59	0.60
	A2	0.65	0.65
	A3	1.26	1.28
Salicylic acid	ABN	4.90	5.29
	A2	3.20	3.39
	A3	6.45	6.78
Neutrals			
Hydrocortisone	ABN	0.53	0.56
	N2	1.48	1.23
	N3	1.76	1.36
Carbamazepine	ABN	0.44	0.37
	N2	1.24	1.04
	N3	1.54	1.33
Triamcinolone	ABN	0.13	0.13
	N2	0.33	0.30
	N3	0.40	0.38
Prednisolone	ABN	0.42	0.37
	N2	1.14	1.00
	N3	1.38	1.20
Propyl-4-hydroxybenzoate	ABN	2.98	2.96
	N2	13.01	10.48
	N3	14.05	12.78
Griseofulvin	ABN	0.08	0.08
	N2	0.32	0.24
	N3	0.41	0.33
Bases			
Benzocaine	ABN	2.75	2.77
	B2	0.89	0.88
	B3	2.72	2.77
Tamoxifen	ABN	0.35	0.33
	B2	0.70	0.64
	B3	3.09	2.95
Trimethoprin	ABN	0.67	0.62
	B2	3.43	3.17
	B3	3.89	3.69

ABN = 2% Tween 80; A2 = 10 mM sodium bicarbonate; A3 = 2% Tween 80, 10 mM sodium bicarbonate. N2 = 10% Solutol; N3 = 2% Tween 80 + 10% Solutol. B2 = 10 mM acetic acid; B3 = 2% Tween 80, 10 mM acetic acid.

Table 3
Solubility of selected Wyeth compounds measured using NMR and HPLC methods using D_2O.

	Formulation	HPLC solubility (mg/mL)	NMR solubility (mg/mL)	Suspension pH
Acids				
Compound 1	ABN	0.19	0.20	6.00
	A2	0.11	0.10	8.30
	A3	0.91	1.35	7.30
Compound 2	ABN	0.86	0.75	3.50
	A2	0.78	0.76	7.40
	A3	3.88	4.20	6.00
Compound 3	ABN	0.12	0.10	6.80
	A2	~0.4 μg/mL	–	8.80
	A3	0.12	0.11	8.10
Bases				
Compound 4	ABN	0.12	0.10	7.60
	B2	3.03	2.88	5.20
	B3	4.29	4.41	5.70
Compound 5	ABN	0.18	0.16	7.20
	B2	0.66	0.83	4.60
	B3	2.38	2.33	5.00
Compound 6	ABN	0.25	0.22	7.00
	B2	0.19	0.13	4.10
	B3	0.72	0.74	4.50
Neutrals				
Compound 7	ABN	0.08	0.07	6.80
	N2	0.26	0.21	7.00
	N3	0.29	0.25	6.90
Compound 8	ABN	0.04	0.05	6.70
	N2	0.18	0.17	7.10
	N3	0.23	0.17	6.80
Compound 9	ABN	0.34	0.27	6.80
	N2	1.17	0.91	7.10
	N3	1.39	1.19	6.90

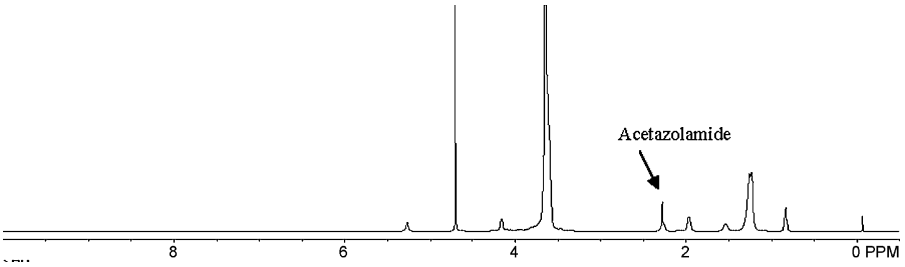

Fig. 2. NMR vs. HPLC solubility data for the Wyeth research compounds listed in Table 3.

the combination of the two. For basic compounds, the combination of 10 mM acid and 2% Tween 80 usually increased the solubility of the compounds. For neutral compounds the solubility was usually higher using the combination of the 2% Tween 80 and 10% Solutol surfactants in solution.

The solubility of Wyeth Compound 3 (0.4 μg/mL, measured by HPLC method) in 10 mM bicarbonate could not be obtained using NMR method due the relatively low sensitivity of the NMR technique, and will be discussed further.

Table 4
Solubility measured in H_2O solutions.

Compound	Formulation	HPLC solubility (mg/mL)	NMR solubility (mg/mL)
MPBA	2% Tween 80 in H_2O	0.41	0.36
Ketoprophen	2% Tween 80 in H_2O	2.05	1.96
Tamoxifen	2% Tween 80 in H_2O	0.36	0.29
Grizeofulvin	2% Tween 80 in H_2O	0.10	0.08
Carbamazepine	2% Tween 80 in H_2O	0.23	0.40
Benzocaine	2% Tween 80 in H_2O	3.05	2.78

4. Discussion

NMR is a powerful technique for solution sample analysis. It provides detailed information of each component in the solution and can also be used for quantitative analysis (Huynh-Ngoc and Sirios, 1973). Quantitation can be carried out using an internal standard using isolated aromatic resonances, without a calibration curve, which makes using NMR for quantitation convenient and provides a time saving over the HPLC-UV method. With NMR, there is also lesser of an issue with dynamic range found in HPLC-UV with higher concentration samples. With more concentrated samples, an HPLC-UV system will yield peaks that are non-quantifiable due to topping out of the detector. This leads to the further step of making sample dilutions prior to analysis. This is not the case with NMR. The added expense of an NMR system versus an HPLC-UV could be prohibitive

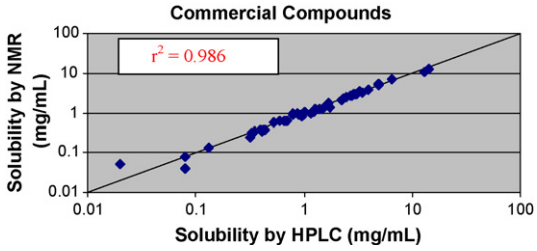

Fig. 3. ^{1}H NMR spectra of Tween 80 and Solutol in D_2O.

to some. However, those labs that have NMR system availability can easily perform this method. As far as sample requirements, we have performed the NMR solubility method with as little as 1 mg per analysis.

In this study, TSP was used as the internal standard. TSP has a single proton signal that is isolated from the signals of the formulation excipients and the compounds of interest, and therefore, eliminates the potential for interference with peaks resulting from the compound of interest. The TSP can be prepared in bulk D_2O facilitating the process. Also, with the internal standard present, a calibration curve is not required as is with an HPLC method which will ultimately save analysis time. Since the NMR method is non-destructive, although some recovery process would be required, samples can be reanalyzed over time to yield information on the stability of a compound in any formulation.

In these studies, deuterium oxide was used in most cases. The cost of D_2O is low considering the small volume used in the sample preparation. The experiments are easy to setup and the process can be automated. Alternatively, one can use H_2O solutions and use water suppression to suppress the water signals during the NMR data acquisition. Table 4 shows the results from solubility studies using H_2O instead of D_2O. In each sample a small amount of D_2O (10%) was added to provide a lock signal for the NMR experiment. Solvent suppression was achieved using a 1D NOESY sequence, which gives good suppression results and a flat baseline. The flat baseline is particularly important for integration of the compounds that have low solubility. In our experience, an excitation sculpting method also yields good results (Hwang and Shaka, 1995).

One can even further simplify the process by adding the internal standard while preparing the formulation vehicles. This can further reduce the time used for sample preparation if multiple compounds are tested in the same vehicle. As with the HPLC-UV method, all of the NMR experiments are amenable to automation.

The NMR method is most useful for compounds containing aromatic protons, which is the case for the majority of small molecule drugs. For compounds lacking any aromatic protons, the NMR method may prove challenging if the compound proton signals overlap with the excipient signals and the solubility of the compound is low. Cases where compounds lacking aromatic signals or UV chromophores that might be amenable to the NMR method are carbohydrates or lipids. Fig. 3 shows the proton NMR spectra of the formulation vehicle Tween 80 and Solutol. Both excipients have signals in the aliphatic region, but no signals in the aromatic region. Therefore, the solubility of any compounds that contains aromatic protons or non-overlapping signals with the excipient can be easily measured. Even in the case where the compounds only have overlapping or partially overlapped signals, it is still possible to extract the solubility information, but the accuracy for low soluble compounds could be limited. Acetazolamide used in this study is an example. The compound does not have aromatic signals and it has a methyl singlet in the aliphatic region. The signal is partially overlapped with the signal of the excipient at 2.28 ppm (Fig. 4). As a result, the integral at 2.28 ppm is the sum of the acetazolamide methyl protons and the excipient protons. Since TSP concentration is kept constant in the formulation and the ratio of the excipient and TSP is known, the integral ratio of the acetazolamide with TSP can be derived by subtracting the integral value of the excipient from the total integral value at 2.28 ppm. The concentration of acetazolamide in the solution can therefore be calculated. However, if the solubility of the compound is very low and the signal is completely buried under the vehicle signal the solubility of the compound cannot be determined accurately.

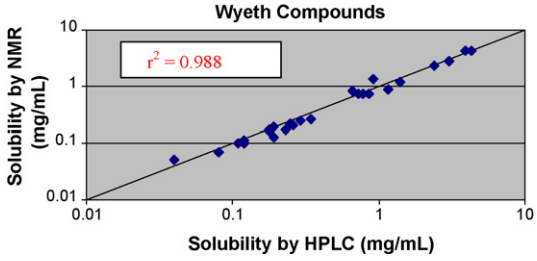

Fig. 4. ^{1}H NMR spectrum of acetazolamide in 2% Tween 80.

One potential draw back of NMR solubility analysis is the relatively lower sensitivity compared with UV detection and mass spectrometry techniques, which limits analysis of samples having low solute concentrations. However, the focus of our application is to evaluate the solubility of compounds in formulations that impart moderate solubility, rather than determine the aqueous solubility limit. Therefore, the relative low sensitivity of NMR technology is not an issue for the purpose of our application. The detection limits under our experimental conditions is approximately 40 μM, (the detection limit can be improved by using higher field NMR or CryoProbes). Solutes that cannot be detected, i.e. those having solubility less than 40 μM in the formulation (Compound 3 for example) will be classified as insoluble, and the formulation vehicle will be considered as unsuitable for the compound. The absolute solubility value is not important for insoluble compounds as additional formulation efforts will be required for successful *in vivo* evaluation.

5. Conclusion

From the results of this study, it is clear that ^{1}H NMR can be used to determine the solubility of compounds in various *in vivo* formulations. In comparing the HPLC-UV results to the results obtained from ^{1}H NMR, it is evident that the NMR experiment can be used to accurately determine the solubility drug-like molecules with moderate solubility in fairly complex media. The simple vehicles used in this initial study enabled proof-of-concept for the NMR method and may serve as a starting point for a useful panel of vehicles for Discovery compounds. The key feature of the NMR method is its ability to determine a starting point for *in vivo* dosing using a very small amount of material, which is often crucial at the Discovery stage. The NMR method is fast, and sample preparation is simple if the formulation materials are made ahead of time in larger batches. In addition, the process can be easily automated. Therefore, solubility determination by NMR provides an easy and practical approach to screening Discovery formulations.

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