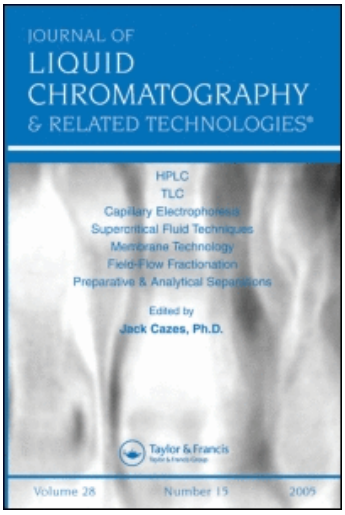

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ESTIMATING THE LIPOPHILICITY OF NATURAL PRODUCTS USING A POLYMERIC REVERSED PHASE HPLC METHOD

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ESTIMATING THE LIPOPHILICITY OF NATURAL PRODUCTS USING A POLYMERIC REVERSED PHASE HPLC METHOD

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□ *The integration of physicochemical profiling screens such as Log P into natural products drug discovery programs is emerging as an approach to front-load drug-like properties of natural product libraries for high-throughput screening. In this study a fast-gradient HPLC method using a polystyrene-divinylbenzene PRP-1 column was developed to estimate the lipophilicity of marine natural products. An excellent correlation was found between the results of the experimental determined and the literature log P values for a diverse set of commercially available drugs using the PRP-1 column. The log P of a series of 24 marine natural products were evaluated using the new method and a good correlation was observed between the experimentally determined and software calculated log P values. Some discrepancies were observed between the measured value of log P and the software calculations of the natural products containing halogens atoms. The method is rapid, insensitive to impurities, and requires very little compound and is amenable for integration into a natural products drug discovery research program.*

Keywords drug discovery, lipophilicity, natural products, polystyrene-divinylbenzene, reversed-phase HPLC

INTRODUCTION

Lipophilicity, expressed as the logarithmic value of the octanol/water partition coefficient ($\log P_{oct}$), is a fundamental parameter that models the biological partition behavior of drug molecules.[1] For example, drug absorption and $\log P_{oct}$ are directly related because of passive diffusion across the cell membrane. We can estimate absorption, even permeability and distribution of drug candidates in body by $\log P_{oct}$. If the Log P value is below 0 or above 5, drug candidates usually have intestinal and central nervous system (CNS) permeability problems or low solubility and poor oral bioavailability. Actually, the $\log P_{oct}$ corresponding to the 90th

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percentile of drug candidates that reached phase II clinical trails is between 0 and 5.[2] Therefore consideration of Log P_{oct} in the process of drug development can prioritize leads from high-throughput screening and reduce the failure rate of drug candidates during development.[3,4]

Reversed-phase liquid chromatography (RP-HPLC) has been widely used to estimate Log P_{oct}.[4,5,6] All of these methods achieve satisfactory correlations with Log P_{oct}, even for very hydrophobic compounds (Log $P_{oct} > 6$). Valkó et al. proposed a rapid method for measurement of Log P *via* reversed-phase HPLC and established a general solvention equation for Log P values and Chromatographic Hydrophobicity Indices with acetonitrile (CHI_{ACN}).[7,8,9] The main advantage of an HPLC-based lipophilicity measurement is that the partition coefficients can be obtained from time measurements instead of concentration determination. The result of this is that retention time (t_R) is independent of the compound concentration/amount injected into the chromatographic system. Consequently, impurities do not affect the measurements, and the low solubility of some compounds does not affect the measurements. Another important feature of this method is that very little compound is required and the Log P of a mixture of several compounds can be obtained from a single injection. These characteristics make HPLC a practical method to determine drug lipophilicity in the early stages of drug discovery. HPLC-based methods suffer some disadvantages such as short linear range of Log P_{oct} and retention time, and the lipophilicity assessment of charged compounds such as basic drugs are unreliable. The discrepancies of log P measurements of charged compounds have been attributed to silanophilic interactions with free silanol groups on silica based C_{18} columns, which results in increases in retention times.[10]

The integration of physicochemical profiling screens such as Log P into natural products drug discovery programs is emerging as an approach to front-load lead-like properties of natural product libraries for high-throughput screening.[11] We have been utilizing polystyrene-divinylbenzene (PS-DVB) chromatographic stationary phases (Diaion HP-20 and PRP-1) for the initial fractionation of crude extracts and the isolation and purification of marine natural products.[12] Unlike silica based stationary phases such as C_8 or C_{18}, the polymer-based chromatographic resins have no stationary phase coating, is a rigid, macroporous, cross-linked polymer that lacks any polar sites and so does not suffer from irreversible binding of polar solutes. In addition polymeric resins are also chemically inert in most organic solvents, can be used at a wide range of pH values (pH 1–13), and has been found to afford improved separation of basic compounds.[13] These characteristics make PS-DVB based separation media a versatile stationary phase for the separation of a broad range of compound classes in a wide range of applications.[14] Although polymeric stationary phases

such as octadecyl-poly(vinyl alcohol) (ODP), and PS-DVB based stationary phases (ACT-1, PRP-1, and PLRP-S) have been used to estimate lipophilicity (Log P) using isocratic HPLC methods with varied success, there is no rapid gradient method available to estimate lipophilicity ($\text{Log P}_{\text{oct}}$) of compounds using a PRP-1 column.[10,15,16]

In this study we have developed a fast-gradient HPLC method using a polystyrene-divinylbenzene PRP-1 column to estimate the lipophilicity of natural products. We believe that the evaluation of log *P* in natural products chemistry will be useful to optimize the generation of drug-like natural product screening libraries, to prioritize leads and improve the success rate of natural products at the later stages of the drug discovery process.

EXPERIMENTAL

Materials

All solvents were HPLC grade and were supplied by Sigma-Aldrich. Water used throughout the study was purified with a Milli-Q water purification system from Millipore (Millipore, Bedford, MA, USA). All reference compounds were obtained from Sigma (St. Louis, MO, USA) and Aldrich (Milwaukee, WI, USA) with high purity (>98%). The marine natural products were isolated from marine organisms collected from the coast of Florida and the Bahamas.

Instrumentation

A Shimadzu (Kyoto, Japan) quaternary low pressure gradient system was used for the HPLC measurements. The solvents were degassed using a DGU-20AT degassing unit and mixed in an FCV-20AL mixer. An LC-20AT pump equipped with a Shimadzu SCL-20AT System controller, permitting automated operation, was used to deliver the mobile phase to the analytical column. Sample injection was performed using a Rheodyne 7725i injection valve (Rheodyne, Cotati, California, U.S.A) equipped with a 20 µL sample loop. Detection was achieved using an UV-SPD-M20A diode array detector and a Shimadzu ELSD-LTII detector. Data acquisition was performed using EZStart chromatography software package version 7.4.

Chromatographic Conditions

Samples were prepared by dissolving 0.1 mg/mL of the solute in 50% (v/v) acetonitrile and 50% (v/v) 50 mM ammonium acetate buffer.

An aliquot of the solution (20 μL) was injected onto the HPLC system. The stationary phase consisted of a Hamilton PRP-1 column (5 μm, 150 mm × 4.6 mm). The flow rate was 1 mL/min. The mobile phase was filtered through a 0.5 μm Fluoropore membrane filter (Millipore, USA) membrane before use. Retention times t_r were measured at least from three separate injections, dead time t_0 was the retention time of sodium nitrate. For the isocratic mode, the mobile phase consisted of different mixtures of acetonitrile and 25 mM $AcONH_4$ adjusted at pH 4.5, 7.2, 9.8 in the range 0 – 100%. For the fast gradient mode, the following gradient program was applied: 0 – 1.5 min, 0% acetonitrile; 1.5 – 16.5 min, 0 – 100% acetonitrile; 16.5 – 18.5 min, 100% acetonitrile; 18.5 – 23.0 min, 100 – 0% acetonitrile; 23.0 – 25.0 min, 0% acetonitrile. The dead time (t_0) was measured by injecting sodium nitrate together with the sample.

Measurement of the Hydrophobicity Index (φ_0) of the Reference Compounds in Isocratic Mode

Theophylline, 5-phenyl-1*H*-tetrazole, benzimidazole, colchicine, 8-phenyltheophylline, indole, acetophenone, propiophenone, butyrophenone, and valerophenone were used as reference compounds that covers the $\log P_{oct}$ range from $-0.02 - 3.26$. These compounds are not ionized at pH 7.4, so their distribution coefficient is equivalent to their $\log P_{oct}$ values. The standard solutions were injected onto the system and their retention times were recorded. The standard mixture contains reference compounds at a concentration of approximately 0.1 mg/mL in acetonitrile:buffer, (50:50) (v/v). The retention times of some of the components varied with pH. This is because some of the components are charged at low or high pH. The retention time of the acetophenone, propiophenone, butyrophenone and the valerophenone should be constant, respectively at all pH values. We used longest t_R under mobile phase at pH 4.5, 7.2, 9.8 to measure hydrophobicity index (φ_0).

For each reference compound, the average retention time (t_R) of three consecutive injections of 20 μL of sample was used to calculate the $\log k'$ values ($\log k' = \log[(t_R - t_0)/t_0]$).

At least 5 mobile phases with different organic-phase concentrations were applied for each reference compounds. Then the $\log k'$ values were plotted against the applied acetonitrile concentration. The slope (S) and the intercept ($\log k'_w$) value were calculated based on $\log k'$ values obtained by a minimum of five organic-phase concentrations. The correlation coefficients of the linear fit were always higher than 0.99. The isocratic chromatographic hydrophobicity index (φ_0) was calculated as $\log k'_w/S$.

Setup CHI-t_R Standard Curve in Gradient Mode with Reference Compounds

Reference compounds were dissolved in an acetonitrile/buffer mixture described above. With gradient chromatography, the retention times (t_R) were measured with mobile phases with three different pH values described above. We used the longest retention times (t_R) of each compound to calculate CHI. A standard curve was created by plotting the highest t_R of each reference compound against its isocratic hydrophobicity index, φ_0, obtained above. An equation relating t_R to φ_0 is also generated from the standard curve. The slope and the intercept of the standard curve were used to convert the gradient retention times of each reference compound to CHI values, φ_0 (predicted value).

Log P_{oct} Calculation

According to the equation:[9]

$$\text{Log } P_{oct} = 0.047 \text{ CHI}_{\text{ACN}} + 0.36 \text{ HBC} - 1.10 \quad (1)$$

Where HBC is hydrogen bond count. The log P was calculated.

Validation of the Method

The fast-gradient HPLC method using the PRP-1 column was validated using a set of 21 commercially available drugs with diverse structures. Using the gradient chromatographic run described above, we used the standard curve to convert t_R measured to CHI value, then we used equation 4 to calculate log P_{oct}. We also obtain the literature log P_{oct} value of the 21 compounds from Drug Bank (http://www.drugbank.ca/). The comparison and correlation analysis between calculated and literature Log P_{oct} were performed.

Estimating the Lipophilicity of Marine Natural Products

The log P of 24 marine natural products were assessed by the fast-gradient HPLC method using the PRP-1 column. Evaporative Light Scattering Detection (ELSD) was used to detect compounds that did not contain a strong UV chromophore. We also used software ChemBioDraw Ultra (CambridgeSoft Corporation) to calculate Log P_{oct}.

RESULTS AND DISCUSSION

According to the HPLC theory, the phase preference of a single solute can be expressed by the capacity factor k ($k = (t_r - t_0)/t_0$). The sample

k values are also related to the volume fraction, φ, of the organic solvent in the mobile phase as:

$$\log k = \log k_w - S\varphi. \quad (2)$$

The intercept log k_w corresponds to the retention in pure water as a mobile phase and represents the commonly employed chromatographic hydrophobicity parameter. S is a solute dependent solvent strength parameter specific to the organic modifier on the stationary phase under consideration. Another retention-related parameter has been introduced recently, the chromatographic hydrophobicity index (CHI), φ_0.[8,17] The φ_0 value represents the volume fraction of the organic solvent in the mobile phase for which the amount of solute in the mobile phase is equal to that in the stationary phase, i.e., the capacity factor is 1 ($\log k = 0$):

$$\varphi_0 = \log k_w / S \quad (3)$$

where φ_0 is equal to the ratio of the intercept and slope of equation 2. In addition to determining the log *P*-values experimentally, they can also be predicted by using a number of log P_{oct} calculation methods.

The isocratic retention times were measured by using various volume percents of acetonitrile in the mobile phase, preferably bracketing the retention when $\log k' = 0$ (that is the retention time was close to the double of the dead time). The isocratic hydrophobicity index, φ_0, was calculated from the slope (S) and the intercept ($\log k'_w$) values of the straight lines obtained by plotting $\log k'$ vs φ (φ_0 = intercept/slope), based on minimum three points and $r > 0.99$. (Shown in Table 1)

The gradient retention time (t_R) (Shown in Table 1) was measured under the gradient profile, which included a 15 min linear acetonitrile

TABLE 1 The CHI and Log *P* Values of 10 Reference Compounds for the Gradient HPLC Calibration for CHI Measurement Using an Acetonitrile Gradient

Reference Compound	φ_0	T_r (Gradient)	CHI	HBC	Log P_{oct} (Calc.)	Log P_{oct} (Literature)
Theophylline	12.57	5.89	11.46	1	−0.2	−0.05
5-Phenyl-1H-Tetrazole	14.29	6.04	12.79	1	−0.14	1.42
Benzimidazole	24.26	7.32	24.03	1	0.39	1.38
Colchicine	32.86	8.61	35.46	1	0.93	0.92
8-Phenyltheophylline	43.88	9.82	46.13	2	1.79	2.05
Acetophenone	64.67	12.03	65.62	0	1.98	1.66
Indole	77.94	13.16	75.59	1	2.81	2.14
Propiophenone	81.45	13.79	81.15	0	2.71	2.19
Butyrophenone	93.18	14.95	91.38	0	3.19	2.73
Valerophenone	98.76	15.95	100.2	0	3.61	3.28

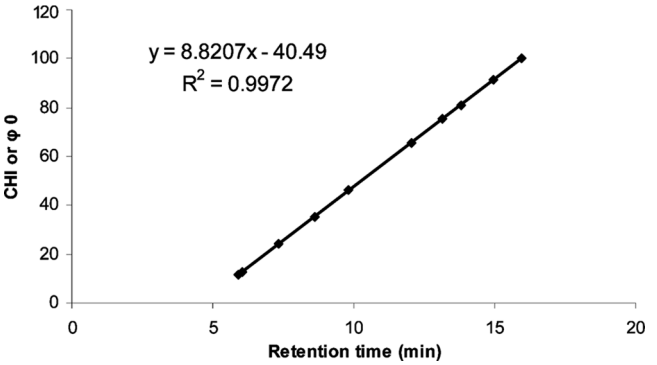


FIGURE 1 Calibration plot for the measurements of the Chromatographic Hydrophobicity Index (CHI) of 10 reference compounds.

gradient from 0 to 100%. The chromatographic hydrophobicity index (CHI) method used fast-gradient reversed phase HPLC to model octanol/water partitioning of a compound by correlating the retention time with the percentage of acetonitrile required to achieve an equal distribution of the compound between the mobile and stationary phases. By plotting φ_0 (obtained in isocratic mode) against t_R (obtained in gradient mode), a standard curve (Figure 1) and equation (Equation 4) were generated. Based on this equation, CHI of each reference compound was calculated (Shown in Table 1).

$$\begin{aligned} \text{CHI} = \varphi_0(\text{predicted value}) &= 8.8207\text{t}_\text{R} - 40.49 \\ \text{R}^2 &= 0.9972. \end{aligned} \tag{4}$$

We used 21 commercially available drugs (Table 2) to validate the method. The set represents a wide range of chemical structures and lipophilicities. The plot of the calculated and literature $\log P_{oct}$ values can be seen in Figure 2. The correlation coefficient is 0.9664.

Paired t test results :

The two-tailed P value equals 0.7732.

$\text{t} = 0.2922, \text{df} = 20, \text{standard error of difference} = 0.053, \text{t} < \text{t } 0.05.$

The difference between the calculated and literature $\log P_{oct}$ values are considered to be not statistically significant. It can be seen that an excellent correlation was found and it provided experimental confirmation of the hypothesis that a linear gradient retention time can be used as a measure of compounds' lipophilicity using a polymeric PRP-1 column.

TABLE 2 Log P_{oct} Values of 21 Drug Compounds Calculated from Gradient Retention Times (t_R), CHI Values and Hydrogen Bond Count (HBC) Using Gradient Elution on a PRP-1 Column and Literature log P_{oct} Values

Drug	T_r (min)	CHI	HBC	Log P_{oct}	Log P_{oct} (Literature)
Adenine	5.21	5.12	2	−0.14	−0.45
Caffeine	5.66	9.43	2	0.06	−0.07
Cimetidine	6.87	19.80	3	0.91	0.46
Acetaminophen	7.06	21.76	2	0.64	0.9
Sumatriptan	7.31	23.83	3	1.09	1.1
Hydrocortisone	9.12	39.78	3	1.85	1.69
Bendroflumethiazide	10.12	48.76	3	2.27	2.09
Lidocaine	11.31	59.03	2	2.40	2.48
Diazepam	12.07	65.16	2	2.71	2.8
Dibucaine	16.38	104.15	1	4.15	4.29
Tinidazole	7.95	29.41	0	0.28	0.12
Trimethoprim	8.46	33.93	2	1.21	1.36
Triamterene	8.11	30.83	3	1.43	1.24
Terbutaline	6.52	16.73	4	1.13	1.57
Tripelennamine	14.5	87.48	0	3.01	2.65
Tolazoline	10.21	49.45	1	1.59	1.21
Benzthiazide	11.20	58.22	3	2.72	2.92
Oxybutynin	16.36	103.97	2	4.51	4.68
Atropine	9.9	46.70	2	1.81	1.83
Hetacillin	7.59	17.35	4	1.52	1.86
Scopolamine	8.73	36.33	3	1.69	1.66

For application of this method for the assessment of the log *P* of natural products, we used 24 biologically active marine natural products isolated in our laboratory (Shown in Table 3). Since the number of hydrogen bonds of a compound, i.e., the number of protons available for hydrogen bonding, can influence calculated log P_{oct}, we also have the potential to use this method to estimate the lipophilicity range of natural products, even

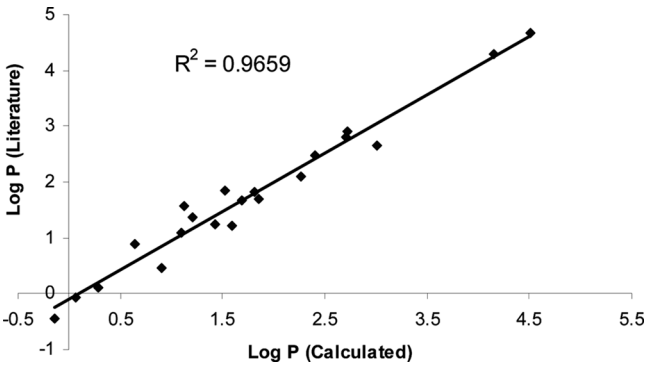
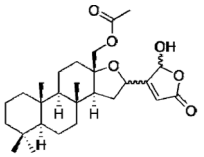
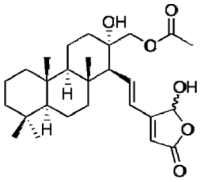
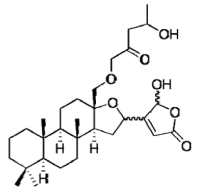
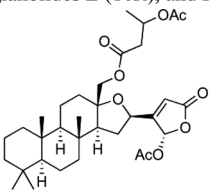
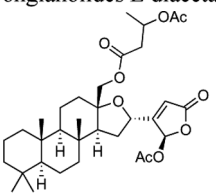
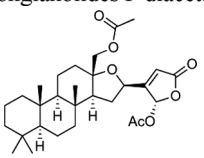


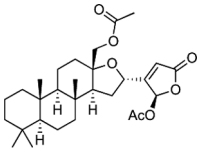
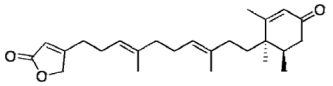
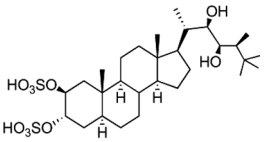
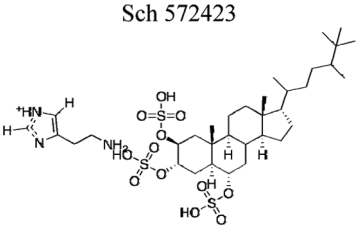
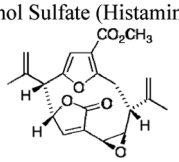
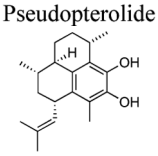
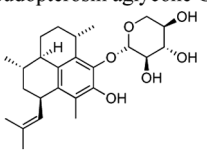
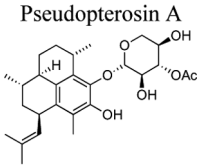
FIGURE 2 Correlation between calculated and literature log P_{oct} for 21 drugs.

TABLE 3 Log P_{oct} Values Determined from Gradient Retention Times (t_R), CHI Values and Hydrogen Bond Count (HBC) Using Gradient Elution on a PRP-1 Column and Software Calculated Log P_{oct} for a Series of Marine Natural products

Compound	t_R (min)	CHI	HBC	Log P_{oct}	Log P_{oct} (Calc.)
 Lintenolide A(16*R*), and B(16*S*)	15.89	99.80	1	3.95	4.36
 Spongianolide A	13.3	76.84	2	3.23	4.5
 Spongianolides E (16*R*), and F (16*S*)	14.74	89.61	2	3.78	3.52
 Spongianolides E diacetate	16.33	103.70	0	3.77	3.98
 Spongianolides F diacetate	17.94	117.98	0	4.44	3.98
 Lintenolide A acetate	16	100.78	0	3.64	4.59

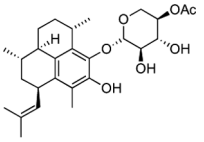
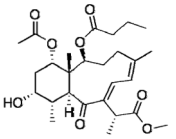
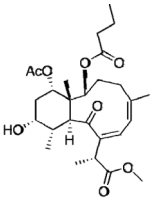
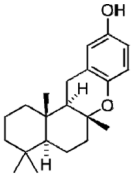
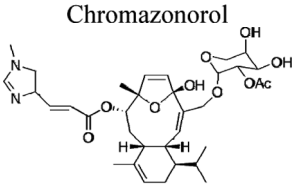
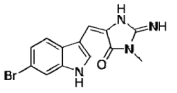
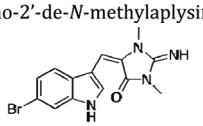
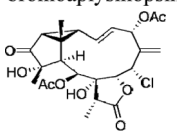
(*Continued*)

TABLE 3 Continued

Compound	t_R (min)	CHI	HBC	Log P_{oct}	Log P_{oct} (Calc.)
 Lintenolide B acetate	15.85	99.45	0	3.57	4.59
 Cyclolinteinone	13.91	81.91	0	2.75	5
 Sch 572423	7.44	24.89	2	0.77	0.5
 Halistanol Sulfate (Histamine Salt)	9.16	40.12	2	1.51	3.52
 Pseudopterolide	13.9	82.16	0	2.76	1.51
 Pseudopterosin aglycone G-J	18.19	120.19	2	5.27	5.47
 Pseudopterosin A	14.22	84.00	3	3.97	4.17
 Pseudopterosin C	15.83	99.27	3	4.59	4.4

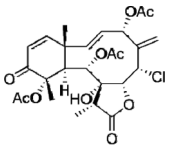
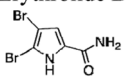
(*Continued*)

TABLE 3 Continued

Compound	t_R (min)	CHI	HBC	Log P_{oct}	Log P_{oct} (Calc.)
 Pseudopterosin D	16.05	101.22	3	4.68	4.4
 Briareolate ester G Δ^8 (*E*)	13.63	79.77	1	3.01	2.65
 Briareolate ester G Δ^8 (*Z*)	14.48	87.30	3	4.08	2.65
 Chromazonorol	17.7	115.85	1	4.70	5.52
 Eleutherobin	10.73	54.06	4	3.08	2.11
 6-bromo-2′-de-*N*-methylaplysinopsin	10.48	51.84	2	2.16	1.52
 6-bromoaplysinopsin	12.31	68.06	2	2.06	1.75
 Erythrolide A	9.75	45.37	3	2.11	0.32

(*Continued*)

TABLE 3 Continued

Compound	t_R (min)	CHI	HBC	Log P_{oct}	Log P_{oct} (Calc.)
 Erythrolide B	10.32	50.42	2	1.74	0.74
 4,5-dibromopyrrole-2-carbamide	9.85	46.26	2	3.90	1.02

through we may not know its structure.

P value and statistical significance:

The two-tailed P value equals 0.8137.

By conventional criteria, this difference is considered to be not statistically significant.

Confidence interval:

The mean of Group One minus Group Two equals 0.0975

95% confidence interval of this difference: From -0.7304 to 0.9254

Intermediate values used in calculations:

$t = 0.2370$

$df = 46$

standard error of difference = 0.411.

Although natural products are considered to be an enormous resource of drug candidates and can provide countless opportunities to discover new drugs, interest in the development of natural products by the pharmaceutical industry has declined.[18–20] A major factor for the decrease in natural products is that modern drug discovery research requires rapid screening, hit identification and hit to lead development. If natural products are to be considered again as an invaluable resource of lead compounds by the pharmaceutical industry natural products programs need to comply with the current timelines of the current drug discovery paradigm established with the high-throughput screening of synthetic chemical libraries.

In 2008, Quinn et al. prepared a screening library of 814 natural products in which 85% of the compounds had no Lipinski violations and demonstrated the possibility of generating natural libraries that conform to the physicochemical properties required in today's drug discovery environment.[11,21] According to Lipinski's "Rule of Five", drug candidates should possess suitable hydrophobicity (partition coefficient $\log P$ less than 5).[2] Thus, integration of drug-like properties screening into the early stages of drug development could potentially speed up the drug development process. The shake-flask procedure is the standard method for determining $\log P_{oct}$'s in the range of $-2-4$.[22] However, it is time and labor consuming and requires relatively large amounts of pure compounds. So, the traditional shake-flask method is not suitable for natural product research in which sample is limited. The RP-HPLC method is an indirect way to estimate $\log P_{oct}$ values in the range of $0-6$ and has become a standard procedure. The advantages of this approach such as, speed, insensitivity to impurities, reduced sample handing and sample sizes makes it amenable for natural products drug discovery research.

Valkó introduced a chromatographic hydrophobicity index based on a fast-gradient RP-HPLC method, and also established equation of $\log P$ calculation.[9] Based on his work, we applied this methodology using a polymeric PRP-1 column to estimate lipophilicity. The result of this study validated the use of the PRP-1 polymeric reversed phase fast gradient HPLC method for determining lipophilicity and showed that we can estimate the lipophilicity of natural products with good accuracy.

The estimated $\log P$'s of the 24 marine natural products isolated in our laboratory matched the requirement of drug-like criteria ($0 < \mathrm{Log}\, P_{oct} < 5$). We did see some large differences in the experimentally determined $\log P$ values and the calculated values. The most significant differences were for the natural products containing bromine and chlorine. The large variation in natural products containing bromine has been previously reported.[11] In the future, we believe integration of a HPLC method to estimate lipophilicity into our natural product isolation process will allow us to optimize the generation of natural product libraries for HTS and develop an efficient polarity-optimized approach for the discovery of potential bioactive natural products.

CONCLUSION

In this study a fast-gradient HPLC method using a polystyrene-divinylbenzene PRP-1 column has been demonstrated to be useful for the estimation of lipophilicity. An excellent correlation was found between the results of the experimental determinations and the literature values of

log P for a diverse set of commercially available drugs. We also found a good correlation between the experimentally determined and software calculated log P's for a diverse set of marine natural products. Some discrepancies were observed between the measured value of log P and the software calculations of the natural products containing halogens atoms. The method is rapid, insensitive to impurities, and requires very little compound and is amenable for integrated into a natural products drug discovery research program.

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