Original Article

DETERMINATION OF OCTANOL-WATER PARTITION COEFFICIENT OF NOVEL COUMARIN BASED ANTICANCER COMPOUNDS BY REVERSED-PHASE ULTRA-FAST LIQUID CHROMATOGRAPHY

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ABSTRACT

Objective: The present study aims at the development of a reversed phase ultra-fast liquid chromatography (RP-UFLC) method for measurement of the lipophilicity (log *P*) between n-octanol and water for the newly synthesized coumarin derivatives in our laboratory.

Methods: The synthesized compounds were dissolved in methanol and analyzed using XTerra RP18 column as the stationary phase and a mixture of methanol (0.25% v/v octanol) and buffer as the mobile phase with isocratic elution.

Results: In this study we concentrated on the relationship between a reversed-phase ultra-fast liquid chromatography (RP-UFLC) retention parameters and log *P* of our synthesized compounds. Furthermore, a good correlation and very close values were obtained between the experimentally determined log *P* values and values obtained from Chemdraw.

Conclusion: The developed method was found to be insensitive to any of the impurities present and moreover it requires very little sample for analysis.

Keywords: RP-UFLC, Synthetic, Coumarin, Log *P*, Octanol-Water

INTRODUCTION

Lipophilicity (log *P*) parameter is a very significant physiochemical property of a synthetic compound. It influences the oral absorption, protein binding, permeability, metabolism and penetration of drugs, as there exists a direct correlation between lipophilicity of the compounds and their solubility. Lipophilicity of the compounds also plays a vital role in the transport of the compounds in the body [1-3]. Generally it is understood that highly lipophilic compounds favor a good metabolism, which in turn leads to high drug clearance levels. Lipophilicity is directly proportional to high plasma protein binding levels. In lipophilic compounds, drug distribution is higher. This may be because of a higher amount of the compound that is binded to the tissue [4, 5]. Lipophilicity of a compound is based on the distribution of the drug between two immiscible phases; thereby we can measure the solubility of the compound between these two phases. It represents the affinity of the drug moiety to the lipophilic environment. IUPAC defines lipophilicity as the distribution pattern of the compounds in biphasic systems such as solid/liquid or liquid/liquid.

The new hypothesis followed in current drug discovery research is that there is an increased rate in finding the biologically active molecules. This is because of the latest technologies available such as high throughput screening technologies and combinatorial chemistry. However, the screening in the drug discovery process has shifted from hit and lead drug discovery to lead optimization, followed by the identification of potentially active drug-like molecules. In this identification process, knowing the physico-chemical properties of lead optimized molecules at early stage of drug development is required. In the modern phase of drug discovery, physico-chemical properties have become one of the most important parameters during the drug discovery process as they directly influence both pharmacokinetics and pharmacodynamics parameters. These physico-chemical properties are mainly responsible for failure of the lead compounds in the preliminary clinical phase of evaluation. Therefore the early determination of physico-chemical properties like lipophilicity of the lead molecules can reduce the associated problems [6].

Meyer and Overton [7] detailed about both lipophilicity and hydrophobic binding importance of the molecule in their theory. Similarly, Hansch and Fujita have developed an experimental method for the determination of lipophilicity, it is expressed as the logarithm of the partition coefficient (log *P*) between two different immiscible phases, i. e an aqueous and other being the lipophilic phase. In this regard, many different solvent systems have been used to estimate the lipophilic property; one such most common system used was octanol-water system. Even though it's difficult to work with 1-octanol as it has a noxious smell and easily forms an emulsion with water, but it has a unique property, i. e it has a hydroxyl functional group hydrogen-bond donor and acceptor. This functional group property of 1-octanol makes it to exhibit similar properties of the biological membrane distribution processes. This is because the biological membrane also contains the polar functional groups which would be able to form the hydrogen bonds. Octanol-water biphasic system partition values were found to be suitable for showing serum protein binding and also showing lipophilic interactions with biological membranes which contains a large amount of protein [8].

A detailed literature survey showed that, in recent years many papers have been reported, that have showed the development of many higher throughput methods to determine lipophilicity in industries as there are many novel compounds synthesized [9-11]. Also, a large number of data base is available for the determination of log *P* values by different calculation methods [12]. Various methods such as shake-flask, microshake-flask, and various chromatographic methods were the most commonly employed methods. In spite of these available methods, different instruments and multiple measurements were needed to obtain the log *P* value. Hence these methods require a lot of time along with a large amount of the sample, which is generally not available in the early phase of drug discovery. In order to overcome these limitations reversed-phase liquid chromatography (RPLC) method for the determination of lipophilicity was developed. It was officially recommended by the Organization for Economic Co-operation and Development (OECD) guidelines in 1989. This method offered simple, reproducible, quick analytical techniques. It is also insensitive to impurities and needs a

very small amount of sample in comparison to the time-consuming and tedious shake flask or slow-stirring methods [13, 14]. Estimation of the partition coefficient is based on the retention factor (log *K*) [15-18]. Hydrophobic reversed-based silica-based stationary phases are most frequently used for the determination of lipophilicity [19]. Many methods have already been reported for the determination of log *P*, which used the conventional octadecyl silica columns, octanol in the chromatographic system and other hydro organic mobile phases [20, 21].

When structurally unrelated compounds were taken for log *P* determination in a highly efficient reverse stationary phase along with hydro organic mobile phases, the obtained data was found to have a very weak correlation between octanol/water partition data and chromatographic partition data because of the difference between the properties of the partitioning solvent. Hence to cover a wide range of lipophilicity, mobile phase compositions should be adjusted. In this case, the obtained log *K'* value is extrapolated to 0% organic phase concentration and is considered for determination of log *P* [22].

Generally, mixtures of water or aqueous buffer along with the organic modifiers like methanol (hydrogen bond donor and acceptor), tetrahydrofuran (less polar hydrogen bond acceptor) or acetonitrile, (hydrogen bond acceptor of high polarity) are the most generally used mobile phases. Largely, methanol is preferred as an

organic modifier as it doesn't significantly affect the polar interactions of the solutes or alter the hydrogen bond network of the water molecules. Conversely, if compounds to be analyzed are highly lipophilic in nature, methanol leads to longer retention time. In these cases, tetrahydrofuran and acetonitrile can used as mobile phase in place of the methanol to shorten the retention time and to broaden the range of lipophilicity measurement [23]. According to the reported lipophilicity study data, different percentages of methanol were used as an organic modifier, normally ranging from 30-70%. Here, the advantage of this HPLC method is that, it not only models the octanol/water partition coefficient, even it also replicates the biological partition directly. Even though many computational methods are available for the determination of log *P*, the values are not very accurate as the values keep changing depending on the type of software used. Detailed literature survey revealed that there are several HPLC methods have been reported for the determination of the log *P*, but In the current paper, we describe an efficient and convenient method for the analytical determination of log *P* values for a series of newly synthesized anticancer coumarin moieties [24] by the RP-UFLC method with the use of Morpholine Propane Sulfonic (MOPS) buffer along with methanol/octanol mixtures in the different proportions which was not reported till date. The structures of the representative compounds are given in fig. 1, have shown good anticancer activity.
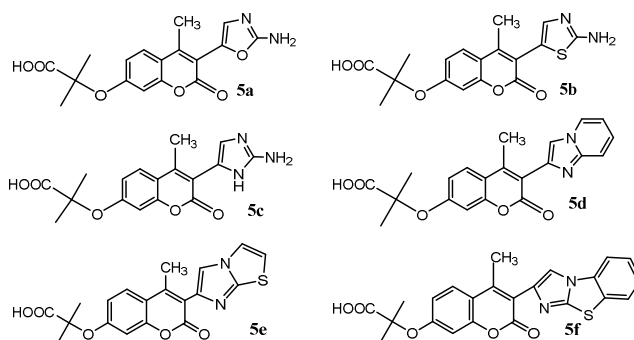

Fig. 1: Structure of coumarin analogues (5a-5f)

MATERIALS AND METHODS

Materials

All the chemicals and solvents such as, HPLC grade methanol, 1-octanol and 3-morpholinopropane-1-sulfonic acid (MOPS) were purchased from Sigma Aldrich. Water used for the study was purified by Milli-Q water purification system.

Instrumentation

All the chromatographic runs were conducted on a Shimadzu UFLC-20AD with a 35 MPa capacity binary pump VL (1260), SIL-20ACHT Autosampler and SPD-M20A Diode array detector at room temperature using XTerra RP18 column (5 μm, 4.6 mm * 150 mm). Data acquisition was performed by LC-Solution software.

Buffer preparation

Octanol was first saturated with water, for this one litre of MilliQ water was taken and saturated with 250 ml of 1-octanol for 24h at 25-27 °C. Then the aqueous layer was separated using separating funnel. 4.18 g of MOPS was weighed and dissolved in the above separated aqueous layer and the volume was made up to 1 litre with an adjustment of pH to 7.4.

Test sample preparation

The synthesized compounds 5a-5f were column purified followed by preparing its stock solutions. 10 mg of the compounds were weighed separately and dissolved in 5 ml of methanol in 10 ml volumetric flask and the volume was further made up with methanol to attain the stock solution of concentration 1 mg ml^{-1}(1000 μgmL^{-1}). These samples were filtered through the syringe filters of 0.25 μm. From

these stocks, final sample concentration of 10 μgmL^{-1} was prepared and injected in to UFLC system.

Chromatographic conditions

The stationary phase consisted of XTerra RP18 column (5 μm, 4.6 mm * 150 mm). Different trials were carried out to run samples for obtaining precise data. The flow rate was kept at 1 mlmin^{-1}. Mobile phase was filtered through a 0.5 μm membrane filter before the use. A mixture of methanol (0.25% v/v octanol) and buffer was used in isocratic as mobile phase. Methanol: buffer at the different ratios of 75:25, 80:20, 85:15 (v/v) was used to elute the samples. Signals were detected at λ max of 254 nm. The sample run time was kept for 20 min. 10 μL of sample was injected, methanol was used as a blank.

Measurement of lipophilicity (Log *P*) of the synthetic compounds

For each sample, the retention time was measured at three different mobile ratios with respect to blank. Retention time (t °) was measured by injecting the methanol together with sample. Capacity factor (*K'*) was calculated for each run by using the equation given below.

$$ K' = \frac{(t^R - t^0)}{t^0} $$

Equation 1: Calculation of log *P* value

Where, *K'* is the capacity factor of the solute at a given organic solvent concentration, t^R is retention time of sample and t ° is the retention time of blank (methanol). The logarithm of *K'* was extrapolated to a 0% concentration of methanol in the graph which is plotted by taking log *K'* in y axis and percentage ratio of methanol in x-axis [24]. Regression equation was generated from the graph for

each sample run. From the regression equation, log K' at 0% methanol (y axis intercept) was calculated as the log *P* of the test compound. Obtained experimental log *P* values were compared with the C log *P* calculated virtually using ChemDraw Pro 12.0.

RESULTS AND DISCUSSION

Experimental log *P* values of synthesized compounds were determined by reverse phase UFLC. The logarithm of capacity factor (log *K'*) was plotted against different concentrations of organic solvent (Methanol) and log *P* was calculated at 0% composition of organic modifier. As the methods involves the isocratic elution, isocratic retention times were measured by various volume percent of methanol in the optimized mobile phase. The isocratic lipophilicity index was calculated from the retention time of the samples and the capacity factor. First only the blank (methanol) was injected to find the retention time of the methanol (fig. 2). This was followed by the test compounds (10 μgmL-1) injections, at the different ratios of the mobile phases (graphical representation for one of the compounds 5a shown in fig. 3-6). Fig. 3, 4 and 5 shows the retention time of blank along with the sample at 75, 80 and 85 % of organic modifier respectively. Fig. 6 represents the overlay chromatogram of compound 5a showing the different retention times of methanol and drug at different ration of methanol (75, 80 and 85).

The experimental log *P* values obtained for each compound were compared with their calculated C log *P* by ChemDraw Pro 12.0 software, results of all the compounds are summarized in table 1. In this method, the stationary phase simulates the biological lipophilic membrane and retention of the compounds is correlated with its lipophilicity.
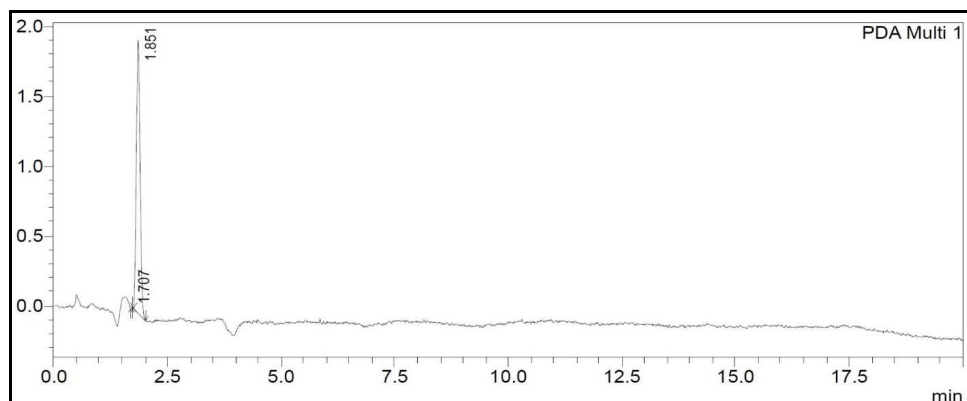

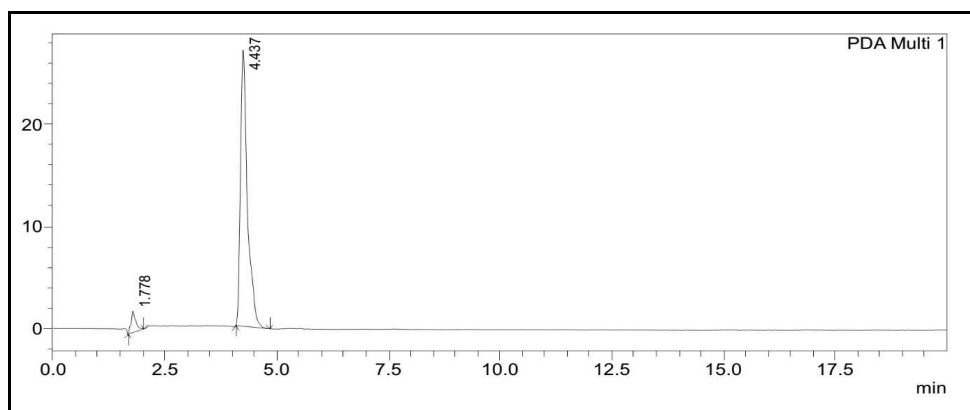**Fig. 2: Blank chromatogram of showing the retention time of methanol**

Fig. 3: Chromatogram of compound 5a showing the retention time of methanol and drug at methanol: buffer (75:25)
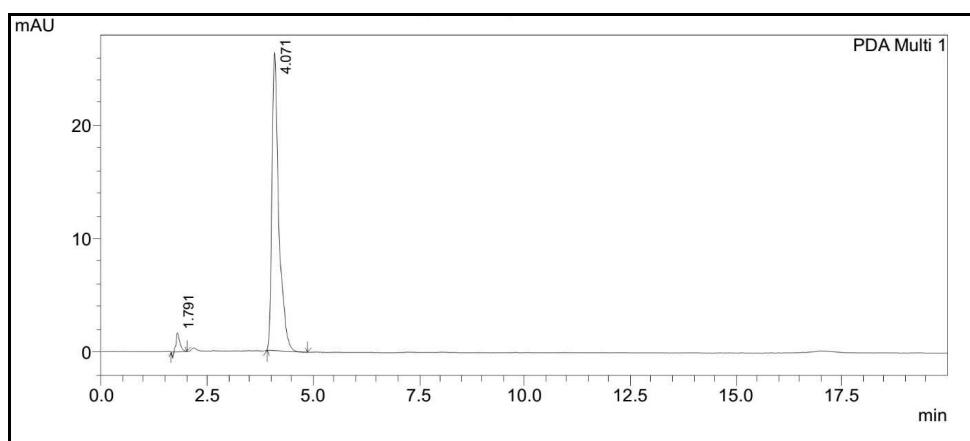

Fig. 4: Chromatogram of compound 5a showing the retention time of methanol and drug at methanol: buffer (80:20)
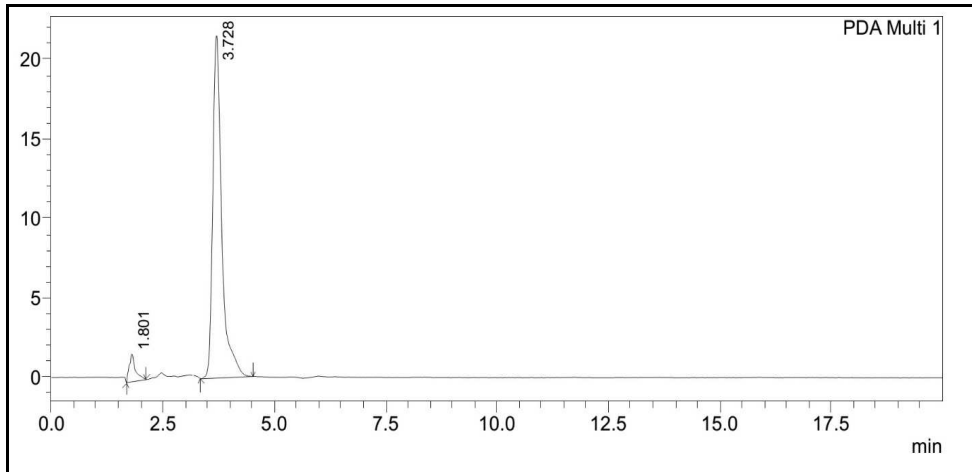
Fig. 5: Chromatogram of compound 5a showing the retention time of methanol and drug at methanol: buffer (85:15)
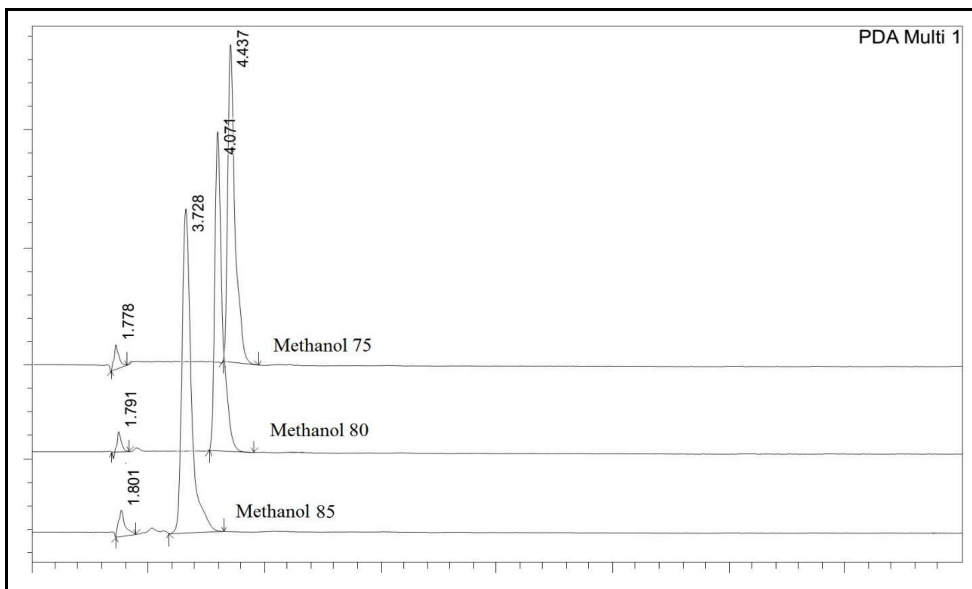

Fig. 6: Overlay chromatogram of compound 5a showing the different retention time of methanol and drug at different ration of methanol (75, 80, 85)
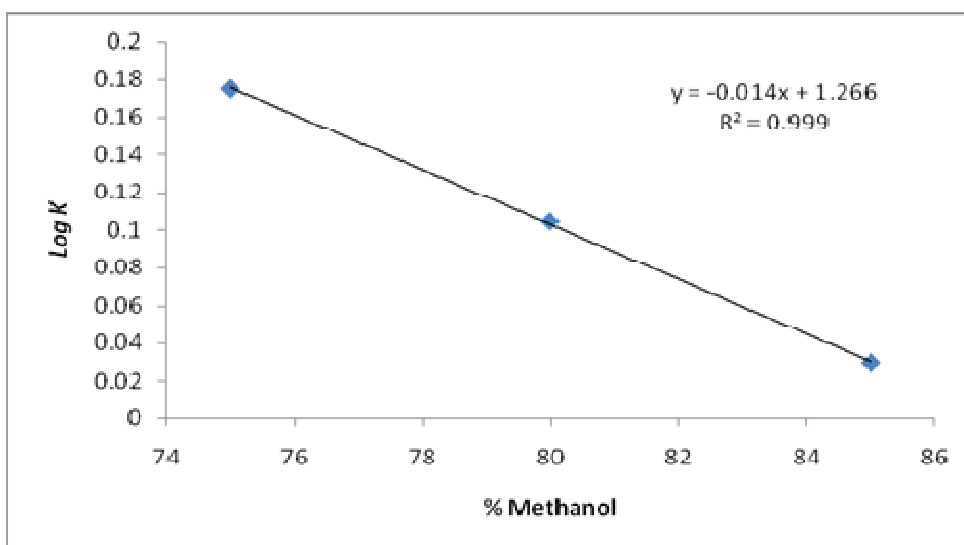

Fig. 7: Determination of log*P* of compounds 5a from standard plot (% methanol *vs* log k')
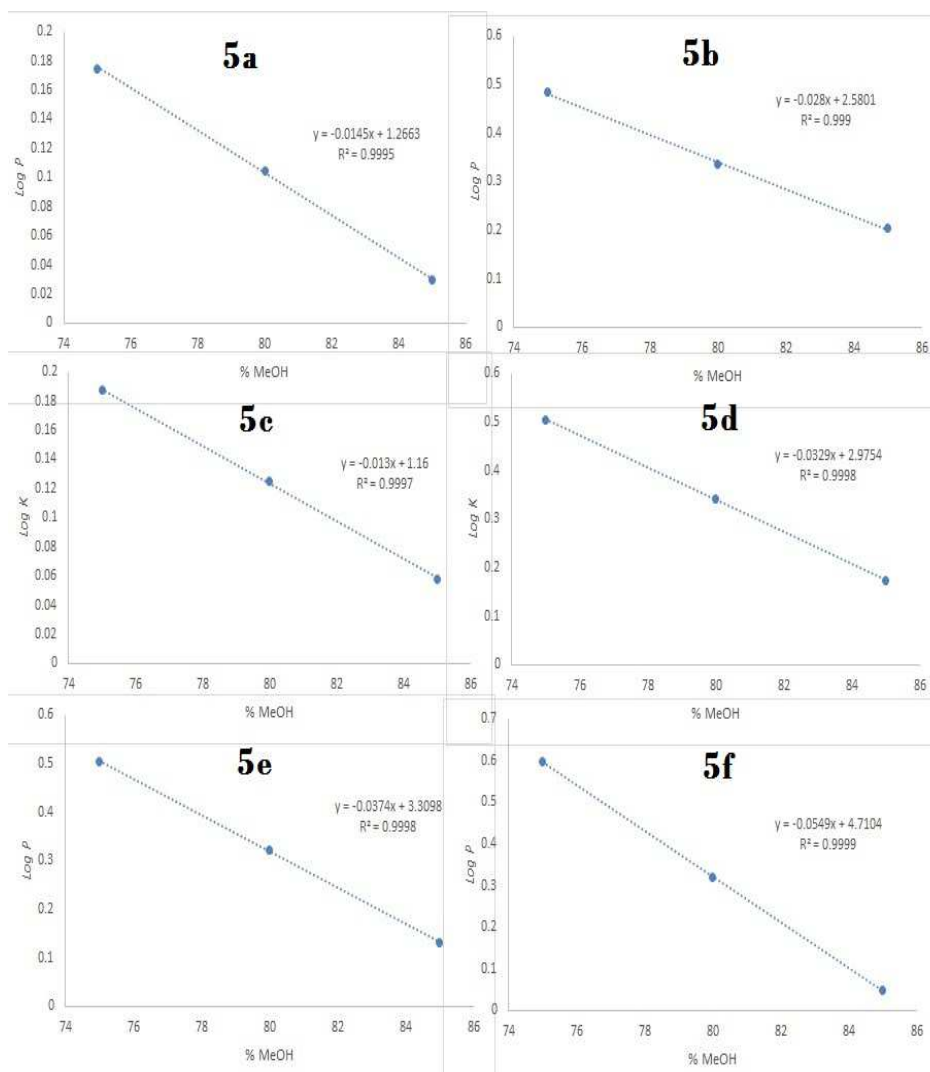
Fig. 8: Determination of log *P* of all the test compounds 5a-5f from standard plot (% methanol vs log k')

Table 1: Log *P* determination by UFLC method and comparison with Clog *P*

Comp.	Mobile phase ratio (methanol: buffer)	Rt of methanol (t °)	Rt of compounds(tᴿ)	*K*`	Log *K*`	Regression equation	R²	Log *P**	Clog *P*#
5a	75: 25	1.778	4.437	1.495501	0.174787	y =-0.0145x+1.2663	0.9995	1.27	1.17
	80: 20	1.791	4.071	1.273032	0.104839				
	85: 15	1.801	3.728	1.069961	0.029368				
5b	75: 25	1.762	7.121	3.04143	0.483078	y =-0.028x+2.5801	0.999	2.58	2.54
	80: 20	1.774	5.616	2.165727	0.335604				
	85: 15	1.784	4.632	1.596413	0.203145				
5c	75: 25	1.702	4.324	1.540541	0.187673	y =-0.013x+1.16	0.9997	1.16	1.11
	80: 20	1.764	4.116	1.333333	0.124939				
	85: 15	1.781	3.817	1.143178	0.058114				
5d	75: 25	1.786	7.471	3.183091	0.502849	y =-0.0329x+2.9754	0.9998	2.98	2.85
	80: 20	1.797	5.749	2.199221	0.342269				
	85: 15	1.812	4.513	1.490618	0.173366				
5e	75: 25	1.771	7.431	3.195935	0.504598	y =-0.0374x+3.3098	0.9998	3.31	3.25
	80: 20	1.782	5.523	2.099327	0.32208				
	85: 15	1.796	4.223	1.351336	0.130763				
5f	75: 25	1.765	8.731	3.946742	0.596239	y =-0.0549x+4.7104	0.9999	4.71	4.36
	80: 20	1.792	5.523	2.082031	0.318487				
	85: 15	1.807	3.823	1.115661	0.047532				
Reference (Doxorubicin)	75: 25	1.602	4.214	1.630462	0.212311	y =-0.014x+1.2678	0.9952	1.26	1.27††
	80: 20	1.713	4.136	1.414478	0.150596				
	85: 15	1.748	3.811	1.180206	0.071958				

*log *P* estimated from Reverse phase UFLC experiment; #Clog *P* calculated from ChemDraw Pro 12.0, †† log *P* value found in literature drug bank (https://www. drugbank. ca/drugs/DB00997)

The results indicate that there is a small but not statistically significant difference between the log *P* estimated from experimental method and the calculated (C log *P*) values. Even the results shows that there is an excellent correlation coefficient of values as in all the cases R^2 values were found to be>0.99 indicating that this method's linear gradient retention time can be used for the measurement of lipophilicity of the compounds using XTerra RP18 column.

Any novel drugs to be considered as a suitable drug candidates according to the Lipinski's "rule of five", they should possess a suitable partition coefficient value of less than five. Hence, implementation of drug-like properties screening at the very early stages of drug development will possibly speed up the drug development process. Generally traditional shake flask method was commonly employed for the determination of log *Poct* for compounds ranging 2-4. But, this process is time and labor consuming and also comparatively it requires large amounts of pure compounds. Therefore, the traditional shake flask method is not considered as an ideal method for the novel synthetic compounds as a very small amount of the sample will be available at the novel discovery stage. Hence we have used the RP-UFLC method to determine the log *P* of our compounds. The estimated log *P* values of all the six compounds in our laboratory were obeying the Lipinksi rule for selection of drug candidate (i. e log *P*<5).

Method validation

To find out the accuracy of our developed method and to know whether it's giving a good linear correlation equation or not we have compared the log *P* values of doxorubicin as the reference compounds which are estimated by the same procedure reported above, with that of the reported literature from drug bank [26] log *P* value. Even we have compared the experimental log *P* values with calculated log *P* from ChemDraw software to check the accuracy of the method.

CONCLUSION

In this study a fast isocratic UFLC chromatographic method has been developed by using short column-XTerra RP18 column for determination of a novel synthetic compound's lipophilicity. The results of this study showed that there was a good correlation between the experimentally determined log *P* values and software calculated log *P* values. The method was found to be insensitive to impurities, rapid, specific, reproducible and requires very small amount of samples in comparison to other methods.

CONFLICT OF INTERESTS

Authors declare no conflict of interest

AUTHORS CONTRIBUTION

The first, second and third author have contributed equally in the work like designing the methodology, analysis and interpretation of results. The fourth author contributed in writing and revision of the manuscript.

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CONFLICTS OF INTERESTS

Declared none

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