



AlphaLogD determination: An optimized Reversed-Phase Liquid Chromatography method to measure lipophilicity on neutral and basic small and Beyond-Rule-of-Five compounds

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ARTICLE INFO

Article history:

Received 13 November 2021

Revised 21 March 2022

Accepted 11 May 2022

Available online 13 May 2022

Keywords:

High performance liquid chromatography

Superficially porous particle

Shake-flask method

Lipophilicity

Beyond-rule-of-5

ABSTRACT

Lipophilicity can be measured with different methods, such as Shake-Flask or liquid chromatography. HPLC presents the advantage of overcoming solubility issues and therefore extending the range of lipophilicity to high values. A specific HPLC method, called ELogD, had been developed 20 years ago on a C₁₆-amide stationary phase, enhancing hydrophobic and hydrogen bond interactions to mimic octanol-water partition. The emergence of novel stationary phases and the need for a less complex mobile phase have led to the development of a new HPLC assay called alphaLogD, applicable to neutral and basic compounds at pH 7.4, that combines superficially porous particles with a high number of equilibria between solutes and stationary phase, leading to a lower number of isocratic methods to determine the logk'_w at a higher throughput. Statistical studies have been run to successfully evaluate the alphaLogD method compared to the Shake-Flask method and to allow this lipophilicity measurement into the so-called Beyond-Rule-of-5-molecules space.

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1. Introduction

Lead Discovery is an iteration of optimizations of different parameters, mainly by improving potency through chemical structure modifications. These modifications are aimed to modulate *in vitro* physicochemical properties with the goal of optimizing *in vivo* oral bioavailability. Lipophilicity is one of the first physicochemical properties integrated in medicinal chemistry design, as it impacts passive permeability, metabolism, excretion, oral absorption and toxicity [1–8]. In addition, physical parameters such as solubility, the flexibility of a molecule based on the presence of rotatable bonds and on the ratio of sp³ carbons, the presence of polar groups, and the presence of Intramolecular Hydrogen Bonding are related to lipophilicity [9–11]. Finally, lipophilicity is a powerful parameter used to modulate potency via the LipE concept, allowing the study of the hydrophobic effect of a structural change on both lipophilicity and potency [12,13].

The importance of lipophilicity on drug design emphasizes the need for accurate determination of this property. There are multiple *in silico* tools that are commercially available and customizable for the determination of lipophilicity. These computational models can be inaccurate when asked to calculate the lipophilicity of new entities that are not published, and they require regular training by introducing these new entities, which can be demanding in terms of time and computing power.

Different analytical techniques, such as solvent/water partitioning by shake-flask, partitioning in micelles by capillary electrophoresis, and liquid chromatography have been developed and miniaturized to adapt to the throughput and low amounts of compound available at the early discovery stage [14,15].

Shake-flask is an accurate, quantitative method that evaluates the amount of compound in each phase and stands as the “gold standard” in lipophilicity measurements providing lipophilicity values up to 4.5 [16,17]. However, the shake-flask technique still shows limitations for compounds of high lipophilicity, as most of the compounds will reside in the upper organic phase with limited quantification in the lower aqueous phase. In addition, low

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solubility of highly lipophilic compounds can generate significant variability in the quantification of a compound and in the final lipophilicity value. These limitations present a need for more accurate determinations of lipophilicity values, especially for very hydrophobic compounds.

Liquid chromatography on the other hand is a qualitative method, highlighting hydrophobic interactions with the lipophilic stationary phase relative to a non-retained entity. As it is less sensitive to solubility, reversed phase HPLC offers an extended range of lipophilicity values based on retention times mainly related to compound interactions and conformations in a specific environment [18]. Several conditions have been developed on different lipophilic supports to try to cover a wider range of lipophilicity with one unique method but with some limitations on the class of studied compounds [19–21].

The ELogD method, amenable to neutral and basic compounds at pH 7.4, involves a C₁₆ lipophilic support with embedded amide functions for higher efficiency with regard to hydrophobic interactions [22]. This reliable and reproducible assay has been developed with a complex mobile phase that contains decylamine, a masking agent to reduce secondary interactions of the solute with the support, 3-morpholinopropane-1-sulfonic acid (MOPS) as an ion-pairing agent to ensure the retention of positively charged entities, and octanol to enhance the energy of interactions present in the octanol/water system. This mobile phase has proven to be detrimental to the HPLC instrument, with the crystallization of the decylamine over time, and to limit the shelf-life of the stationary phase with the saturation of the sites of the C₁₆ amide support coated with MOPS. The need for reproducibility and reliability has led to the selection of a new generation of stationary phases, such as Superficially Porous Particle (or SPP) that contains a solid, non-porous silica core covered by a porous shell layer. SPP enhances the speed of equilibria between the stationary and the mobile phases, leading to reduced resistance to mass transfer, minimal compound diffusion, and higher column efficiency [23]. As a result, SPP allows the use of smaller particles and higher flow-rates without generating stronger back pressure. This optimized SPP technology combined to C₁₆ lipophilic chains and an embedded amide function has led to the development of the Express RP-Amide column to generate lipophilicity data of quality similar to ELogD with significant reproducibility and a less complex mobile phase. Finally, the developed conditions on the Express RP-Amide stationary phase allow for the measurement of high lipophilicity (logP ≥ 5) and open new opportunities to better support the chemical space expansion towards highly lipophilic compounds, so-called Beyond-Rule-of-5 molecules.

2. Material and methods

2.1. Material for ELogD method [22]

The ELogD HPLC method uses the Supelcosil LC-ABZ (RP-amide) column (Supelco), 5 μm particle size, 50 mm x 4.6 mm.

The mobile phase contains decylamine CH₃(CH₂)₉NH₂ (CAS 2016–57–1, from TCI, purity > 98%), 3-morpholinopropane-1-sulfonic Acid (MOPS) C₇H₁₅NO₄S (CAS 1132–62–1, from J.T. Baker, purity ≥ 99.5%), Sodium Hydroxide (Purity > 99%), 1-Octanol CH₃(CH₂)₇OH (Purity ≥ 99% Fisher), Optima HPLC grade water (Fisher), Optima HPLC grade Methyl alcohol (Fisher).

The aqueous phase is prepared by adding 0.05% v/v of octanol to water, 0.15% v/v N decylamine, 20 mM of MOPS, and the pH is adjusted to 7.4 with the ammonium hydroxide.

The organic phase contains 0.25% v/v of octanol in methyl alcohol.

Table 1
ELogD methods.

Method range	ELogD _{oct} range	Flow-rate (mL/min)	% MeOH
Low	<1	0.5	15,20,25
Middle	1–3	1	40,45,50
High	>3	2	60,65,70

2.2. Material for alphaLogD method

The alphaLogD HPLC method uses the Express RP-amide (Supelco), 2.7 μm particle size, 50 mm x 4.6 mm.

The mobile phase contains Ammonium Acetate CH₃CO₂NH₄ HPLC grade (EMD Millipore), Ammonium Hydroxide (Fisher), 1-Octanol CH₃(CH₂)₇OH (Purity ≥ 99% Fisher), Optima HPLC grade water (Fisher), Optima HPLC grade Methyl alcohol (Fisher).

The aqueous phase is prepared by adding 0.05% v/v of octanol to water, and ammonium acetate at a concentration of 50 mM. The pH is adjusted to pH7.4 with the addition of ammonium hydroxide.

The organic phase contains 0.25% v/v of octanol in methyl alcohol.

2.3. Sample preparation

All standards used to build the calibration curves are from Sigma-Aldrich with purity ≥ 98% and are described in Table 3. The standards are dissolved in DMSO (USP, Spectrum) at a concentration of 10 mM and are diluted down to 1 mM with either DMSO or a mixture of water/methanol 50/50 v/v.

2.4. Instrumentation and software

The HPLC instrument is an Agilent 1100 piloted by Chemstation Software (Version C.01.06) equipped with a quaternary HPLC pump (Model G1311A) with a micro vacuum degasser (model G1322A), a micro-well plate autosampler WPALS (Model G1367A) with an injection loop of 20 μL, a Column thermostatic column compartment (Model G1330B), and a UV Diode Array Detector (Model G1315B). The temperatures of column compartment and autosampler are both maintained at 23 °C.

Statistical Analyses: Linear regressions, ANOVAs, parallel lines analysis, and Bland-Altman plots were generated using SigmaPlot version 14.5, from Systat Software, Inc., San Jose California USA, (www.systatsoftware.com).

2.5. Methodology applied for lipophilicity measurement

2.5.1. ELogD methodology [22]

The ElogD methodology is described with a set of three ranges of isocratic methods, listed in Table 1. Each range of methods is related to the lipophilicity range, that is primarily estimated by in-silico calculation tools before any experimental measurement. An extrapolation to 0% of methanol is then performed from each of the method set and the ELogD_(octanol/water) is calculated with a calibration curve built on standards of known lipophilicity.

2.5.2. AlphaLogD methodology

Comparative studies run by Carrupt [21] between gradient and isocratic mobile phases using methanol as organic solvent have confirmed that optimal results are obtained in isocratic mode at similar flow-rate, and specifically for compounds of high lipophilicity.

The lipophilicity measurements are therefore run with isocratic methods at different contents of organic solvent for a further extrapolation to 0% of methanol from each of the method sets, and

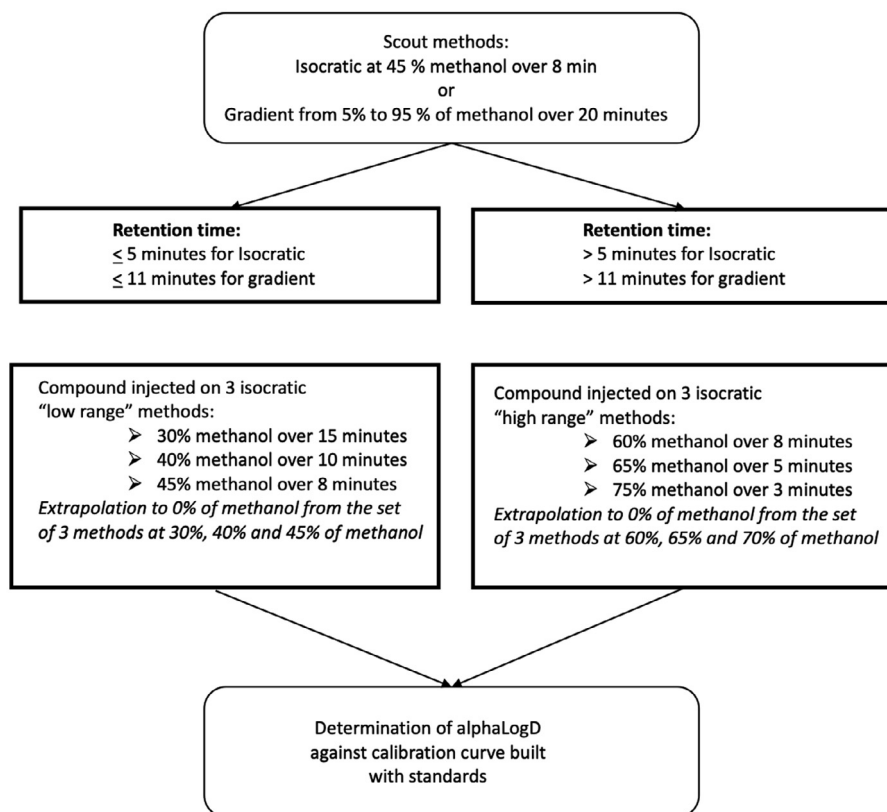


Fig. 1. AlphaLogD decision tree.

the alphaLogD at pH7.4 is calculated with a calibration curve built on standards of known lipophilicity in octanol/water.

Each isocratic method is built with the pumping system programmed to deliver constant volumes of each aqueous and organic solvent, and is delivered at 2 mL/min.

Each compound is analyzed following a logical approach based on its retention time for a given method, as described in the decision tree in Fig. 1.

A scout method at 45% of methanol is first applied for a total run time of 8 min, regardless of any predicted or calculated lipophilicity.

- Any compound with a retention time below or at 5 min is then injected in two additional isocratic methods, the 40% of methanol method for a total run time of 10 min, and the 30% of methanol method for a maximum run time of 15 min. The set of these three isocratic methods constitutes the so-called the “low range” and is applied for compounds of measured lipophilicity below 4.
- Any compound with a retention time higher than 5 min in the scout method is injected in three different isocratic methods, with a higher content of organic solvent, the 60% of methanol method for a run time of 8 min, the 65% of methanol method for a run time of 5 min, and the 75% of methanol method for a run time of 3 min. This set of three methods represents the “high range” applied for compounds with a measured lipophilicity equal to and above 4.

The optional use of a “scout gradient” from 5% to 95% of organic phase in 20 min at a flow-rate of 2 mL/min can be applied instead of the “Scout isocratic method” to ensure the total elution of compounds of high lipophilicity.

- Compound eluted in this gradient at a retention time below or at 11 min is then injected in the “low range” of isocratic methods at 30%, 40% and 45% of methanol.

- Compound eluted in the gradient at the retention time higher than 11 min is studied in the high range of isocratic methods at 60%, 65% and 70% of methanol.

3. Theory and calculations

Lipophilicity models by Reversed-Phase Liquid Chromatography have been proven to be indirectly related to the Shake-Flask model where the compound partition between octanol and water $\log K_{OW}$ is driven by an ensemble of diverse types of interactions, as described by the Linear Solvation Energy Relationship, LSER established by Abraham [24] defined by Eq. (1):

$$\text{Log}K_{OW} = c + eE + sS + aA + bB + vV \quad (1)$$

Each specific intermolecular interaction is represented by the product of solute descriptor with the complementary system constant related to the solute. These solute descriptors respectively highlight the excess molar refraction E , the polarizability S , the effective hydrogen-bond acidity A , the effective hydrogen-bond basicity B , and the McGowan’s characteristic volume V . The constants stand for the system contributions related to the solute, such as e for the capacity of the system to interact with the electron lone pair interactions, s for the ability to form dipole-dipole interactions with the solute, a and b for the capacity of forming hydrogen bonds, v for the ability of the solute to create cavities through cohesion and dispersion interactions in each phase, and c being a system constant. Parallel to the Shake-Flask partition, the LSER model can be applied to a reverse-phase liquid chromatographic system with each intermolecular interaction contributing to the retention of the solute. In both cases, each system constant is calculated with multiple linear regression analyses for a selected group of solutes with known descriptors. The resulting $\log k'$ is the qualitative and quantitative description of the intermolecular interactions in the partition process between octanol and water or in the

Table 2

Comparison of system constants of LC-ABZ and Express RP-Amide stationary phases with system constants of octanol-water partition.

Separation system	Separation constants				
	ν	e/ν	s/ν	a/ν	b/ν
Octanol-water [26]	3.81	0.15	-0.28	0.01	-0.9
Supelcosil LC-ABZ ^a [26]	3.48	0.12	-0.27	-0.01	-0.89
Express RP-Amide ^b [27]	4.15	0.09	-0.23	-0.03	-0.84
Express RP-Amide ^c [28]	2.23	0.07	-0.17	0.03	-1.12
Ascentis C ₁₈ ^d [28]	2.30	0.12	-0.32	-0.11	-0.91

^a Supelcosil LC-ABZ system:

- Embedded RP-Amide stationary phase, coated with octanol,
- Mobile phase: 20 mM MOPS pH 7.4 saturated with octanol -15% to 70% of methanol containing 0.25% v/v octanol.

^b Express RP-amide:

- Embedded RP-Amide stationary phase,
- Mobile Phase: 20 mM Sodium Phosphate buffer pH 7 saturated with octanol- isocratic methods from 40 to 55% of Methanol.

^c Express RP-amide:

- Embedded RP-Amide stationary phase,
- Mobile Phase: 20 mM Sodium Phosphate buffer pH 2- isocratic method 75/25% of acetonitrile.

^d Ascentis C₁₈:

- Mobile Phase: 20 mM Sodium Phosphate buffer pH 2- isocratic method 75/25% of acetonitrile.

equilibrium of the solute between the mobile phase and the stationary phase in a liquid chromatographic system [25].

A comparative study of LSER system constants calculated from the octanol-water partition and from two chromatographic systems involving the Supelcosil LC-ABZ and the Express RP-Amide stationary phases, respectively, highlights the similarities of the interactions of the two chromatographic processes with the octanol-water system in the lipophilicity determination [26–28] (Table 2, Rows (a) and (b)). The magnitude of each system constant is related to the importance of the interactions in the partition or retention process, and the positive or negative sign is indicative of the interactions with either the stationary phase or the mobile phase in the chromatographic system. The interactions study on the RP amide support emphasizes the positive contribution of the Hydrogen Bond Acidity (or Hydrogen Bond donor) of the solute with the stationary phase compared to the C₁₈ support (Table 2, Rows (c) and (d)), with the amide phase being weakly basic compared to the other embedded phases [28].

The compared ratios of the system constant between the RP-amide chromatographic system and the Octanol-Water partition are nearly identical and therefore a correlation model can be built between partition and retention, defined by Eq. (2) [26]

$$\log K_{ow} \text{ or } \log P = p + q \log k' \quad (2)$$

$\log K_{ow}$ = partition coefficient between octanol and water = Lipophilicity. $\log k'$ = solute retention between stationary phase and mobile phase in a reversed-phase liquid chromatography system. p and q = linear regression coefficients.

The solute retention $\log k'$ on the stationary phase is directly related to its interactions between the stationary phase and the mobile phase and is expressed as the capacity factor.

A change in the mobile phase composition will induce a change in the retention time, and we can apply the Snyder Linear Solvent Strength model (LSS) to assume a direct linear relationship between the solute retention and a binary mobile phase composition, as shown in Eq. (3):

$$\log k' = \log k'_w - S\Phi \quad (3)$$

$\log k'_w$ = extrapolated value of $\log k'$ at 100% of water.

S = Solute dependent solvent strength parameter.

Φ = ratio of organic modifier in the mobile phase of the chromatographic system.

Applying the theory regarding retention of a solute in a chromatographic system and based on our previous knowledge of chromatographic lipophilicity determination on LC-ABZ stationary phase, we are developing a new methodology on the embedded C₁₆-amide column Express RP-Amide with Superficially Porous Particle to generate alphaLogD on neutral and basic compounds.

4. Results and discussion

4.1. Linear solvent strength model

The LSS concept has been validated through the interactions of tetracaine of known lipophilicity of 2.29 and eluted on the Express RP-Amide column with isocratic mobile phases containing 50 mM Ammonium Acetate adjusted to pH 7.4 with ammonium hydroxide, and 0.05% v/v octanol for the aqueous phase and 0.25% v/v octanol in methanol for the organic phase. Seven isocratic methods containing respectively 20%, 30%, 40%, 45%, 60%, 65%, and 75% of organic content have been screened and the $\log k'$ of tetracaine is reported as a linear function of the organic solvent strength (Fig. 2), confirming the use of Eqs. (2) and (3) for the respective determination of $\log k'_w$ and the final LogD for charged entities or LogP for neutral ones.

With a pKa measured at 8.78, the basic tetracaine is partially ionized in the mobile phase at pH 7.4 and, in addition to the hydrophobic interactions with the lipophilic chains of the stationary phase, the presence of the hydrogen donor contributes to the retention of the compound based on its interactions with the carbonyl group of the amide function of the stationary phase [26]. The disruption of the linearity of the regression, however not always reproducible, could be interpreted as hydrogen bonding within the system [amide support/mainly aqueous mobile phase/solute] in the zone between 20% and 45% of methanol. On the other side, the polarization of the stationary phase in presence of increasing content of methanol, as well as increased hydrophobic interactions of lipophilic compounds with the C₁₆ chains of the support explains the second part of the curve, from 45% to 75% of methanol.

4.2. Choice of standards

The main goal of the study is to create a linear model between the distribution in an interaction-based system, such as Reversed Phase Liquid Chromatography, and the partition between two non-miscible liquid phases, such as octanol-water, for compounds of known diverse lipophilicities. The choice of the standards is based on the potential combination of least one hydrogen donor at the studied pH and of lipophilic chains to create interactions with the RP-Amide stationary phase that will result in different retention times. The selected standards, mainly basic, have an extended range of measured pKa leading to the presence of neutral and ionized forms in the mobile phase at pH 7.4 (Table 3). A set of 20 standards on a lipophilicity range from -1 to 6, described in the literature, are selected (Fig. 3) and studied in the Express RP-Amide system.

4.3. Correlation model between partition and retention

Each solution of standard, initially dissolved in DMSO, is diluted down to 1 mM in either 50/50 v/v or 25/75 v/v water/methanol mixture. The DMSO present in the injected solution is used as the void volume marker and its corresponding retention time (t_0) is included in the calculation of $\log k'$. The tetracaine is injected and eluted in each isocratic method from 20% to 75% of methyl alcohol. Based on their known lipophilicity, the standards of lipophilicity below 4 are injected in the low ranges of methanol from 20% to 45%, and those compounds with lipophilicity above 4 are injected

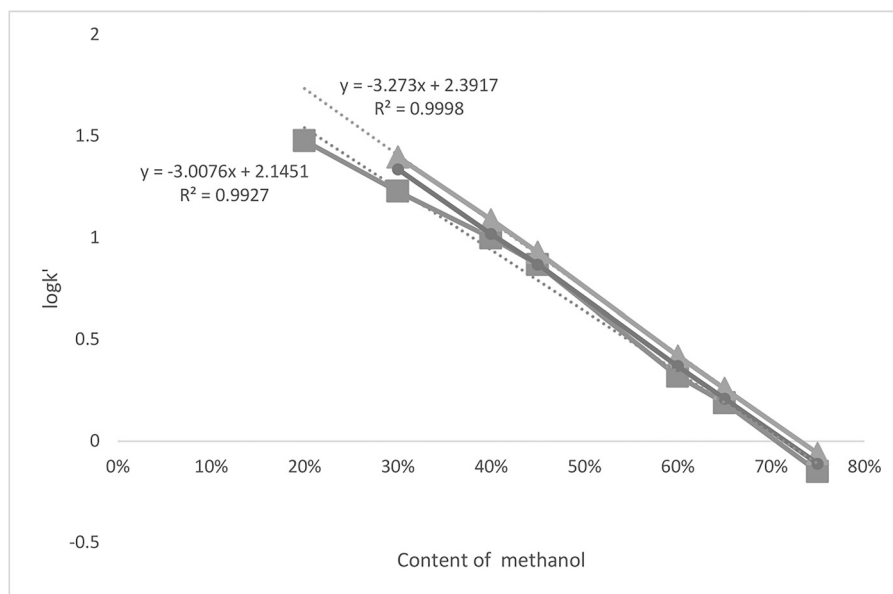


Fig. 2. Correlation of retention time of tetracaine with content of methyl alcohol in the mobile phase on 3 different calibration curves captured at different times.

Table 3
Standards used for the calibration curve of alphaLogD.

Compound	CAS no	MW	# H donor	pKa * measured	Literature logD[20]	ELogD [20]	AlphaLogD
Procainamide	51-06-9	235.3	2	2.739.42	-0.91	-0.72	-0.98
Allopurinol	315-30-0	136.1	2	9.20	-0.44	-0.06	-0.86
Acebutolol	37,517-30-9	333.4	3	9.70	-0.29	-0.53	0.27
Metrodinazole	443-48-1	171.5	1	2.49	-0.02	0.08	-0.31
Antipyrine	60-80-0	188.2	0	< 1.7	0.38	0.29	0.02
Acetaminophen	103-90-2	151.2	2	9.38	0.51	0.31	0.10
Alprenolol	13,655-52-2	249.3	2	9.72	0.97	0.59	1.53
Triamterene	396-01-0	253.3	3	6.39	1.21	1.14	1.01
Hydrocortisone	50-23-7	362.5	3	> 12	1.55	1.57	1.29
Quinidine	56-54-2	324.4	1	4.368.69	2.04	1.61	1.93
Tetracaine	94-24-6	264.4	1	1.938.78	2.29	2.49	2.75
Omeprazole	73,590-58-6	345.4	1	6.349.07	2.30	2.03	2.22
Imipramine	50-49-7	280.4	1	9.68	2.40	2.53	2.73
Clozapine	5786-21-0	326.8	1	4.107.94	3.13	3.60	3.48
Triflupromazine	146-54-3	352.4	1	9.39	3.61	3.69	3.85
Bifonazole	60,628-96-8	310.4	0	6.28	4.77	5.24	4.90
Diethylstilbesterol	56-53-1	268.3	2	9.77	5.07	4.95	4.83
Clotrimazole	23,593-75-1	344.8	0	5.99	5.20	4.91	4.67
Tolnaftate	2398-96-1	307.4	0	< 1.2	5.40	5.46	5.25
Amiodarone	1951-25-3	645.3	1	7.85	6.10	6.33	6.42

* pKa measured by Capillary Electrophoresis.

Table 4
Linear regression of the 3 calibration curves.

	Calibration 1	Calibration 2	Calibration 3
Regression	$y = 1.0279x + 0.3989$	$y = 0.9804x + 0.515$	$y = 1.0105x + 0.4375$
df	20	20	20
R ²	0.970	0.976	0.976
Standard Error of Estimate	0.388	0.329	0.345
Analysis of Variance	$F = 573.282P < 0.001$	$F = 802.633P < 0.001$	$F = 729.403P < 0.001$

Power of performed test with alpha = 0.050.

in the high ranges of methanol from 60% to 75%. Each solution is injected 3 times, and three different lots of Express RP-Amide stationary phase are being tested.

All the chromatographic conditions are similar to the ones applied for the study of tetracaine. The $\log'k_w$ of each compound is calculated with Eq. (3) from a curve built with at least three different solvents strengths.

Each linear regression and analysis of variance (ANOVA) statistics are reported in Table 4. The equality of the three regressions is shown as pair-wise comparisons tests for parallel lines, which includes tests for equality of slopes and intercepts, reported in Table 5. The slopes and y intercepts of the three curves are not significantly different, so they can be pooled to build one average calibration curve with the LogD in octanol as a direct function of the retention of each studied standard on the Express RP-Amide

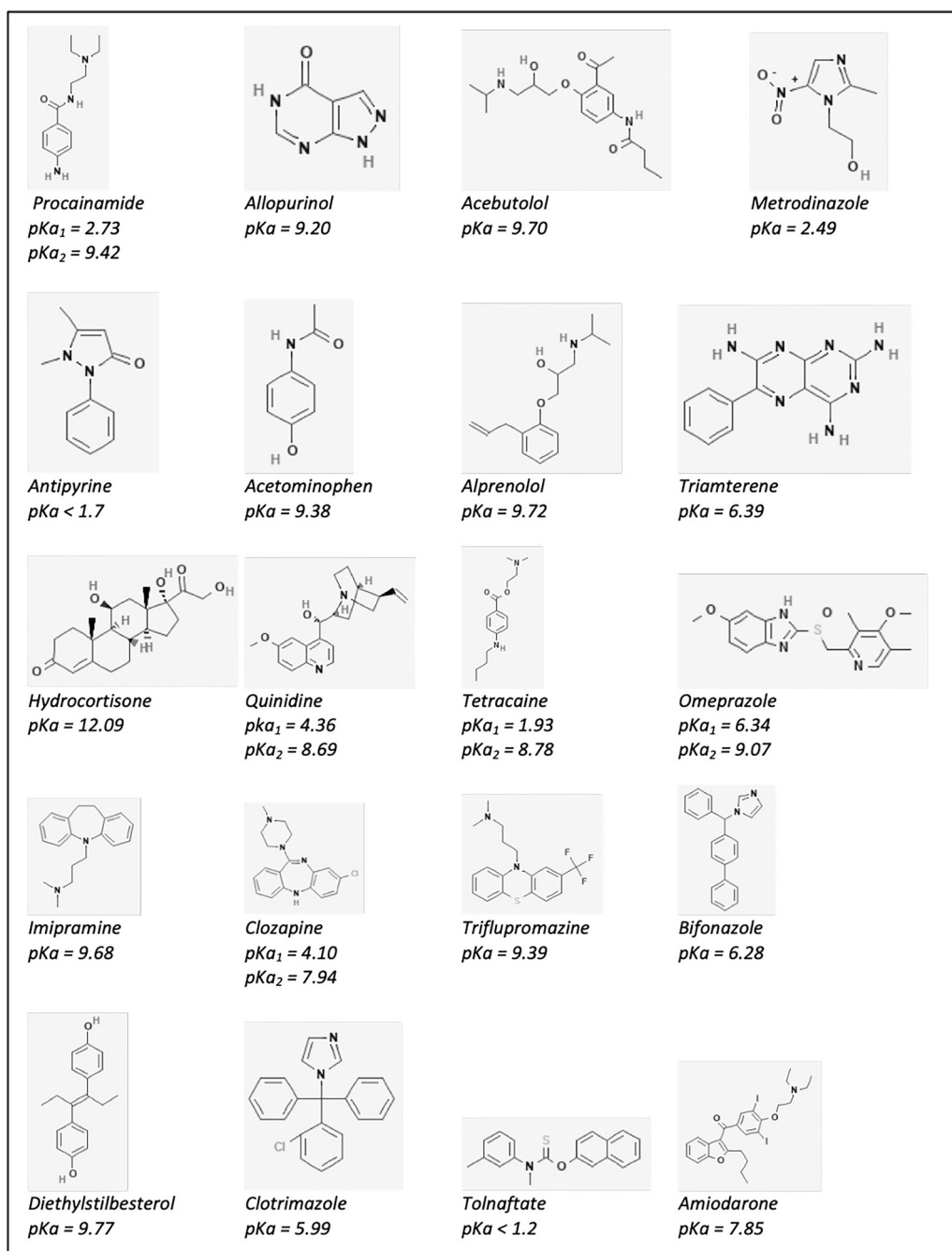


Fig. 3. Structures of 20 standards selected for the development of alphaLogD method.

Table 5
Pair-wise comparison tests for equality of slopes and Intercepts.

	Between Curve 1 and Curve 2	Between Curve 2 and Curve 3	Between Curve 3 and Curve 1
Test for Equality of Slopes	$F = 0.7490P = 0.3925$	$F = 0.3495P = 0.5581$	$F = 0.0938P = 0.7612$
Test for Equality of Intercepts	$F = 0.0728P = 0.7889$	$F = 0.0489P = 0.8262$	$F = 0.0039P = 0.9507$

stationary phase (4):

$$\text{LogD}_{\text{Oct}7.4} = 1.009(\pm 0.022)\text{log}k'_{\text{wExpress}} + 0.435(\pm 0.06) \quad (4)$$

4.4. Discussion on the alphaLogD method

4.4.1. Interpretation of interactions on the express RP-Amide phase

The slope of the Eq. (4) highlights differences of energies and forces, between the distribution (HPLC) and the partition (Shake-

Flask) systems. The slope value close to one implies similarity of these energies between the two systems and indicates a good correlation between the octanol-water partitioning system and the chromatographic interactions of the solute with the mobile phase and with the RP-amide stationary phase. The intercept highlights the presence of secondary interactions in the chromatographic system, despite the embedded amide function and the presence of octanol that is supposed to reduce the hydrogen bond interactions of

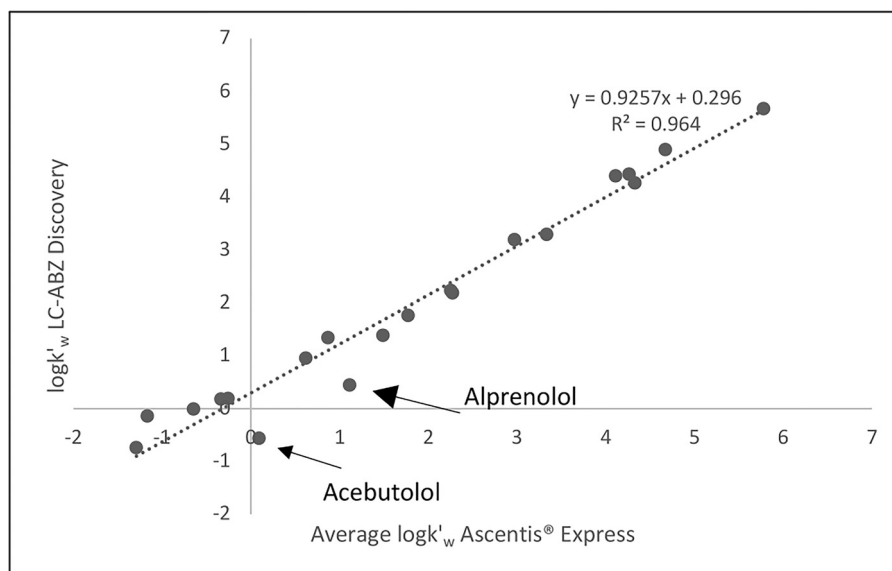


Fig. 4. Correlation of $\log k'_w$ Express RP-Amide with $\log k'_w$ LC-ABZ-Discovery on standard compounds.

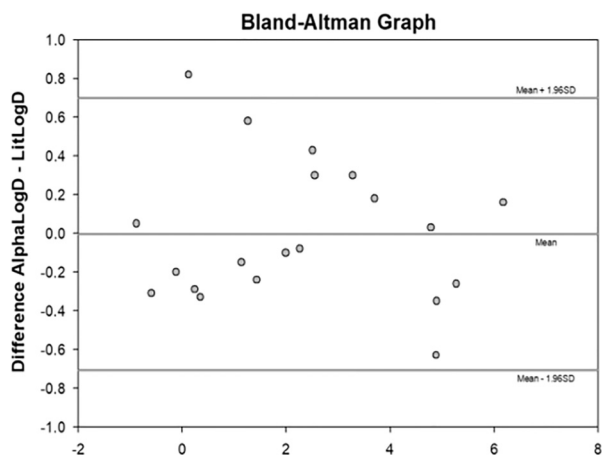


Fig. 5. Plot of the differences between alphaLogD method and Literature LogD.

the residual silanols of the stationary phase with the solute [29]. One could argue that the presence of decylamine (used on EL-ogD system) would reduce these interactions, as the intercept on the EL-ogD calibration is slightly lower than the one on the Express RP-Amide (0.21 for ABZ-Discovery and 0.45 for Express RP-Amide), but the influence of these secondary interactions on the final lipophilicity values obtained on alphaLogD is not significant enough to justify the use of a reagent that is significantly detrimental to the robustness of the entire HPLC system due to recrystallization of the decylamine in the aqueous phase over time.

The chromatographic distribution process of the solute between the mobile phase and the stationary phase seems to be enhanced by two main types of interactions. In the low lipophilicity range, the retention is mainly governed by the hydrogen bonding interactions between the solutes that have hydrogen bond donors and the amide function of the stationary phase that is hydrogen bond acceptor due to the presence of the carbonyl group. It has been described that polar embedded stationary phase can enhance the retention of polar compounds in Reversed phase HPLC even with a high ratio of aqueous phase promoting high retention of phenols [28,30].

In the high lipophilicity range, the hydrophobic interactions represent an additional contribution to the solute retention and

explain why the embedded RP-amide phase is considered more retentive than a regular C_{18} support [28].

4.4.2. Positive effect of fused-core particle

The Express RP Amide support is made of purified $2.7\text{-}\mu\text{m}$ superficially porous silica particles that are constituted of $1.7\text{-}\mu\text{m}$ solid silica cores and $0.5\text{-}\mu\text{m}$ thick shells of 9 nm pores which have been developed to allow highly efficient and fast separations, supporting high flow-rates while generating low back pressure [31]. The structure of the superficially porous particles induces a reduction of the longitudinal diffusion by 20 to 30%, as now 20% of the column volume is occupied by non-porous silica thereby preventing the solute from axial diffusion. In addition, the thin layer of porous particles reduces eddy dispersion inducing a quick mass transfer of the solute in the chromatographic system leading to shorter retention times, sharper peaks and higher column efficiency compared to classic regular porous silica. Fused core particles enhance the linearity of the LSS model over the range of isocratic methods in the high range of polar organic content, as the low back pressure reduces the electric field that is usually created by the alignment of mobile phase dipoles at high pressure and that is responsible for the increase in retention times [32].

4.4.3. Ammonium acetate versus MOPS

Ion-pairing chromatography is a very powerful technique to separate entities based on their ionized forms, as the ion-pairing agent creates a layer over the hydrophobic surface to add a second dimension to the retention of the solute by creating a complex that is simultaneously dissociated in the aqueous phase. The lipophilicity measurement on the ABZ-Discovery is completed in the presence of Morpholino Propane Sulfonic acid (MOPS) for positively charged entities. The Express RP-Amide chromatographic system works in the absence of MOPS and only contains the ammonium acetate at a concentration of 50 mM that could be enough to "ionize" the upper layer of the stationary phase.

A comparison of $\log k'_w$ of the same compounds on both ABZ Discovery and Express RP-Amide stationary phases shows a good correlation between the two systems (Eq. (5)):

$$\begin{aligned} \text{Log}k'_w(\text{ABZ} - \text{Discovery}) \\ = 0.9257 \log k'_w(\text{Express RP} - \text{Amide}) + 0.296 \end{aligned} \quad (5)$$

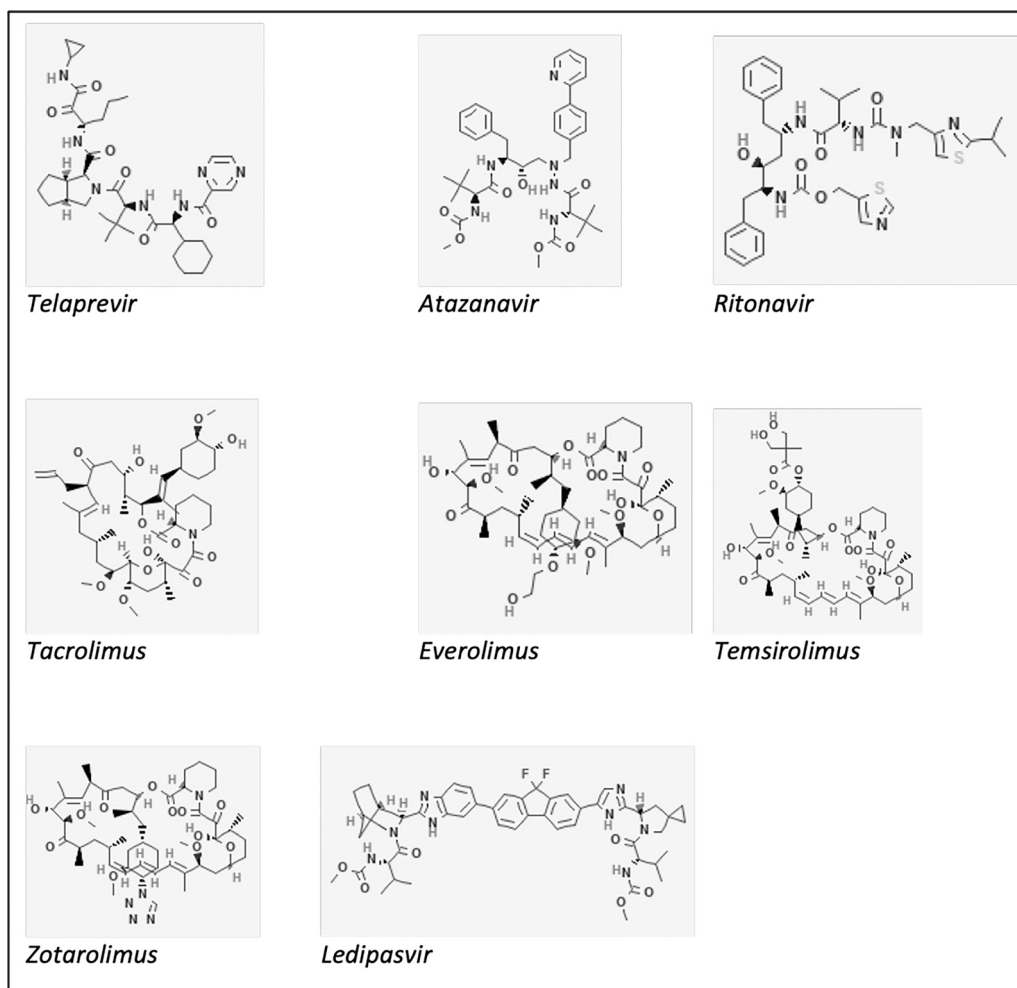


Fig. 6. Structure of "Beyond Rule of 5" molecules used as calibration standards.

Table 6
Calculated and measured properties of "Beyond rule of 5" molecules.

	MW (g/mol)	Rotatable Bounds	TPSA (Å)	# Hydrogen donors	Out of compliance Ro5	Measured pKa	Calc LogP (ACD)	ELogP [32]	alphaLogP**
Telaprevir	679.8	14	180	4	2	11.84	3.93	4.4	4.48
Atazanavir	704.9	18	171	5	4	4.29	5.20	4.7	4.81
Ritonavir	720.9	18	202	4	3	1.92	5.28	4.9	5.09
Tacrolimus	804	7	or178	3	2	3.30-9.90	3.96	6.1	N/A
Everolimus	958.2	9	205	3	2	10.40*	3.35	6.7	6.8
Temsirolimus	1030	7	242	4	2	9.96	2.96	6.9	7.00
Zotarolimus	966.2	7	219	2	2	9.81	3.55	N/A	6.59
Ledipasvir	889	12	175	4	3	4.32	6.77	N/A	6.99

* ACD calculated pKa.

** Calculated with global calibration curve.

There is a similar retention of positive entities in the presence of ammonium acetate on the fused-core support compared to the presence of MOPS on the porous ABZ-Discovery support, despite the different hydrophobicity between these two entities. It can be explained by the high rate of exchanges on the fused core support between the ion-pair that is formed with the positive form of the solutes and the acetate counter-ion and the dissociated forms in the mobile phase. The low hydrophobicity of the acetate counter-ion does not hide as much as the MOPS the embedded amide function of the support. It therefore enhances the retention of entities that have a significant number of hydrogen donors, such as the positively charge entities of the acebutolol and alprenolol that have respectively 3 and 2 hydrogen donors in their ionized state (Fig. 4).

It is important to highlight the use of ammonium acetate buffer to control the pH. It significantly simplifies the composition of the mobile phase and ensures a higher stability of the chromatographic system, a longer shelf-life of the column and the option of coupling a mass spectrometer detector for added value to the lipophilicity determination [21].

4.4.4. Evaluation of alphaLogD measurement against literature values

A further evaluation of the alphaLogD method against the Shake-Flask method is run on the residuals between alphaLogD and Literature LogD values with the Bland Altman analysis. The Normality test of Shapiro-Wilk shows a normal distribution of differences between alphaLogD and literature LogD values. The Bland

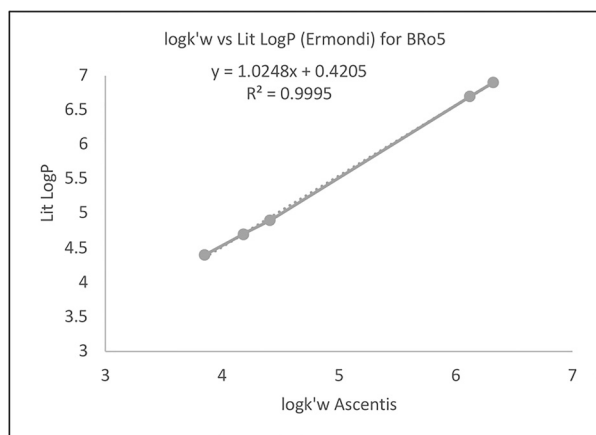


Fig. 7. Correlation $\log k'_w$ Express RP-Amide with literature ELogP [32].

Altman Analysis (Fig. 5) indicates that the alphaLogD values are on average 0.0045 lower than the literature values. In addition, the study of agreement limits leads to the conclusion that 95% of the alphaLogD measurements fall between +0.6989 and - 0.7079 of the literature values.

These results show that the alphaLogD method is comparable to the Shake-Flask method. The range of alphaLogD might appear wide when compared to the Shake-Flask. It is important to remember that the Shake-Flask method is highly dependent on compound solubility in both the aqueous and organic phases, and that could induce significant variability in the extreme ranges of lipophilicity.

4.5. Lipophilicity measurement of beyond rule of 5 compounds

The quick exchanges enhanced by Semi-Porous Particles between solute and stationary phase, added to the exceptional mass transfer enabled by the Fused-core particles lead to high efficiency of compound elution and result in sharp peaks, allowing study of entities highly retained on lipophilic support [31]. The chromatographic system developed with the Express RP-Amide is then tested on the so called Beyond Rule of 5 molecules that are se-

lected based on calculated properties that do not comply with the Lipinski Rule of 5 (Table 6), with at least 2 out-of-compliance rules out of 5 [33]. The applied chromatographic conditions are similar to the ones used for the small molecules with the use of isocratic methods in the high range of methanol due to the high predicted lipophilicity.

The difficulty of this specific study does not reside in the choice of the Beyond Rule of 5 standards nor in the measurement of the lipophilicity by chromatography but in finding lipophilicity data in literature that can correlate to the experimental $\log k'_w$. With predicted high lipophilicity and resulting low solubility, most of these Beyond Rule of 5 compounds are not measurable by the shake-flask method. The calculated values don't always integrate the 3D aspect, as for the macrocycles (Fig. 6), and the values of reference we use for this study are chromatographic data measured on the ELogD system [34].

The correlation of $\log k'_w$ (Express RP-Amide) with ELogP (as all the species are neutral at pH 7.4) on the compounds presents excellent similarities of energies of interactions between the two systems as shown in Fig. 7 and, as a result, we can build a calibration curve including small and large molecules (Fig. 8).

4.6. Application on research compounds: comparison of lipophilicity measurements from ELogD and alphaLogD methods

Following the methodology of first applying the scout method at 45% of methanol, which places the compounds into the appropriate low or high range, the final alphaLogD method was tested on a pool of 324 research compounds of unknown structures and ionization stages and is compared on the ElogD method (Fig. 9).

The analysis of alphaLogD data compared to the ELogD data shows a general good correlation between the two methods in the low lipophilicity range as well as in the high range.

The systematic application of the rule for compounds that elute below 5 min in the scout method are directed to the "low range" set of methods, allows a quick and reliable determination of lipophilicity up to 4. Conversely, the study of compounds in the set of "high range" when they elute above 5 min in the scout method, allows the determination of high lipophilicity values above 4.

The outliers can be explained by the initial mis-prediction of the LogD that triggers the choice of inappropriate set of methods

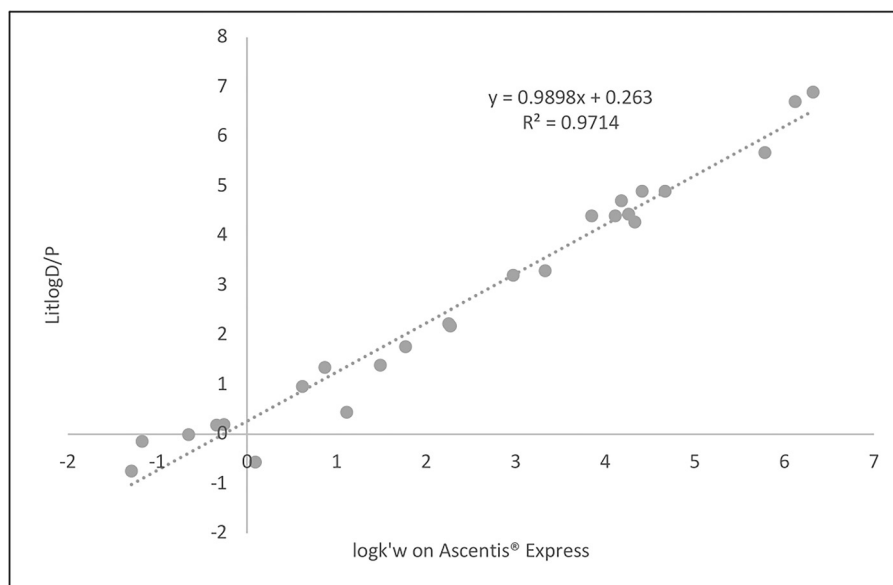


Fig. 8. Calibration curve for alphaLogD determination including small and large molecules.

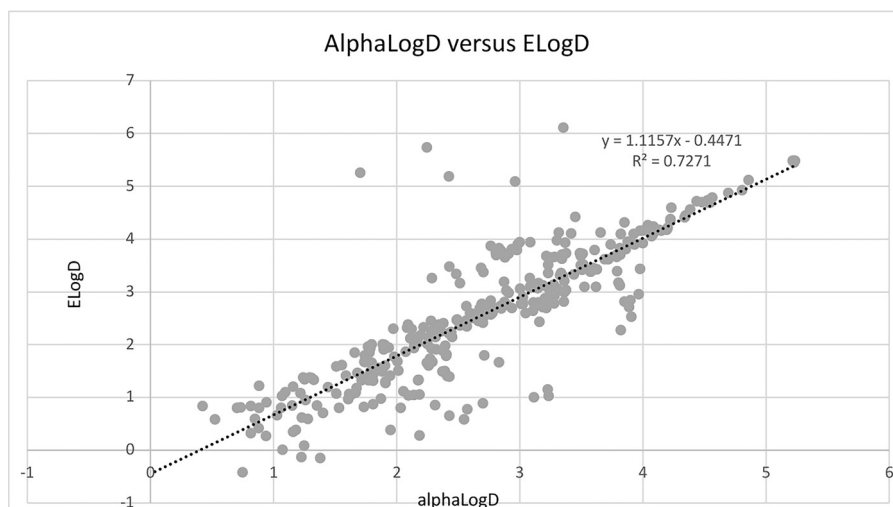


Fig. 9. Research compounds lipophilicity measurement with alphaLogD versus ELogD.

for ELogD versus the alphaLogD methodology, where the choice of method is uniquely based on a compound's interactions with the support at 45% of methanol.

5. Conclusion

The HPLC alphaLogD method has been successfully developed to ensure a sustainable and reliable determination of lipophilicity by introducing the advantageous SPP support, allowing higher flow-rate and reducing analysis time. The method optimization has led to a less complex system than ELogD, by removing reagents like N-decylamine and MOPS, that have a detrimental effect on the stationary phase and equipment in a very short term. Keeping the approach of determining the $\log k'_w$ with isocratic methods at different contents of methanol, the alphaLogD methodology doesn't rely on predicted lipophilicity values to drive the selection of different ranges of isocratic methods, but is based on interactions of the compounds with the support at the given amount of 45% of organic solvent. The retention time in this 45% scout method will then help assign the range of isocratic methods to be applied for the lipophilicity determination. An initial gradient can also be applied to ensure the total elution of highly lipophilic compounds and confirm the choice of high range of isocratic methods for the further lipophilicity determination. This methodology presents the advantage of selecting the most appropriate range of mobile phases for a compound of interest, which significantly increases the throughput of analysis by 40%. The wide range of measured lipophilicity values from -1 to 7 with the alphaLogD assay represents a reliable tool to design a series of compounds with data delivered with a single assay.

Finally, the use of hyphenated HPLC to Mass Spectrometry is now made possible by the absence of MOPS and phosphate buffer in the mobile phase, and provides the opportunity for higher throughput by studying a mixture of compounds of potential different lipophilicities, as well as providing higher integrity data by identifying the main compound from any potential impurity.

Credit author statement

Dan Katz: Investigation, Methodology, validation, data curation, reviewing and editing. **Kate Fike:** Data curation, Formal analysis (statistical), reviewing and editing. **Steve Placko:** Data curation, reviewing and editing. **Justin Longenberger:** Data curation, reviewing and editing. **Laurence Philippe-Venec:** Conceptualization, writing-original draft. **Andrew Chervenak:** Conceptualization,

methodology, data curation, reviewing and editing, project administration and supervising.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We thank Aimee Kestranek and Kate Favre for their constant support and for allocating time to the team to run the method development and optimization.

We thank Wendy Roe and Cory Muraco for Millipore Sigma for giving us access to a free Superficially Porous Particle Express RP-Amide column to allow us starting the alphaLogD method development and optimization.

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