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A High-Throughput Method for Lipophilicity Measurement

Zhi Chen and Stephen G. Weber*

Department of Chemistry, University of Pittsburgh, Pittsburgh, Pennsylvania

Abstract

A high-throughput method has been developed for lipophilicity measurement. It measures the partition coefficient of a solute between a polymer phase and an aqueous phase (P_{pw}) in a 96-well format. The polymer is plasticized poly(vinyl chloride) (PVC), which is widely used as a material for clinical containers and ion-selective electrodes. The composition is 2:1 (w/w) dioctyl sebacate (DOS) and PVC. With 6 repeats, $\log P_{pw}$ values of 15 solutes have been determined in one 96-well microplate in 4 hours. A linear relationship between $\log P_{pw}$ and $\log P_{ow}$ (octanol-water partition coefficient) values exists with a correlation coefficient of 0.979. The slope and intercept of the $\log P_{pw}$ vs. $\log P_{ow}$ plot are statistically indistinguishable from 1 and 0, respectively. Similar to the HPLC method, by using the correlation line as a calibration curve, the measured $\log P_{pw}$ values can be used to predict $\log P_{ow}$. This protocol is faster than the HPLC method. Moreover, it is straightforward to extend the protocol to the determination of the distribution coefficient and pK_a of charged solutes. We show that the $\log P_{pw}$ of the neutral form of racemic econazole is $4.83(\pm 0.06)$, for the cationic form (presumably as a dihydrogen phosphate ion pair) $1.68(\pm 0.04)$, and the pK_a is $6.15(\pm 0.04)$. This method has great flexibility as well, and is potentially fully automated.

Lipophilicity represents the affinity of a molecule or a moiety for a lipophilic environment.^{1, 2} Over a century ago, Overton³ and Meyer's⁴ pioneering work demonstrated that the lipophilicity of a compound could be related quantitatively to its biological activity. As suggested by Collander⁵ and Hansch,^{6, 7} the logarithmic value of the *n*-octanol/water partition coefficient, $\log P_{ow}$, has been widely recognized in the pharmaceutical, biomedical and environmental sciences to describe the lipophilicity of various compounds. Numerous experimental methods exist to measure $\log P_{ow}$ values. The shake-flask procedure is a standard method⁸ to determine $\log P_{ow}$ in the range of -2 to 4, but it is time and labor consuming, and requires relatively large amounts of pure compounds.^{9, 10} In addition, entrainment and octanol/water emulsions can be severe problems for compounds having a $\log P_{ow}$ value larger than 4.¹¹ The HPLC method is an indirect way to estimate $\log P_{ow}$ values in the range of 0 to 6, and has also become a standard procedure.^{12, 13} A series of reference compounds is injected onto a C18 column. The compounds' retention factors are used to create a calibration curve with their known $\log P_{ow}$ values. Compounds with unknown $\log P_{ow}$ values are then injected and their retention factors are used to predict $\log P_{ow}$ from the calibration curve. However, this technique is only valid for neutral molecules since charged molecules have a far more complex retention behavior than simple partitioning. HPLC and other separation-based methods to determine $\log P_{ow}$ have been reviewed.^{11, 14, 15} Responding to the need for more speed and accuracy, workers have used other clever approaches over the last decade. Valko¹⁶ introduced a chromatographic hydrophobicity index based on a fast-gradient RP-HPLC method. Tsang¹⁷ made silica-encapsulated magnetic nanoparticles to adsorb a tiny amount of *n*-octanol and measured the sample partition coefficient from water to the new phase. Solid-phase microextraction (SPME) has also been applied by several research groups to estimate $\log P_{ow}$.¹⁸⁻²⁴ All of the above methods achieved satisfactory correlation with $\log P_{ow}$, even for

very hydrophobic compounds ($\log P_{ow} > 6$).²⁴ There are also some theoretical approaches to calculate $\log P_{ow}$ of a compound, but their reliability for new chemical entities and/or novel structures is not satisfactory.²⁵

With the development of combinatorial synthesis, high-throughput technologies have become the paradigm in pharmaceutical industry. Although accuracy remains a goal, small sample usage, ultra-fast analysis, and automation, are becoming important factors for the determination of physico-chemical properties in early drug discovery.¹¹ To increase the throughput of $\log P_{ow}$ measurement, an attempt has been made to transfer the traditional shake-flask method to a 96-well format.²⁶ However, restrictions of the shake-flask method still remained, and the yield was limited to one plate per day due to the detection bottleneck. An indirect method based on transportation experiments through octanol layers in 96-well filter plates has also been introduced,²⁷ yet an unstable octanol membrane could be a problem for reproducibility. Recently, our group developed a high-throughput screening method for chiral selectors, which was modeled after the protocol for biological screening of combinatorial libraries.²⁸ This method was based on target partitioning between a selector-containing polymer-film phase and an aqueous phase. The polymer used was plasticized poly(vinyl chloride) (PVC). Due to its wide use in plastic containers such as catheters and infusion bags, the partitioning of drugs, environmental contaminants and food components into PVC has caught the interest of many researchers.²⁹⁻³⁵ Plasticized PVC is also the material for ion-selective electrodes, and the lower detection limit of these electrodes can be dependent on the solute lipophilicity in plasticized PVC.^{36, 37} Plasticized PVC films have seen application to microextraction.³⁸⁻⁴⁰ Moreover, studies on PVC containers have shown a reasonable linear correlation between P_{pw} (polymer-water partition coefficient) and P_{ow} .^{33-36, 41} Here we introduce such a high-throughput method to measure P_{pw} in a 96-well format. A linear correlation between $\log P_{pw}$ and $\log P_{ow}$ for 15 standard references has been established, which can be used as a calibration curve for indirect determination of $\log P_{ow}$. This application has also been extended to the determination of distribution coefficient and pK_a of a charged solute.

EXPERIMENTAL SECTION

Chemicals and Materials

HPLC grade tetrahydrofuran (THF) and acetonitrile were purchased from Aldrich (Milwaukee, WI). Poly(vinyl chloride) (PVC, high molecular weight, Selectophore) and dioctyl sebacate (DOS, Selectophore) were purchased from Fluka (Ronkonkoma, NY). Water used in all the experiments was purified with a Milli-Q Synthesis A10 System (Millipore, Bedford, MA). All the solutes were purchased from commercial sources with purities greater than 99%. Costar polypropylene 96-well microplates (flat-bottom, 330 μ L well-volume), BD Falcon UV-transparent 96-well microplates (370 μ L well-volume) and thermal adhesive sealing films were purchased from Fisher Scientific Co. (Pittsburgh, PA).

Equipment

A Deep Well Maximizer (or BioShaker) (Model: M-BR-022 UP, made by TAITEC, Japan, and distributed by BIONEXUS, Inc., Oakland, CA) was used to speed up the solute distribution kinetics and control the temperature for better reproducibility. UV spectra were acquired with a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA) in UV-transparent microplates. An X-LCTM (Jasco Inc., Japan) HPLC system was used to measure the retention factors of 15 standard compounds on a C₁₈ column (Prevail C18, 4.6 $\times$ 100 mm, particle size: 3 μ M, Alltech Associates, Inc., Deerfield, IL). For high-throughput determination of econazole concentration, the same HPLC system with a UPLCTM C₁₈ column (1.0 $\times$ 50 mm, particle size: 1.7 μ M, Water, Milford, MA) was used.

General Procedure for P_{pw} Determination

Chart 1 gives the sequence of operations for the P_{pw} determination. The plasticized PVC films were prepared in polypropylene 96-well microplates. Aliquots of the solute solution were then dispensed. The plates were covered by adhesive sealing films and incubated in a shaker (500 rpm, 25 °C). In order to determine the equilibration time, a kinetic study was performed. The concentration of solute remaining in the aqueous phase was measured as a function of time. All other data generated were at equilibrium. To determine the solute concentration, the supernatant from each well was transferred to a UV-transparent microplate. A microplate reader was used for UV absorbance measurement. The partition coefficient, P_{pw} , was then calculated as:

$$P_{pw} = \frac{C_0 - C_1}{C_1 \cdot \Phi} \quad (1)$$

Here C_0 is the initial solute concentration in the aqueous phase, C_1 is the concentration at equilibrium, and Φ is the phase ratio.

Preparation of Plasticized PVC films

PVC (1.67 g) and DOS (3.33 g) were dissolved in 200 mL THF in a volumetric flask. A multi-channel pipette was used to dispense the solution to the wells of a polypropylene 96-well microplate. The plate was placed in the hood for 6 hours for evaporation of the THF, and the films were formed at the bottom of each well. The volume of each film was estimated as:

$$V_{film} = \frac{5\text{g}/200\text{mL} \times V_{solution}}{d_{film}} \approx \frac{V_{solution}}{100\mu\text{L}} \times 2.5\mu\text{L} \quad (2)$$

Here $V_{solution}$ is the volume of the THF solution used in each well, and d_{film} is the density of the film which is estimated as 1 g/mL. In this paper, 100 μL of the THF solution was dispensed in each well, so the volume of each film was about 2.5 μL .

Protocol to determine the P_{pw} values of the 15 reference solutes

Fifteen compounds were dissolved in water at the concentration of 0.5 mM. The solutions were then filtered. As shown in Table 1, aliquots of the solutions were dispensed to the polypropylene microplate in which the plasticized PVC films were made. The volume of the dispensed aqueous solution in each well was 200 μL , and 6 repeats were performed. The plate was then sealed by an adhesive film and equilibrated in the shaker (500 rpm, 25 °C) for 4 hours. At the same time, aliquots of 100 μL of each standard solution were dispensed to a UV-transparent microplate. Again, 6 repeats were performed. The UV absorbance of each standard was measured at its maximum absorbance wavelength by the microplate reader. Note that for all the measurements, same amount of water in the wells was used as reference for background deduction. For standard i , A_{i0} ($i = 1$ to 15) is defined as its average absorbance. The UV absorbance of the standards at partition equilibrium was measured similarly: After 4 hours, from each well of the polypropylene plate, 100 μL of the supernatant was transferred to a UV-transparent plate. The corresponding UV absorbance data were again collected by the microplate reader. A_i is defined as the average absorbance for standard i at equilibrium. As shown in Table 1, there was no solute in wells H7-12. The UV absorbance in those wells (A_b) was due to small aqueous solubility of DOS. Thus, the partition coefficient for standard i could be calculated as:

$$P_{pw} = \frac{A_{i0} - (A_i - A_b)}{(A_i - A_b) \cdot \Phi} \quad (3)$$

Here, $\Phi = 2.5 \mu\text{L} / 200 \mu\text{L} = 1 / 80$.

The protocols to measure the P_{pw} values of other solutes are similar.

The HPLC method to determine the k values of the 15 reference solutes

The retention factors of the 15 standard references were measured on the Alltech C_{18} column. The mobile phase was water/acetonitrile (50/50), with a flow rate of 0.6 mL/min. The back-pressure was about 2550 psi. Two repeats were performed for each solute.

Protocol to determine the D_{pw} values of econazole at different pH

To measure distribution coefficient, D_{pw} , the protocol had some minor modifications. The solute was initially prepared in the polymer film. The solute econazole was dissolved in THF at 200 μM . Each well received 200 μL of the solution. The PVC/DOS solution was then dispensed and the same procedures as previously described were followed to make the films. For distribution experiments, 10 mM phosphate buffer solutions with various pH values were added to the films. The econazole concentration in the supernatant at equilibrium (C) was determined both by HPLC and by UV absorbance as above. The distribution coefficient at a specific pH could be calculated as:

$$D_{pw}^{pH} = \frac{C_0^{pH} - C^{pH}}{C^{pH} \cdot \Phi} \quad (4)$$

Here, $C_0 = 200 \mu\text{M}$.

HPLC method to determine econazole concentration

To minimize the ionized form, econazole samples in acidic buffer ($\text{pH} < 4$) were diluted 20 times by 25% ACN + 37.5 mM Na_2HPO_4 solution; samples in near-neutral buffer ($6 < \text{pH} < 8$) were diluted twice by 50 mM Na_2HPO_4 solution. The column used was the Water UPLCTM C_{18} column. The mobile phase was 50 mM Na_2HPO_4 /acetonitrile (65/35), with a flow rate of 0.2 mL/min. The back-pressure was about 6400 psi. To ensure reproducibility, the full-load injection mode was used (injection volume: 30 μL ; loop volume: 5.7 μL). The peak area of econazole was used for the calibration and determination of its concentration. The time per analysis is about 60 seconds.

RESULTS AND DISCUSSION

When a solute partitions from the aqueous phase to the membrane phase, its UV absorbance in the aqueous phase should continually decrease until equilibrium. As shown in Figure 1, four hours is needed for the partitioning of solutes to reach equilibrium. Careful inspection of the curves shows that the equilibration time is longer for more hydrophobic compounds. In this paper, all the solutes (except econazole) have their $\log P_{ow}$ values in the range of 0 to 3.2, so all the partitioning experiments were performed for 4 hours, if not stated otherwise.

The Organization for Economic Co-operation and Development has recommended the 15 compounds listed in Table 2 as the reference substances for the HPLC method.¹³ As shown in Figure 2, they span a wide range in hydrogen bond basicity and acidity. Figure 3 shows the correlation between the $\log P_{ow}$ values and the $\log P_{pw}$ values for these 15 compounds. The least-squares regression equation of $\log P_{pw}$ vs. $\log P_{ow}$ (Table 2) leads to:

$$\log P_{pw} = 0.933(\pm 0.054) \log P_{ow} + 0.185(\pm 0.108) \quad \begin{array}{cccc} n & r & s.e. & p \\ 15 & 0.979 & 0.170 & < 0.0001 \end{array} \quad (5)$$

where n is the number of compounds studied, r is the correlation coefficient and $s.e.$ is the standard error of the regression. This regression line can be used as the calibration curve for indirect determination of $\log P_{ow}$ as in the HPLC method. Briefly, for compounds that have unknown $\log P_{ow}$ values, measure their $\log P_{pw}$ values and use the regression line to predict their $\log P_{ow}$ values.

Figure 4 gives the correlation between the $\log k$ (RPLC) and $\log P_{ow}$ values of the same 15 reference solutes. The least-squares regression equation is:

$$\log k = 0.41(\pm 0.04)\log P_{ow} - 0.49(\pm 0.08) \quad \begin{matrix} n & r & s.e. & p \\ 14 & 0.952 & 0.110 & <0.0001 \end{matrix} \quad (6)$$

Note that the data point of 4-acetylpyridine is not used for regression analysis since it is an obvious outlier. The linear correlation coefficient would decrease to 0.879 if that point had been included. The r values of the two correlations (after discarding the outlier) are similar, indicating that the precision of our method is similar to the HPLC method for indirect determination of $\log P_{ow}$. However, as the slope of the HPLC method is lower, the precision in the slope of the polymer-based method is better than that of the HPLC method based on comparing the relative standard deviation of the two slopes. Besides lipophilicity, H-bonding also plays an important role in determining the P_{ow} value of a solute. As a result, due to the diversity of the H-bonding ability of PVC/DOS (1:2) ($\beta = 0.53$, $\alpha = 0$),⁴⁰ n-octanol ($\beta = 0.45$, $\alpha = 0.33$)⁴² and C18 ($\beta = 0$, $\alpha = 0$), the linear correlations are not perfect among systems when the solutes have various numbers of H-bond donors and acceptors. Note that P_{ow} is just a term to picture lipophilicity. Actually, to describe lipophilicity, the ideal partition system should use an organic phase that is free of polar binding forces, and a hydrocarbon solvent such as cyclohexane or hexadecane⁴³ should serve as a better reference than octanol. However, some practical issues hindered their popularity. Furthermore, one of the most important reasons making P_{ow} a standard term to describe lipophilicity is the large number of data measured.

Studies in drug partitioning behavior in polymers have shown that the slope of the $\log P_{pw}$ - $\log P_{ow}$ correlation line has an inverse linear relationship to the polarity or the solubility parameter of the polymer,^{44, 45} which can be used to predict drug solubility in polymers. It also reflects how sensitive the solvent system is to changes in lipophilicity of solutes.⁷ In equation 5, the slope is close to 1 ($t = 1.24$, $p = 0.24$), indicating that the polymer material (PVC:DOS = 1:2) ($\pi^* = 0.64$)⁴⁰ has a similar polarity to octanol ($\pi^* = 0.40$).⁴² The intercept of the $\log P_{pw}$ - $\log P_{ow}$ correlation line can be used as a measure of the polymer lipophilicity. The intercept of equation 5 is close to 0 ($t = 1.71$, $p = 0.11$), indicating that DOS plasticized PVC (PVC:DOS = 1:2) has a similar lipophilicity to octanol. The intercept is also linearly related to the solubility of water in the polymer phase.^{7, 44, 45}

In another application, $\log P_{pw}$ values of the three positional isomers of nitroanisole were measured and correlated to their experimental $\log P_{ow}$ values. The linear regression equation is:

$$\log P_{pw} = 1.054(\pm 0.041)\log P_{ow} + 0.210(\pm 0.082) \quad \begin{matrix} n & r & s.e. & p \\ 3 & 0.9992 & 0.013 & 0.025 \end{matrix} \quad (7)$$

For isomers that have the same functional groups, the partition behavior is more similar than for a set of compounds that have different functional groups. The measured $\log P_{pw}$ values, as well as the experimental $\log P_{ow}$ values from the literature, and the estimated $\log P_{ow}$ values from online-software calculations, are listed in Table 3. Obviously, the order of the three experimental $\log P_{ow}$ values of the isomers matched the order of the three $\log P_{pw}$ values very well: 3-nitroanisole > 4-nitroanisole > 2-nitroanisole. For the calculated $\log P_{ow}$ values, the orders did not match. This mismatch of the calculated data with the experimental data confirms

our statement in the introduction, that theoretical calculations of $\log P_{ow}$ sometimes are not very accurate.

For a homologous series of solutes, the free energy for the partitioning process linearly increases with increasing number of methylene groups in a straight chain. Figure 5 confirms this relationship by examining the $\log P_{pw}$ and $\log P_{ow}$ values of four alkyl 4-hydroxybenzoates. Since methyl 4-hydroxybenzoate has no methylene group, only three points from each data set were used for the regression analysis. The least-squares regression equations are:

$$\log P_{pw} = 0.480(\pm 0.012)m + 1.373(\pm 0.025) \quad \begin{matrix} n & r & s.e. & p \\ 3 & 0.9997 & 0.016 & 0.015 \end{matrix} \quad (8)$$

$$\log P_{ow} = 0.550(\pm 0.012)m + 1.927(\pm 0.025) \quad \begin{matrix} n & r & s.e. & p \\ 3 & 0.9998 & 0.016 & 0.013 \end{matrix} \quad (9)$$

The two slopes are not significantly different ($t = 4.12$, $p = 0.15$). The free energies required for a methylene group to transfer from the organic phases to the aqueous phase are $2.74(\pm 0.06)$ kJ/mol from the polymer phase to water and $3.13(\pm 0.06)$ kJ/mol from octanol to water.

Compared to the HPLC method, which requires about 5-20 minutes for determining the retention factor of one sample, our new technique is more suitable for a large number of solutes, for instance, combinatorial libraries. The time needed to prepare one plate of PVC films is about 6 hours. Increasing the number of plates will not significantly change the processing time since the bottleneck of the film-making procedure is THF evaporation. The equilibration time is 4 hours, regardless of the number of plates. Dispensing solutes to one plate takes about 15 minutes, and UV absorbance reading adds another 15 minutes. As described above, the time scale for both methods is about the same if the $\log P$ values of only 30 samples are to be determined. However, if the analysis of more compounds is required, this technique will save time. Moreover, it has the potential to build a larger database for P_{pw} than for P_{ow} .

The method assumes that partitioning only occurs between the two phases: aqueous and polymer. Loss of solute to the microplates due to sorption can lead to misinterpretation. Polypropylene is a good packaging material for drug-like compounds because the sorption effect is small or nonexistent.⁴⁶⁻⁴⁸ We tested the concentration stability of the solutes in the polypropylene plate in the absence of a polymer film and did not see any measureable loss. Solute adsorption on the surface of the plasticized PVC film should also be considered. We have calculated that if a monolayer of well-packed solute (M.W. ~ 200) had been adsorbed on the film surface, only about 0.3% of the solute would be lost from the aqueous solution at 0.5 mM. If a lower solute concentration is used, this could become more of a problem. When using the UV-transparent plate, buffering sometimes may be needed since mass loss of basic compounds to polystyrene materials can occur.⁴⁶ Attention to volatile solutes should also be paid: we have found that one of the reference compounds, ethylbenzene requires timely covering of the well-plate and quick action to measure the UV absorbance.

Figure 6 shows the estimated measurement error associated with the calculated $\log P_{pw}$ value, based on assumptions that: 1. Concentrations are measured by optical absorbance in the

aqueous phase; 2. $A_{i0} = 1$, $\frac{s_{A_{i0}}}{A_{i0}} = 0.015$; 3. $A_b = 0.06$, $s_{A_b} = 0.006$; 4. $\frac{s_{A_i}}{A_i} = 0.037$ ($s_{A_i} \geq 0.003$); 5. four repeats are performed for each measurement. $s_{A_{i0}}$, s_{A_b} and s_{A_i} are the standard deviations

of A_{i0} , A_b and A_i , respectively. The selected values of $\frac{s_{A_{i0}}}{A_{i0}}$, A_b , s_{A_b} , and $\frac{s_{A_i}}{A_i}$ are the averages obtained from experiments. P_{pw} values are calculated from equation 3 with various A_i values. The standard deviation, s , is primarily a result of pipetting errors during the processes of film preparation, solute dispensing, and supernatant transferring, but not from the determination of

UV absorbance. With a more precise liquid handling technique, it is possible to reduce the pipetting errors, as well as the propagated error of $\log P_{pw}$. At one fixed phase ratio, it is possible to obtain precise measurement results ($s_{\log P_{pw}} < 0.2$. $s_{\log P_{pw}}$ is the standard deviation of $\log P_{pw}$) for about 2.6 $\log P_{pw}$ units. Changing the phase ratio will greatly extend the applicable range, e.g., by doubling or halving the phase ratio, the boundaries will be 0.3 units larger or smaller, respectively. This strategy is similar to changing the strength of the mobile phase or the length of column to adjust the retention time when using the HPLC method. Increasing determination repeats, which is very easy when using a microtiter plate, will also help to decrease measurement error. Therefore, depending on the acceptable precision, an operator performing the protocol can flexibly choose the necessary phase ratio and determination repeats.

This high-throughput technique can also be applied to determine the distribution coefficient (D_{pw}) of a substance at various pH values. Some minor modifications of the protocol, as shown in the experimental section, made the measurement even more convenient and extended the range of $\log D_{pw}$ accessible at a single phase ratio. Figure 7 shows an example of the application. Econazole (Chart 2) is a basic compound that has two ionizable groups, with only one of them ionizable at $\text{pH} \geq 1$ ($\text{pK}_a = 6.69$, $\log P_{ow} = 5.32$, calculated using Advanced Chemistry Development (ACD/Labs) Software V8.14 for Solaris). Thus its $\log D_{pw}$ value increases with increasing pH of the buffer, and can be further elucidated by the $\log D$ estimation formula for bases:

$$D(\text{pH}) = \frac{P}{1 + 10^{\text{pK}_a - \text{pH}}} + \frac{P'}{1 + 10^{\text{pH} - \text{pK}_a}} \quad (10)$$

where P is the partition coefficient of the neutral form while P' is that of the ionized form. The equilibration time was 6 hours. Two parallel experiments were performed, using the plate reader and HPLC to determine the econazole concentration at equilibrium, and two sets of $\log D_{pw}$ values were correspondingly acquired. Applying nonlinear least squares curve fitting, as shown in Figure 7, the $\log P_{pw}$, $\log P'_{pw}$, pK_a values of econazole can all be obtained, which are $3.53(\pm 0.03)$, $1.69(\pm 0.04)$, $5.41(\pm 0.06)$, respectively, when the plate reader was used, and $4.83(\pm 0.06)$, $1.68(\pm 0.04)$, $6.15(\pm 0.04)$, respectively, when the HPLC was used. Apparently the latter set of data is more accurate. The two fitting curves almost overlap at low pH, but have a clear variance when $\text{pH} > 7$, where $\log D_{pw}$ values are larger than 3.5. This is in good agreement with our previous calculation about the applicable range of $\log P_{pw}$ when using the plate reader as the detection tool. The plate reader can only quantitate the econazole concentration above 10 μM , which limits the measurable range of $\log D_{pw}$ to 3.5; while HPLC on-column preconcentration helped to quantitate econazole concentration as low as 0.03 μM , with the extension of the measurable range of $\log P_{pw}$ and $\log D_{pw}$ to 5.7. The estimated error associated with $\log P_{pw}$ (or $\log D_{pw}$) is again shown in Figure 6, based on assumptions that: 1. Solute concentrations in the aqueous phase are measured by HPLC with on-column preconcentration;

2. $C_0 = 1$, $\frac{S_{C_0}}{C_0} = 0.015$; 3. $\frac{S_C}{C} = 0.037$ (when $C > 0.0001$); 4. four repeats are performed for each measurement. C_0 is the initial relative concentration of the solute and C is the relative solute concentration at equilibrium. S_{C_0} and S_C are the standard deviations of C_0 and C , respectively. P_{pw} values are calculated from equation 1. For more straightforward comparison, the selected

values of $\frac{S_{C_0}}{C_0}$ and $\frac{S_C}{C}$ are the same as $\frac{S_{A_{i0}}}{A_{i0}}$ and $\frac{S_{A_i}}{A_i}$, as in the previous estimation of $\log P_{pw}$ error when using the plate reader to measure solute concentration. Apparently, with HPLC as the detection tool, the errors at high $\log P_{pw}$ are much smaller. For quantitative analysis of the solutes, one HPLC run needs about 30 to 60 seconds. Although slower than the plate reader, it is still reasonably rapid. Again, depending on the desired application, an operator performing

the protocol can flexibly choose the most suitable detection technique. Since the traditional HPLC method for lipophilicity measurement is usually inaccurate for charged compounds, the applicability of our system for the determination of log D and pK_a values is another advantage over it.

CONCLUSIONS

We have successfully developed a new method for lipophilicity measurement using high-throughput technologies. This method measures the partition coefficient of a substance between a polymer phase and an aqueous phase (P_{pw}) in 96-well microplates. With 6 repeats, log P_{pw} determination of 15 compounds can be performed simultaneously. A linear relationship between log P_{pw} and log P_{ow} has been established. By using the correlation line as a calibration curve, the measured log P_{pw} values can be used to predict log P_{ow} . This method proven to be more accurate than some software for the estimation of log P_{ow} . It can also help to understand the partition mechanism among different solvent systems. Compared to the HPLC method, our protocol is more time-saving for combinatorial libraries, and it is not only applicable for the partition coefficient determination of neutral compounds, but can also be utilized as a tool for distribution coefficient and pK_a measurement. This method has great flexibility as well, as instruments exist to make it fully automated.

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Figure 1.
Kinetic UV absorbance of solutes in the aqueous phase during the partition equilibration process.

Figure 2. Hydrogen bond acidity (α_2^H) and basicity (β_2^H) map of the 15 reference solutes (■);^{42, 49, 50}. (*) Estimated (2-chloroaniline: $\alpha_2^H = 0.25$, $\beta_2^H = 0.31$; 3-chloroaniline: $\alpha_2^H = 0.30$, $\beta_2^H = 0.30$. 2,3-dichloroaniline should be slightly more acidic but less basic than both of them).

Figure 3.
Linear correlation of $\log P_{pw} - \log P_{ow}$ for the 15 reference solutes.

Figure 4.
Linear correlation of $\log k - \log P_{ow}$ for the 15 reference solutes.

Figure 5. Linear correlation of the $\log P_{pw}$ and $\log P_{ow}$ values versus the number of methylene groups in alkyl 4-hydroxybenzoates.

Figure 6.
Estimated error of $\log P_{pw}$ at various phase ratios (Φ).

Figure 7.
Log D_{pw} of econazole at various values of pH.

Add PVC/plasticizer in THF

Evaporate THF

Add target, equilibrate (const. T)

Transfer target to a UV- transparent plate, measure absorbance of target

Chart 1.
General procedure for P_{pw} determination.

Chart 2.
Econazole structure.

Table 1
Layout of the 15 reference solutes in the 96-well microplate.

	1	2	3	4	5	6	7	8	9	10	11	12
A			4-Acetylpyridine						Acetophenone			
B			Aniline						Methyl benzoate			
C			Acetanilide						4-Chlorophenol			
D			Benzyl alcohol						Ethyl benzoate			
E			4-Methoxyphenol						2,3-Dichloroaniline			
F			Phenol						Ethylbenzene			
G			Benzonitrile						Benzophenone			
H			Phenylacetoneitrile						Water			

Table 2

The 15 reference solutes and their values of $\log P_{ow}$, $\log P_{pw}$, and $\log k$.

Number	Standards	$\log P_{ow}$	$\log P_{pw}$	$\log k$
1	4-Acetylpyridine	0.5	0.70 ± 0.07	0.234 ± 0.015
2	Aniline	0.9	1.28 ± 0.20	-0.130 ± 0.002
3	Acetanilide	1.0	1.06 ± 0.11	-0.046 ± 0.002
4	Benzyl alcohol	1.1	0.95 ± 0.32	-0.052 ± 0.002
5	4-Methoxyphenol	1.3	1.42 ± 0.06	-0.082 ± 0.002
6	Phenol	1.5	1.48 ± 0.09	-0.002 ± 0.002
7	Phenylacetoneitrile	1.6	1.50 ± 0.06	0.288 ± 0.001
8	Benzonitrile	1.6	1.66 ± 0.06	0.278 ± 0.001
9	Acetophenone	1.7	1.84 ± 0.11	0.278 ± 0.001
10	Methyl benzoate	2.1	2.45 ± 0.04	0.464 ± 0.001
11	4-Chlorophenol	2.4	2.16 ± 0.05	0.264 ± 0.002
12	Ethyl benzoate	2.6	2.67 ± 0.05	0.658 ± 0.002
13	2,3-Dichloroaniline	2.8	2.81 ± 0.10	0.620 ± 0.001
14	Ethylbenzene	3.2	3.19 ± 0.28	0.913 ± 0.005
15	Benzophenone	3.2	3.17 ± 0.19	0.786 ± 0.006

Table 3

The log P_{ow} and log P_{pw} values of the 3 isomers of nitroanisole.

Targets	log P_{pw}	log P_{ow}		
		Exp.	esc.syrres.com	logP.com
2-Nitroanisole	2.03 ± 0.03	1.73	1.89	1.90
3-Nitroanisole	2.49 ± 0.06	2.17	1.89	1.97
4-Nitroanisole	2.36 ± 0.07	2.03	1.89	2.02