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Determination of log *P* values of new cyclen based antimalarial drug leads using RP-HPLC

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Lipophilicity, expressed by log P, is an important physicochemical property of drugs that affects many biological processes, including drug absorption and distribution. The main purpose of this study to determine the log *P* values of newly discovered drug leads using reversed-phase high-performance liquid chromatography (RP-HPLC). The reference standards, with varying polarity ranges, were dissolved in methanol and analyzed by RP-HPLC using a C18 column.The mobile phase consisted of a mixture of acetonitrile, methanol and water in a gradient elution mode. A calibration curve was plotted between the experimental log *P* values and obtained log k values of the reference standard compounds and a best fit line was obtained. The log k values of the new drug leads were determined in the same solvent system and were used to calculate the respective log *P* values by using the best fit equation. The log *P* vs. log k data gave a best fit linear curve that had an R^2 of 0.9786 with *P* values of the intercept and slope of $1.19 \times 10^{-6}$and 1.56×10^{-10}, respectively, at 0.05 level of significance. Log *P* values of 15 new drug leads and related compounds, all of which are derivatives of macrocyclic polyamines and their metal complexes, were determined. The values obtained are closely related to the calculated log *P* (Clog P) values using ChemDraw Ultra 12.0. This experiment provided efficient, fast and reasonable estimates of log *P* values of the new drug leads by using RP-HPLC.

1. Introduction

Physicochemical profiling, including the determination of solubility, ionization, lipophilicity and permeability of novel drug substances provides information predictive of pharmacokinetic parameters and biological effects. Lipophilicity, often expressed as the logarithm of a compound's octanol/water partition coefficient (log *P*), plays a pivotal role in absorption, distribution, metabolism and elimination (ADME) properties (Testaet al. 2000). Partition coefficients were first characterized by Berthelot and Jungfleisch (1872) to help correlate and analyze the efficacy of biologically active substances. QSAR studies correlated with lipophilicity were established considering the relative potencies of drugs at the site of action, regulation of the drug transportation, protein binding, toxicity, enzyme induction, metabolism, and pharmacokinetics. The relationship between affinity to lipids and biological activity of drug substances was first reported by Overton (1897), Meyerand Baum (1899). Hansch and Steward (1964)reported many examples and reasons for the parabolic relationship between lipophilicity and biological activity. Penniston and co-workers evaluated a mathematical model supporting the parabolic relationship between the molecules that are transported through a series of membranes (lipid barriers) and its log *P* values in a given time. This estimated relationship helps in determining an optimum log *P* value for a drug that is transported to the site of action within the time of a biological assay Penniston (Beckett et al. 1969).

Lipophilicity affects most of the processes in drug action that are closely related with the permeability and solubility of a drug. Hence, determination of lipophilicity is a fundamental requisite during the early stages of the drug development processes (Kaliszan et al.2001). Fig. 1 shows a schematic diagram of drug discovery and development stages indicating the most critical stage where log *P* determination is important.

Oral drug delivery, which is the most common route of drug administration, requires the drug to cross lipid bilayers in the intestinal epithelium. Lipophilic molecules can easily reach the blood stream and a target by passing through the cell membrane and cytoplasm. However, insufficient absorption may lead to reduced bioavailability. The protein binding of a drug that influences both distribution and elimination is also highly dependent on lipophilicity (Lazicek and Lanznickorva 1995). Drug metabolism is influenced by the lipophilicity parameter; more lipophilic drugs are more easily accessible to cytochrome P450 family enzymes and thus more susceptible to metabolism (Lazicekand Lanznickova 1995). Cross et al. (1997) have shown that the toxicity of a series of alcohols was proportional to the number of carbon atoms, and thus the lipophilicity. Nava-Ocampo and Bello-Ramirez (2004) have also revealed that the toxicity of some local anesthetics was substantially increased at log *P* value 3 due to plasma availability of these drugs. A recent study on the known bile acids indicated that the relationship between cytotoxic properties and lipophilicity is complex and indirect (Sharma et al. 2010).

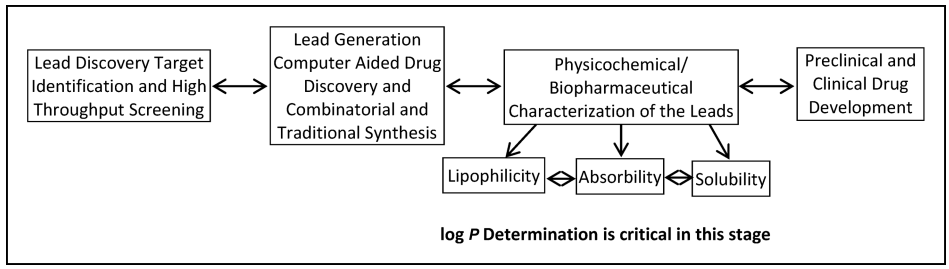

Fig. 1: Stages of drug discovery and development showing the critical stage where log *P* determination is important.

Log *P* values for most of the known drugs are only estimated, yet they are often used as references compounds (Craig et al.1995). Calculating log *P* values has become the key element in predictingthe biological effects of organic chemicals from their physical properties through the use of structure-activity relationships (Gouldet al. 1975).

In recent years, a surrogate method for the determination of log *P* is reversed-phase high-performance liquid chromatography (RP-HPLC), which overcomes the difficulties of the conventional shake-flask method (Walter et al.1977). RP-HPLC is a process in which the drug is injected onto a column of fine particles coated with water insoluble, non-polar oil, and then eluted with a polar solvent such as water or methanol (Veith et al.1979). Log *P* values are determined using the regression line equation between the chromatographic parameter log k and known experimental log *P* values of the reference compounds. Therefore, the important parameter that is to be estimated is the capacity factor (k) characteristic of the specific column and each drug molecule. The choice of stationary phase is critical, because of the direct relationship between retention time and the affinity ofthe test compound. Retention times are specific to individual compounds and are the basis for qualitative analysis. However, they are also necessary in quantitative analysis to identify peaks that are then quantified by using the area or height of the peak identified by the detector. Within the limit of resolution of the elution curves, for a given stationary phase of known length and flow rate, the retention values can be made to vary in proportion to the log *P* values of a series of drug molecules. Modification in the composition of the mobile phase is the most efficient way to vary retention times. That is, the order of the retention values of compounds differ with the composition of the aqueous and the organic solvent used (Schoenmakers et al. 1980). This clearly indicates that the chromatographic conditions play a major role in the estimation of the retention parameters.

Lipophilicity estimation using RP-HPLC is based on the principle of the partition of a solute between a high polarity eluent and a low polarity stationary phase. RP-HPLC has several advantages, like speed of determination, better reproducibility, on-line detection, insensitivity to impurities or degradation products, broader dynamic range, reduced sample handling, and small sample size when compared to other methods (Braumannet al. 1998). The affinity of the drug for the stationary phase is defined by the capacity factor (k). The capacity factors and obtained log *P* values of the reference standards are used to create a calibration curve. The new compounds with unknown log *P* values are then injected and their capacity factors are used to predict the log *P* values by extrapolating them on the calibration curve (Rutkowska et al. 2013).

The most widely used stationary phase for RP-HPLC is the C-18 column. In the case of basic drugs, interference of silanophilic interactions, which are associated with free silanol sites and also include hydrogen bonding, produces great increases in retention times. In addition to the hydrophobic interactions with the solvents, silanophilic interactions arising from the hydrogen bonding and ionic mechanisms between the analyte and the silanol function present on the silica surface play critical role in the reduction of the retention time of the analytes. Thus the silanophilic interaction is an important phenomenon that must be accounted for HPLC method development practices (Nahum and Horvath 1981). This is a considerable drawback in the

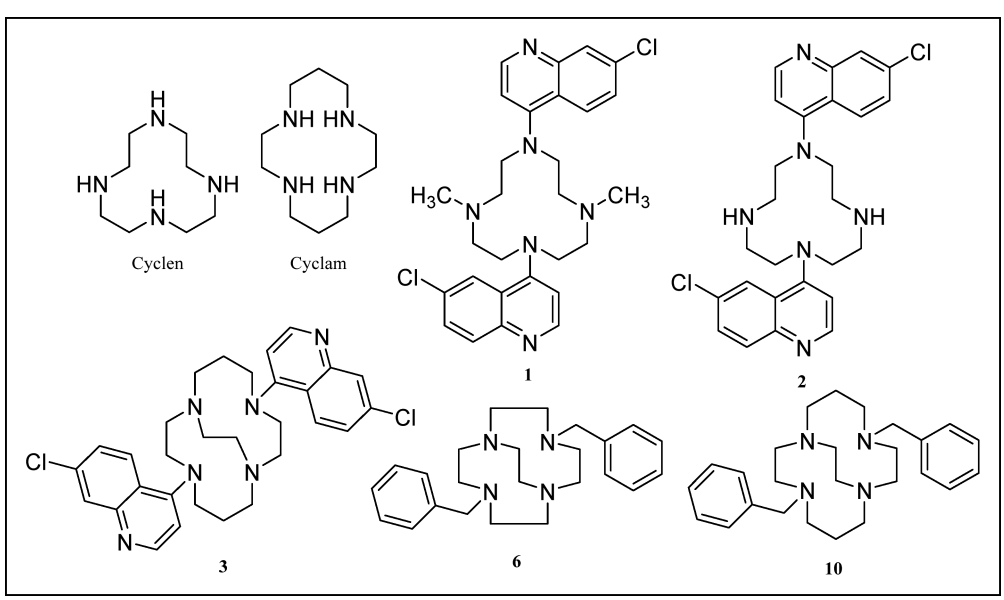

Fig. 2: Structures of cyclen and cyclam and related antimalarial (1–5) and other drug leads (6–12). Compounds 4 and 5 are the $FeClO_4$ and $MnCl_2$ complexes of compound 2, respectively. Compounds 6 and 10 were first published by Weisman et al. (1996) compound 7 is a previously published $FeCl_2$ complex of compound 10 (Hubin et al. 2003*)*. Structures of compounds 8, 9, 11 and 12 are not shown due to potential patent issues.

Table 1: Standard reference drugs and their lipophilicity parameters

Compound	Mol. Weight	Elog P^a	RT	k values	log k	log RT
Penciclovir	253.26	−1.1	4.905	1.366516	0.135615	0.690639
Adenosine	267.24	−1.05	5.623	1.70988	0.232966	0.749968
Metoclopramide	299.80	2.62	9.6405	3.5984	0.55611	0.9841
Tacrine	198.26	2.71	9.195	3.3817	0.52914	0.963552
Quinine	324.42	3.44	9.97467	3.803984	0.576153	0.998899
Quinidine	324.42	3.44	9.90867	3.768369	0.580239	0.996015
Triprolidine	278.39	3.92	11.2983	4.447589	0.648125	1.053013
Promethazine	284.42	4.81	14.668	5.9898	0.77741	1.166371
Amitriptyline	277.40	4.92	13.2887	5.404193	0.732731	1.123482
Chlorpromazine	318.86	5.41	13.648	5.575236	0.746263	1.135069
Thioridazine	370.58	5.9	13.9097	5.705625	0.756303	1.143318
Butenafine	317.47	6.6	18.1495	7.6488	0.88359	1.258865
Amiodarone	645.31	7.57	20.9865	8.9746	0.95301	1.32194

[a] Data taken from the references: Hansch, & Leo,1995; Avdeef et al. 1995; Avdeef 1995.

partitioning mechanism of RP-HPLC (Tayar at al. 1988) and it is overcome by adding masking agents to the solvent or by using columns with reduced free or available silanol sites (Minick et al. 1988).

Mixtures of water (or aqueous buffer) with an organic modifier like methanol (a hydrogen bond donor and acceptor), acetonitrile, (a hydrogen bond acceptor of high polarity) or tetrahydrofuran (THF, a less polar hydrogen bond acceptor), are the most widely used mobile phases in RP-HPLC. Methanol is the most suitable organic modifier for RP-HPLC, as it does not markedly alter the hydrogen bonded network of water or affect polar interactions of solutes (Karger et al. 1976). However, for more lipophilic solutes, methanol leads to long retention times. Therefore, acetonitrile and THF have been used to reduce the retention times and to broaden the range of lipophilicity measurable by RP-HPLC (Horvath and Melander 1977). Acetonitrile prevents adsorption of positively charged amines onto the column, and also acts as a masking agent because it preventsthe solvation of stationary phase with sufficient water (Bechalany et al. 1991).

The main purpose of the present study was to develop a convenient and efficient method of determining the log *P* values of a series of newly discovered cyclen bisquinoline antimalarial drug leads (Khan et al. 2009) and related compounds by RP-HPLC method. All the compounds in this series are basic in nature. The structures of representative compounds are shown in Fig. 2. Compounds 8, 9, 11, and 12, whose structures are not shown due to potential patent issues, are derivatives of the known biologically active bis-tetraazamacrocycles. Analogues of these compounds have shown extreme efficiency at antagonizing the CXCR4 chemokine receptor and are being developed as medicinal compounds, as well as imaging agents (Valks et al.2006).

2. Investigations, and Results

The reference standards and their lipophilicity parameters, such as experimental log *P* (Elog *P*) values from the literature, retention time (RT), k and log k values obtained in this study using the solvent system and stationary phase mentioned earlier are shown in Table 1.

The best fit regression line equation was obtained from the plot between the Elog *P* values and the log k for the calibration mixture is shown in Fig**.** 3. The regression line obtained from this plot gave a best fit linear curve with a R^2 of 0.9786, which indicates that 97.86% of the variability of the data could be explained by the linear regression. The regression line, expressed in the linear form is Y = 11.054x – 3.1162, where the predictor variable X represents the log k, and the outcome variable Y represents the log *P*. The estimated regression parameters are a = − 3.1162 (intercept) and b = 11.054 (slope) with their *p* values as 1.19×10^{-6} and 1.56×10^{-10}, respectively. Effects of both the intercept and slope are statistically significant as the probability *p* value is less than the level of significance 0.05. The log *P* values of new drug leads are determined from this calibration curve (Fig. 3).

With the obtained linear equation, log *P* values of the new drug leads were determined. From the HPLC chromatograms, all the average retention times (RT) of the test compounds and average retention times of methanol were noted. From the obtained retention times, k values and their logarithmic values were calculated and are shown in Table 2. These capacity factors allowed us to determine the log *P* values using the obtained calibration curve from the reference compounds. As discussed below, among four different types of calibration curves, the log *P vs*. log k plot gave the best fit line with the highest R^2 value and was therefore, used to calculate the log *P* values of the compounds. The obtained log *P* values, termed "Elog *P*", are given in Table 3.

The RT values shown in Table 2 are the averages of 3 runs. A standard series method is used to determine the calibration curve which was then used in determining the parameters of the unknown compounds. All of the reference standards Table 4 were measured first to obtain the calibration curve, and then the unknown samples were estimated by extrapolation.

The calibration curve is also plotted between (i) the Elog *P* values and the k values, (ii) the Elog *P* and the log of RT, and (iii) Elog *P* values and RT values. The plots (i) and (iii) gave best fit polynomial line equations with R^2values of 0.9771 (Fig. 4) and 0.9756 (Fig. 5), respectively. As shown in Fig. 7 and 8, plot (ii) gave a best fit linear line with an R^2 of 0.9762 (Fig. 6).

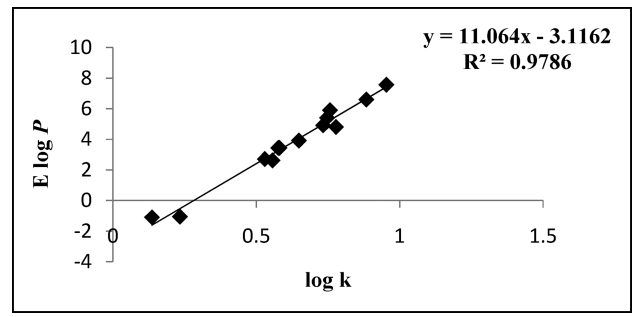

Fig. 3: Calibration curve of Elog *P* verses log k.

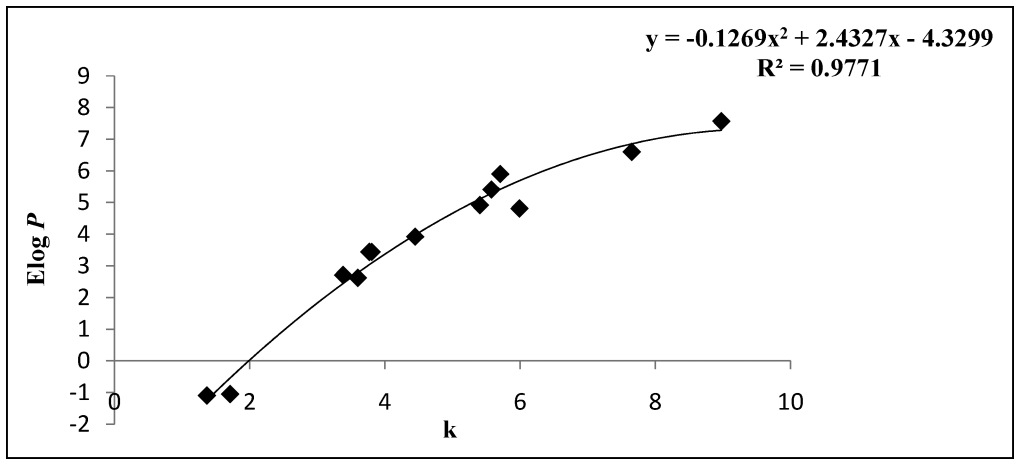

Fig. 4: Calibration curve of Elog *P* versis k.

Table 2: Results obtained from the RP-HPLC chromatograms, calculated capacity factors and the experimental log *P* values

Drug	Mol. Weight	RT	RTo(t_0)	k	log k
CYCLAM	172.28	4.7	2.164	1.171904	0.068892
CYCLEN	200.33	4.5721	2.167	1.109875	0.045274
1	523.51	16.0925	1.98374	7.112202	0.852004
2	495.45	13.0375	1.983779	5.572053	0.746015
3	549.54	10.2405	1.98756	4.152297	0.618288
4	885.66	12.994	1.983278	5.551779	0.744432
5	622.20	12.9575	1.986641	5.522316	0.742121
6	378.56	13.376	2.168	5.169742	0.713469
7	533.36	12.485	2.168	4.758764	0.677494
8	554.87	7.994	2.165	2.692379	0.430136
9	358.31	8.729	2.165	3.031871	0.481711
10	406.62	13.647	2.164	5.306377	0.724798
11	554.87	9.713	2.165	3.486374	0.542374
12	498.76	10.034	2.165	3.634642	0.560462

Hansch et al. (1979) determined the log *P* values using 6 reference compounds with a polarity range of 2.13 to 6.11. They estimated the log *P* values from the linear equation obtained from the calibration curve between the Elog *P* values and the log RT values, where the R^2 was 0.975. They also estimated and

Table 3: Elog *P* and Chem Draw Clog *P* values of the drug leads and related compounds

Drug Lead	log P from plot of log P vs. log k (Elog P)	Chem Draw Clog *P* values
CYCLAM	−2.35398	−1.85
CYCLEN	−2.61529	−2.968
1	6.310372	6.62447
2	5.13771	5.59347
3	3.724538	6.40747
4	5.120196	7.00227
5	5.094627	7.17387
6	4.777619	4.649
7	4.379595	4.462
8	1.642826	2.251
9	2.213447	2.3944
10	4.902966	4.462
11	2.884626	2.251
12	3.084748	2.562

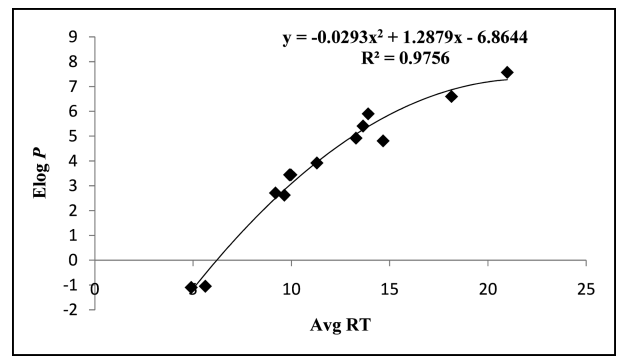

Fig. 5: Calibration curve of Elog *P* verses retention time.

compared the mean standard deviations for the Clog *P* values and Elog *P* values for accuracy. Similarly, we also estimated a calibration curve between Elog *P* values and log RT values, where the R^2 obtained is 0.9762 (Fig. 6). This data indicates that the logarithmic values of the retention times are linearly correlated with the logarithmic values of partition coefficient. Recent studies reported that plots using the capacity factor are more accurate than that of the plots using the retention times. To determine the accuracy of this calibrated liquid chromatographic method, the log *P* values that were obtained from the linear equation (Clog *P*) were compared with those reported in the literature (Elog *P*), as shown in Table 4. The R^2 values obtained from the calibration curve of log k values are always higher when compared to the R^2 values obtained from retention times (Veith

Table 4: Results showing the Clog *P* and Elog *P* values

Drug	Elog P	Clog P
Penciclovir	−1.1	−1.61576
Adenosine	−1.05	−0.53866
Metoclopramide	2.62	3.036601
Tacrine	2.71	2.738205
Quinine	3.44	3.258357
Quinidine	3.44	3.303564
Triprolidine	3.92	4.054655
Promethazine	4.81	5.485064
Amitriptyline	4.92	4.990736
Chlorpromazine	5.41	5.140454
Thioridazine	5.9	5.251536
Butenafine	6.6	6.65984
Amiodarone	7.57	7.427903

Table 5: Mobile phase for log *P* determination: gradient elution mode

Time/solvent	Water (i)	Acetonitrile (ii)	Methanol (iii)
T = 0min	96	0	4
T = 10mins	64	30	6
T = 12min	4	90	6

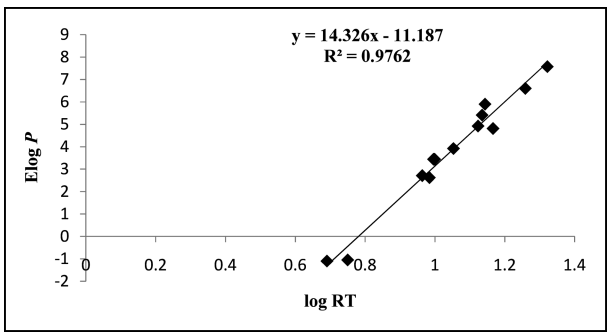

Fig. 6: Calibration curve of Elog *P* versis log RT.

et al.2001). The present method using 13 reference drugs with polarity range of -1.1 to 7.57 plotted three calibration curves of: i) log *P* vs log k, ii) log *P* vs RT, and iii) log *P* vs k. The R^2 values obtained from plots i), ii), and iii) are 0.9786, 0.977 and 0.9701, respectively. It is obvious that plot i) (Fig. 7) has the best fit among all these three types of plots as evident from the respective R^2 values and thus it was used for log *P* determination of the compounds, which is termed Elog *P*.

To estimate the linearity between the Elog *P* values and the Chem Draw log *P* values (Clog *P*) of the test compounds, a curve was plotted between them as shown in the Fig. 8a. A linear curve was obtained with an R^2 of 0.8971. A calibration curve plotted without three outlier compounds (3, 4, and 5) gave a better linear fit with an R^2 of 0.9773, as shown in Fig. 8b.

The deviation from linearity by compounds 3, 4, and 5 can be explained by their chemical structures. The Chem Draw software calculates the values according to their molecular formula and cannot estimate their 3D structures. The crossed bridge of compound 3 is definitely (Hubin et. al. 2001) folding the structure so that nitrogen atoms are exposed to interact easily with the solvent, giving higher polarity than obtained by calculation using ChemDraw. Therefore, their retention times were reduced, while simultaneously reducing their capacity factors and log *P* values. Compounds 4 and 5 are Fe and Mn complexes, respectively for which ChemDraw could not give good estimates of their log *P* values. The RP-HPLC experiment, however, is not limited in this way and helped to get reasonable estimates of log *P* values of all of the compounds. It had been reported previously that HPLC can provide a good estimation of log k values in the

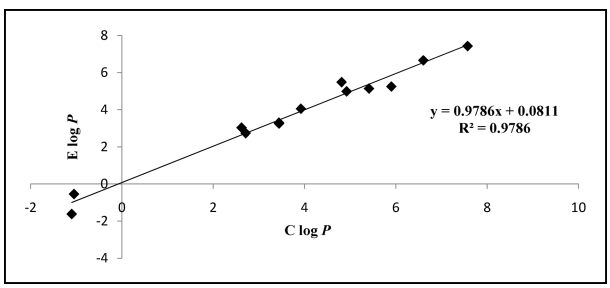

Fig. 7: Calibration curve of Clog *P* verses Elog *P*.

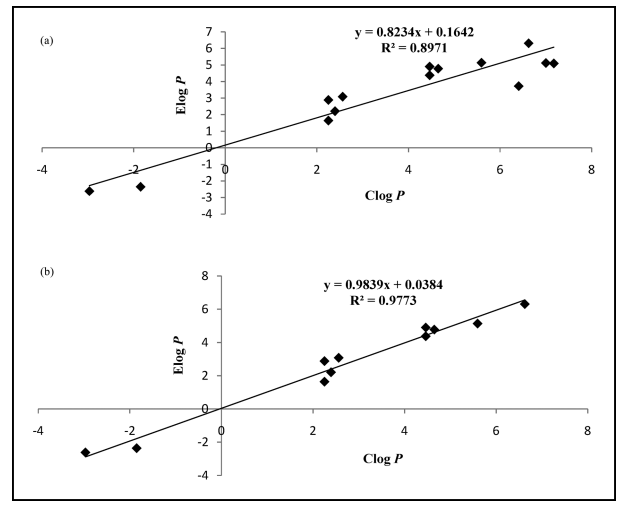

Fig. 8: (a) Plot of log *P* values of the compounds obtained from the plots log *P* vs. log k (Elog *P*) vs. log *P* calculated using Chem Draw (Clog *P*). (b) Plot of log *P* values obtained from the plots log *P* vs. log k (Elog *P*) vs. log *P* calculated using Chem Draw (Clog *P*) without the drug leads 3, 4, and 5.

range of -0.3 to 2.5 (Cimpan et al. 1998). This study showed a good estimate of log *P* values of compounds between 2 to 7 (Seydel and Schaper 1982). Hence, the log *P* values obtained from the linear equation all the drug leads are acceptable.

In short, the results obtained from the present method demonstrate a strong relationship between a compound's retention times and their log *P* values. It also fulfills the objectives of developing a simple method for calculating log *P* from their retention times and also reducing the cost of analysis. Log *P* values of any new drugs, which have approximate log *P* ranges from -1.5 to 8, can be determined using the above calibration line equation obtained from the plot of Elog *P* vs. log k.

3. Discussion

A lipophilicity experiment provided efficient, fast and reasonable estimates of log *P* values of new drug leads by using RP-HPLC. The standard curve obtained by using reference standards will be useful in determining lipophilicity of new drug leads under study in our laboratory. The applied influence of lipophilicity on the elemental properties of chemicals results in ceaseless and intensive research in this field. Advancement of models to anticipate this parameter based on their retention performance and calibration curves has attracted considerable attention. Nevertheless, there is a consistent essentiality for standardization of the methods for lipophilicity estimation. The ability to forecast lipophilicity accurately, despite the predominance of the approach, cannot be explicitly evaluated due to the wide variety of chemical structures, experimental methods, calculations, and partition systems. There are also a variety of log *P* determining methods which are calculated based on different algorithms that are available (Hou and Xu 2003). Development of chromatographic assessments plays a crucial role in areas like medicinal chemistry and molecular pharmacology. The reproducibility and precision of retention parameters is mainly enhanced by the automated chromatographic procedures and also by optimizing conditions. Stationary phases facilitate mimicking of bio-partitioning, moreover, give better insight into permeability phenomenon and the role of molecular properties in the biological activity of similar and unrelated compounds (Rutkowska et al. 2013). A large set of chromatographic data requires the use of a wide range of statistical methods, which help in providing systematic information. As mentioned in our report, all the results for determining lipophilicity are based upon

Table 6: Trials using isocratic mode

Trial	Water (%)	Acetonitrile (%)	Methanol (%)
1	50	50	
2	50	—	50
3	—	50	50
4	70	20	10
5	70	10	20
6	80	15	5
7	80	5	15
8	55	45	5
9	50	30	20

Table 7: Trials using gradient mode

Trial	Time (mins)	Water (%)	Acetonitrile (%)	Methanol (%)
1	0	80	0	20
	10	50	30	20
	12	15	80	5
2	0	90	5	5
	10	55	30	15
	12	10	90	0
3	0	95	0	5
	10	60	30	10
	12	5	90	5
4	0	96	0	4
	10	64	30	6
	12	4	90	6
5	0	98	0	2
	10	66	30	4
	12	2	90	8

statistical methods of interpretation. At the moment, there is a demanding requirement for fast and accurate procedures to quantify the lipophilicity of new drug leads.

Correlation between the chromatographic parameter and the biological activity is the most important phenomenon in QSAR studies. In a few cases, correlation between these two parameters is poor (Spencer et al. 1985) due to unsuitable selection of chromatographic conditions, like taking high proportions of organic modifier in the mobile phase, or poor choice of reference compounds for the calibration curve. Development of more solvent systems to validate the lipophilicity values of compounds are still in progress. A chromatographic system which best correlates with the biological system must be established for an improved operation of HPLC in QSAR studies. The method we used leads to a simpler correlation of the biological activity with a single chromatographic parameter when compared to the conventional QSAR with many complex parameters. For basic drugs, the silanophilic interactions, or the secondary interactions, can interfere with the partition system, inducing excessive and inaccurate lipophilicity. However, there are ways to decrease these interactions and to acquire extrapolated capacity factors, k and log k, which emulate the accuracy of the log *P* values.

The calibration curve that is obtained using our method is very accurate in determining the log *P* values of new drug leads. This can be utilized even with different drugs leads where log *P* values are required. Accuracy can always be compared when the structure of the drug is known. Given the structures of the compounds tested, the presently developed method has proved to be accurate enough to determine the log *P* values. Therefore, log *P* values of a drug under development can be estimated with the HPLC retention times, even if the structure of the drug is not known. The linear equation obtained from the calibration curve plotted between log *P* and log k values provides accurate log *P* values. In this study, all the compounds possess log *P* values between the ranges of 1.9 to 6.3. As discussed earlier, drugs with log *P* values between 2 to 7 are good in absorption and in producing biological action. Therefore, the main drug leads in this study (1–5) fall under this range which indicates that these would presumably possess good ADME properties and are worth further preclinical investigations.

4. Experimental

4.1. Materials

The data set includes 13 drugs (Table 4) of highest quality pharmaceutical grade provided by pharmaceutical companies (Sigma Aldrich, Fisher Scientific and Spectrum Chemicals). Drug leads and related compounds used to determine the log *P* values were synthesized and/or obtained commercially from Strem Chemicals Company Inc. The structures of representative compounds are given in Fig. 2. Methanol and acetonitrile were of HPLC analytical grade purchased from Fisher Scientific, USA. Water used was deionized (DI) and further purified by filtration and degassing.

4.2. Chromatographic conditions

The HPLC system used was an Agilent 1100 series (Hewlett-Packard-Strasse 8, 76337 Waldbronn Germany), equipped with a pump, diode array detector with UV lamp operated at variable wavelengths, autosampler, and thermostat. Data acquisition was performed using Open Lab CDS Chem Station Edition software package with A.01.02 (010) version implemented in the chromatographic system. Detection at 250 nm and 280 nm was standard. However, all the samples were sufficiently detected at 250 nm.

The stationary phase consisted of a Waters X-Bridge C-18 column (4.6 mm × 150 mm, 5.0 μm particle size, pore volume 0.76 cm^3/g) purchased from Waters Corporation (34 Maple Street, Milford, Massachusetts 01757-3696 U.S.A). The packing material was synthesized using Waters patented Hybrid Particle Technology (U.S Patent No. 6,686,035) which contained both inorganic (silica) and organic (organosiloxanes) components. It was operated at constant temperature of 25 °C on both ends of the column. The column was equilibrated for at least one hour before running the samples. A post-run time of 10 minutes for recalibration was set between each samples injection.

The mobile phase consisted of a mixture of DI water (i), acetonitrile (ii) and methanol (iii) in a gradient elution mode as shown in Table 5. The injection volume was 5 μl and the eluent was delivered from the pump at a constant flow rate maintained at 1.0 ml/min at a pressure sufficiently high to overcome the backpressure of the column, approximately 6000 psi as set on the instrument.

4.3. Method development

Lipophilicity determination by RP-HPLC requires a good mobile phase in order to achieve accuracy. Moreover, the mobile phase should not alter the ionization or the pH of the drug. All of the new drug leads in this experiment are basic in nature and thus no acid was used. Several trials had been done in order to achieve a good mobile system. Proper selection of the mobile phase is the second most important step in the development of the separation method. The main requirement for the mobile phase is that it has to dissolve the analyte up to the concentration suitable for detection. Variation of the eluent composition provides great flexibility of HPLC separations. The mobile phase has to be polar, and has to provide a reasonable competition for adsorption sites for the analyte molecules. Therefore, a combination of acetonitrile, methanol and water, as shown in Table 6 was chosen. Many combinations of isocratic modes (Table 6) were estimated, but the results were not satisfactory. The 13 reference drugs were analyzed using these isocratic combinations and their retention times are noted. When compared with the standards, they showed a variation. The chromatographic peaks were wider or split but not of good shape. Gradient modes using different combinations, a few of them are shown in Table 7, eliminated these problems. All of the drug samples were dissolved in methanol at a concentration of 0.5 mg/ml. These samples were then filtered using micro-filters and were transferred to the HPLC sample vial. The calibration samples were selected largely on the basis of log *P* values reported in the literature, and are in general basic in nature to structurally correlate well with the lead compounds. The range of log *P* values of the reference compounds selected was between −1.1 to 7.56. The samples were placed in the autosampler according to the sequence template that is saved in the Open LAB method panel. Using the above HPLC conditions, the retention times of the compounds (t_R) were

noted. Also, the retention time of the solvent (methanol), indicated as t_0, was measured for each test compound.

The capacity factor was calculated by using the equation, $k = (t_R - t_0)/ t_0$; where t_R and t_0 are the retention times of sample and an unretained reference compound, respectively (Terada 1986). Methanol was used in this experiment as the unretained reference compound.

All the HPLC runs were done in triplicate to ensure the reproducibility of the method and the resulting average values of the retention times were considered for the calibration. Using these retention times, the capacity factor k was estimated. A calibration curve was plotted between the literature experimental log *P* values (Elog *P*) and the log k values using Microsoft excel 2010. Using the data analysis tool, regression analysis was estimated to find the coefficient of determination R^2, and the equation of the line. A simple regression analysis model contains only one independent variable, which is explanatory, X_i. (For $i = 1$ to $i = n$ subjects). It is linear with respect to both the regression parameters and the dependent variable. The corresponding dependent variable which is the outcome is labeled. The model is expressed as shown in the linear equation, $Y = m\ X + C + e_i$; where, the regression parameter m is the slope of the regression line and the regression parameter C is the intercept on the y axis. The random error term e_i is assumed to be unrelated, with a constant variance and the mean as 0. For accessibility in deductions and enhanced adaptability in estimation, analyses often receive an additional assumption that the errors are distributed normally. Conversion of the statistical data to achieve normality may be applied (Abraham et al. 1997).

4.4. Method validation

To determine the accuracy of the method and to know whether it gives a good linear equation or not, log *P* values of the all the reference compounds were estimated using the same linear equation obtained from the literature experimental log *P* values and the logarithmic capacity factor values from our data. By comparing the data obtained from the linear equation to that of the literature log *P* values, the strength of the relationship between them can be estimated, which in turn helps in predicting the correct log *P* values for the new drug leads. The calculated log *P* values of the new drugs were also checked for accuracy with the log *P* values obtained from the Chem Draw software using the compound's structure.

Conflicts of interest: The authors confirm that this article content has no conflicts of interest.

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