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REVIEW

Analytical tools for the physicochemical profiling of drug candidates to predict absorption/distribution

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Abstract The measurement of physicochemical properties at an early phase of drug discovery and development is crucial to reduce attrition rates due to poor biopharmaceutical properties. Among these properties, ionization, lipophilicity, solubility and permeability are mandatory to predict the pharmacokinetic behavior of NCEs (new chemical entities). Due to the high number of NCEs, the analytical tools used to measure these properties are automated and progressively adapted to high-throughput technologies. The present review is dedicated to experimental methods applied in the early drug discovery process for the determination of solubility, ionization constants, lipophilicity and permeability of small molecules. The principles and experimental conditions of the different methods are described, and important enhancements in terms of throughput are highlighted.

Keywords Physicochemical profiling · Solubility · Ionization constant · Lipophilicity · Permeability · Experimental models

Abbreviations

ACN	Acetonitrile
ADME	Absorption, distribution, metabolism and elimination
ADMET	Absorption, distribution, metabolism, elimination and toxicity
APCI	Atmospheric pressure chemical ionization
API	Atmospheric pressure ionization
APPI	Atmospheric pressure photoionization
b	Gradient steepness
BBB	Blood–brain barrier
CAD	Corona aerosol discharge
CAPS	3-(Cyclohexylamino)-1-propanesulfonic acid
CCD	Contactless conductivity detection
CE	Capillary electrophoresis
CNS	Central nervous system
CNS+	Compounds crossing the blood–brain barrier
CNS−	Compounds not transported into the brain
CZE	Capillary zone electrophoresis
DAD	Diode array detector
DME	Dimethyl ether
DMSO	Dimethyl sulfoxide
ELSD	Evaporating light scattering detector
EOF	Electroosmotic flow
ESI	Electrospray ionization
FaSSIF	Fasted state simulated intestinal fluid
FeSSIF	Fed state simulated intestinal fluid
HEPES	4-(2-Hydroxyethyl)piperazine-1-propanesulfonic acid
HILIC	Hydrophilic interaction liquid chromatography
HPLC	High-performance liquid chromatography
HTS	High-throughput screening
IAM	Immobilized artificial membrane
ITIES	Interface between two immiscible electrolyte solutions
k	Retention factor
k_i	Retention factor at the initial gradient composition
LC	Liquid chromatography
L_{eff}	Effective length of the capillary
LEKC	Liposome electrokinetic chromatography

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log D^{pH}	Logarithm of the distribution coefficient at a given pH
log k_{IAM}	Logarithm of the retention factor measured on immobilized artificial membrane columns
log k_w	Logarithm of the retention factor extrapolated to 100% water
log P	Logarithm of the partition coefficient
log P_a	Apparent permeability coefficient
log P_e	Effective permeability coefficient
log P^I	Logarithm of the partition coefficient of an ion
log P_{lip}	Logarithm of the partition coefficient in liposomes/water system
log P_{oct}	Logarithm of the partition coefficient in the 1-octanol/water system
LOQ	Limit of quantification
LSER	Linear solvation free-energy relationship
LSS	Linear solvent strength
L_{tot}	Total length of the capillary
LYSA	Lyophilized solubility assay
MEEKC	Microemulsion electrokinetic chromatography
MEKC	Micellar electrokinetic chromatography
MeOH	Methanol
MES	2-Morpholinoethanesulfonic acid
MS	Mass spectrometry
NCE	New chemical entity
NMR	Nuclear magnetic resonance
NQAD	Nano-quantity analyte detector
ODS	Octadecyl-bonded silica
PACE	Pressure-assisted capillary electrophoresis
PAMPA	Parallel artificial membrane permeability assay
PC	Phosphatidylcholine
PC	Polycarbonate
PDMAC	Poly(diallyldimethylammonium chloride)
PK	Pharmacokinetic
pK_a	Ionization constant
pK_a^{app}	Apparent ionization constant
PVDF	Polyvinylidene fluoride
PVS	Poly(vinylsulfonate)
RI	Refractive index
RPLC	Reversed-phase liquid chromatography
SAR	Structure–activity relationship
SDS	Sodium dodecyl sulfate
t_0	Column dead time
t_D	System dwell time
t_{EOF}	Migration time of the electroosmotic flow marker
t_G	Gradient time
t_m	Migration time
t_{mc}	Migration time of the micelle marker
t_{EOF}	Migration time of the EOF marker
t_R	Retention time of a compound
U	Tension
UHPLC	Ultrahigh-pressure liquid chromatography
VEKC	Vesicle electrokinetic chromatography
z	Charge
$\Delta\varphi$	Change in composition of the mobile phase during the gradient
μ_{eff}	Effective mobility

Introduction

Drug research is a complex and time-consuming process. A period of 12–20 years is needed from the discovery of new chemical entities (NCEs) to the marketing authorization of a new drug. This process can be divided into two main phases (cf. Fig. 1): (i) *drug discovery*, which includes target identification, hit discovery, and lead optimization; and (ii) *drug development*, which comprises preclinical and clinical studies. Inappropriate pharmacokinetic (PK) behavior has been recognized as one of the major factors leading to the rejection of NCEs during drug development [1]. Therefore, a great deal of effort has been made to perform these studies as early as possible in the drug discovery process, preferably before the drug candidate enters the lead optimization phase. In this way, only compounds with high potency and suitable PK properties are selected for development [2].

Physicochemical properties (solubility, ionization and lipophilicity) allow the ADME (absorption, distribution, metabolism and excretion) behavior of a compound to be predicted [3, 4]. Solubility is a parameter of prime importance in the drug discovery process. Indeed, drugs must be soluble in order to reach their targets, and poor solubility seems to be the most important cause of rejection during drug discovery and development [5]. Moreover, it severely affects results of in vitro screening bioassays.

Also, the majority of NCEs are ionizable compounds. As the ionization state of a compound affects its solubility and lipophilicity, and thus its pharmacokinetic behavior, early evaluation of its ionization constants is an important step in the discovery process [6]. Lipophilicity, which describes the partitioning of a compound between an aqueous and a lipidic environment, is another essential physicochemical property impacting ADME behavior, as it widely contributes to membrane permeation, solubility, protein binding and metabolism.

Finally, important information on advanced ADME or PK properties such as passive drug permeability across a phospholipidic membrane is also useful. Previously, permeability experiments were generally carried out later in the discovery process, since they were based on low-throughput in vitro methods, often performed with biological materials. Recently, new analytical methods based on artificial mem-

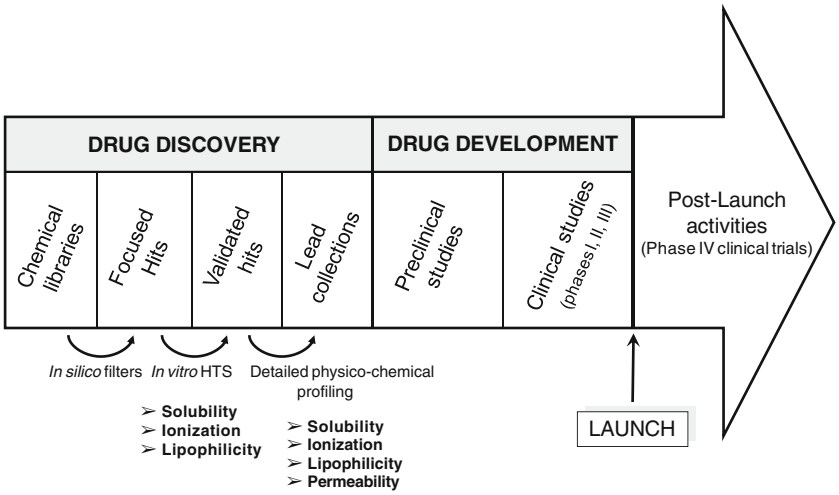

Fig. 1 Scheme of the drug research process

branes or partitioning in an anisotropic medium have emerged. These approaches are now largely implemented in the screening processes used in pharmaceutical research as a first predictor of biological membrane permeability.

Since advances in combinatorial chemistry have drastically increased the number of NCEs, tools for analytical physicochemical and PK properties determination are automated and progressively adapted to high-throughput technologies [6]. Indeed, although in silico prediction methods are gaining in importance, experimental determination remains necessary in order to optimize in silico methods and build chemical libraries of experimental physicochemical data.

The present review is dedicated to experimental methods applied early in the drug discovery process to determine solubilities, ionization constants, lipophilicities and permeabilities of small molecules. The principles and experimental conditions of the different methods are described, and the important enhancements in terms of throughput are highlighted.

Ionization constants

Traditional methods

Potentiometric and spectrophotometric methods are commonly used for pK_a determination.

In potentiometric titration (cf. Table 1), the pH of a vigorously stirred solution is continuously measured with a glass electrode as precisely known volumes of a standardized strong acid or base are added [7]. The pK_a is determined from the difference between the potentiometric titration curve of the tested compound and a blank aqueous titration. When the solubility of a compound in aqueous media is insufficient, co-solvents are added, and titrations at different co-solvent concentrations allow the pK_a to be extrapolated to zero percent co-solvent [8–10]. This method is universal and takes 30–60 min/titration (i.e., 10–30 titrations/24 h) using an automated instrument (e.g., the Sirius GLpKa from Sirius Analytical Instruments, Forest Row, UK). As this technique requires milligram amounts of sample at relatively high concentrations (200–5000 μM) [11], a microscale pH-titrimetric method was described but is rarely used [12]. However, it remains difficult to handle impure or unstable compounds.

Spectrophotometric pK_a determination (cf. Table 1) is based on the change in the absorption of a chromophore near ionizable moieties [4]. This technique was recently used for the pK_a determination of lansoprazole [13] or sartans [14]. The ionization constants are determined from a plot of the absorbance measured at an appropriate wavelength as a function of pH. More recently, Tam and coworkers developed a generalized method called multiwavelength spectrophotometric titration. It requires a diode array detector coupled to an automated pH titrator and a much more complex data treatment [15–19]. Spectrophotometric pK_a determination is usually more sensitive than potentiometry, with sample concentrations ranging from 10 to 50 μM. A microscale spectrophotometric method was also introduced by Morgan et al. [12]. As it takes 30 min/titration, a spectral gradient analysis (SGA) method designed for a 96-well format and based on a pH gradient flow technique with diode array UV detection was developed to increase the throughput [20, 21]. A commercial SGA instrument (from Sirius Analytical Instruments) is available, allowing the determination of pK_a values of one compound in only 4 min (240 samples/24 h) [4]. However, this technique requires a measurable UV chromophore that is dependent on the pH, which is not the case for all pharmaceutical compounds [7,

Table 1 Experimental techniques for pK_a determination

Method	Potentiometry	Spectrophotometry	CZE-UV
Measurement	Ionization profile	Ionization profile	Single points
Quantity	2–10 mg	2–10 mg	<<<1 mg
High purity	Necessary	Necessary	Not necessary
Low/medium-throughput instrumentation	Automatic titrator: GLpKa (Sirius Analytical Instruments, Forest Row, UK)	DAD-UV spectrophotometer coupled to an automatic titrator (various vendors)	Capillary electrophoresis unit (various vendors)
High-throughput instrumentation with 96-well plate technology	-	Spectral gradient analysis, SGA (Sirius Analytical Instruments, Forest Row, UK)	Multiplexed capillary electrophoresis: cePRO 9600 (Advanced Analytical Technologies, Ames, IA, USA) or MCE 2000 (Pfizer Laboratory, Groton, CT, USA)
Miscellaneous	-	Only for compounds with a measurable chromophore that is dependent on the pH	-

11]. Moreover, it is also difficult to deal with impure and unstable compounds.

Capillary electrophoresis

Theory and principles

In recent years, capillary zone electrophoresis (CZE) (cf. Table 1) has emerged as the method of choice for pK_a determination due to some inherent advantages, as reported in several reviews [4, 22–29]. (i) The sample and solvent consumptions are small: 1–50 nl of sample at concentrations of 10–500 μM is usually introduced into the capillary, while the whole capillary volume is about 1 μl (for a conventional capillary of length 50 cm and internal diameter 50 μm). (ii) Separations are performed under high electric fields with a flat flow profile, ensuring very thin peaks and high resolving power. (iii) The instrumentation is fully automated and able to handle impure samples, as it is a separation method. (iv) This technique is universal, since different detection systems can be coupled to CZE [24].

With this approach, pK_a determination is based on the measurement of the effective mobility (μ_{eff}) of the analyte at various pH values. Indeed, the μ_{eff} of a compound depends on the molar fraction of each ionized form present at a given pH. Practically speaking, μ_{eff} can be measured as:

$$\mu_{\mathrm{eff}} = \frac{L_{\mathrm{eff}} \cdot L_{\mathrm{tot}}}{U} \cdot \left(\frac{1}{t_{\mathrm{m}}} - \frac{1}{t_{\mathrm{EOF}}} \right) \qquad (1)$$

where t_m and t_{EOF} are the migration times of the analyte and neutral marker, respectively, U is the applied voltage (V), L_{tot} is the total capillary length (cm) and L_{eff} is the effective capillary length (cm). The variation in electroosmotic flow (EOF) at different pH values is corrected for by using a neutral marker, as described in Fig. 2A.

Then, pK_a values are calculated by nonlinear regression using the relationship between μ_{eff} and pH (Eq. 2). This equation can be applied to different ionizable compounds, depending on the number and nature of the ionizable groups, as described elsewhere [30]:

$$\mu_{\mathrm{eff}} = \sum_{i=0}^{n} \frac{\left[\prod_{j=1}^{\mathrm{i}} 10^{-\mathrm{p}K_{aj}} \right] \cdot 10^{(i-n)\mathrm{pH}}}{\sum_{i=0}^{n} \left[\prod_{j=1}^{\mathrm{i}} 10^{-\mathrm{p}K_{aj}} \right] \cdot 10^{(i-n)\mathrm{pH}}} \cdot \mu_{\mathrm{H}_{n-i}\mathrm{X}^{z-i}} \qquad (2)$$

where i and j are, respectively, the ith and jth dissociation steps, n is the total number of ionizable groups and z in the charge of the fully protonated species H_nX^z. A typical plot of the variation of the effective mobility of the monobasic compound lidocaine as a function of pH is presented in Fig. 2B.

The determination of pK_a values by CZE has mainly been applied to pharmaceutical compounds [28]. Agreement with other methods is generally good (about 0.2 pK_a units), but can be much lower in the case of weak bases (pK_a<3) or weak acids (pK_a>10).

Experimental conditions

To obtain reliable pK_a values by CZE, several general considerations concerning critical experimental conditions, like the choice of the buffer (i.e., its nature and ionic strength), the applied voltage and the detection method, must be taken into account.

First, the buffer nature should be carefully chosen. Rafols et al. published an extended study of this problem [31]. Ammonium buffers should be avoided due to their

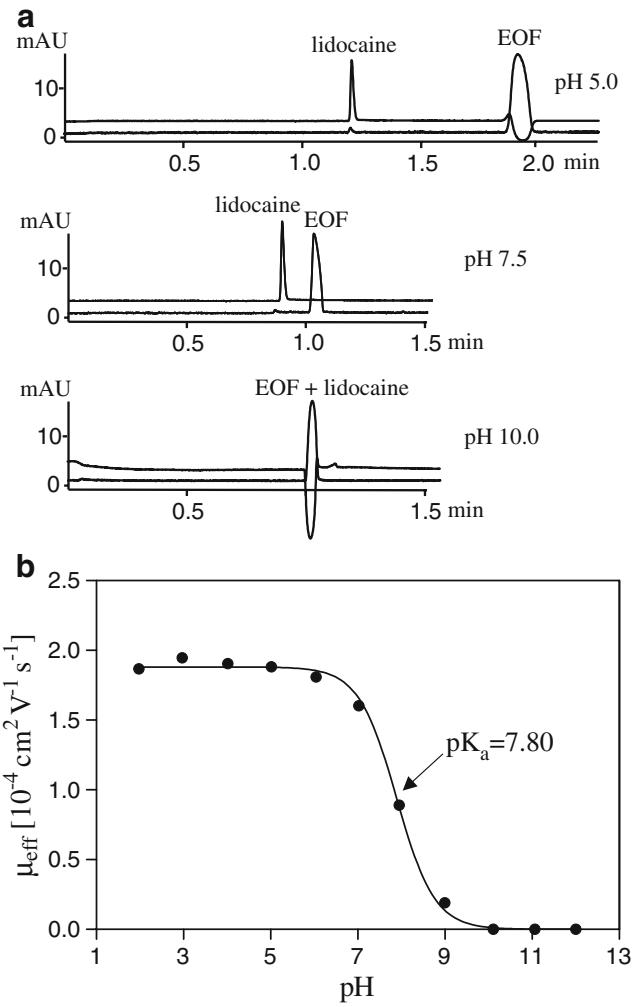

Fig. 2 **A** Electropherograms of the monobasic compound lidocaine and the EOF marker at different pH values: at pH 5.0 the compound is mainly in its cationic form and migrates before the EOF, at pH 7.5 the compound is partially ionized and migrates closer to the EOF, and at pH 10.0 the compound is neutral and migrates with the EOF. **B** The curve of the effective mobility of lidocaine as a function of pH is sigmoidal: at pH<6 the compound is mainly present in its cationic form and μ_{eff} is high, between pH 6 and 10 the curve shows an inflection point corresponding to the pK_a of lidocaine, and at pH>10 the compound is neutral and μ_{eff} is equal to zero

instability, as they are highly volatile. Amino-based buffers like butylammonium, ethanolammonium and diethanolammonium tend to interact with the capillary wall, generating distortions in the curves of μ_{eff} versus pH. Some other buffers interact with certain types of analytes, which could affect their migration behavior [30–35]. For example, it is well known that borate buffers interact strongly with compounds possessing consecutive diols, like catechol. Finally, interactions between mono/dihydrogenophosphate, MES, HEPES and protonated amines may occur when the pH is higher than the pK_a, and similarly between borate buffers and deprotonated amines when the pH is lower than the buffer pK_a. Therefore, taking into account the constraints discussed above, recommended buffers have been extracted from the literature for the whole pH range, and are listed in Table 2. Another important parameter concerning buffers is their ionic strength. A constant ionic strength should be used to avoid μ_{eff} variations and keep activity coefficients constant. If each buffer has a different ionic strength, corrections for ionic strength must be calculated for each μ_{eff} measurement. Since the different formulae used for μ_{eff} corrections are approximate [36, 37], a decrease in the accuracy of the results must be expected [35]. Therefore, it is easier to work at constant ionic strength and then correct the pK_a values determined with activity coefficients for zero ionic strength. The ionic strength is generally set to between 10 and 50 mM to ensure sufficient buffer capacity and low Joule heating. Buffers cover a large pH range (e.g., from 2 to 12), generally with an increment of 1.0 pH unit. For compounds with multiple pK_a values close to one another, more buffers should be used (for example with an increment of 0.5 pH units) to obtain good curve fitting [38].

Temperature variations should be strictly avoided when determining pK_a values, since this parameter is dependent on the temperature [39]. Numerous parameters influence the temperature inside the capillary, such as the applied voltage, the buffer conductivity, the capillary dimensions (diameter, total length) and the thermoregulation system (controlled by an air- or liquid-pooling system). Therefore, the applied voltage should be adjusted to remain in the linear region of Ohm's law and prevent excessive Joule heating [40]. In the absence of active cooling of the capillary, it is possible to correct the measured mobilities to standard temperature with empirical equations [41].

Organic or hydroorganic solvents can alternatively be used to determine dissociation constants of water-insoluble

Table 2 Recommended buffers according to [30]

pH	Buffer	pK_a	Possible interactions
1.5–2.5	Phosphate	2.12	
3.0–4.5	Formate	3.75	
4.0–5.5	Acetate	4.76	
5.5–7.0	MES[a]	6.13	Protonated amines
6.5–8.0	MOPS[b]	7.20	Protonated amines
6.5–8.0	Phosphate	7.20	Protonated amines
7.5–9.0	Tricine[c]	8.15	Protonated amines
8.5–10.0	CHES[d]	9.30	Protonated amines
8.5–10.0	Borate	9.24	Diols
11.5–12.0	Phosphate	12.37	Protonated amines

[a] MES: 2-morpholinoethanesulfonic acid
[b] MOPS: 3-(N-morpholino)propanesulfonic acid
[c] Tricine: N-tris(hydroxymethyl)methylglycine
[d] CHES: 2-(N-cyclohexylamino)ethanesulfonic acid

or sparingly soluble compounds. Methanol and acetonitrile are the organic solvents most commonly used in CZE, with a preference for methanol as it has properties closer to those of water. Numerous papers deal with pK_a determination by CZE in aqueous–organic solvents [33, 42–56] or nonaqueous solvents [57–62]. Practically speaking, the pH of hydro-organic buffers is measured with electrodes calibrated with the usual aqueous standards, which leads to the absolute ${}^{s}_{w}$pH scale (i.e., pH measured in solvent s, relating to the water pH scale) [63]. Then the aqueous pK_a values (i.e., ${}^{w}_{w}$pK_a) can be estimated from the ${}^{s}_{w}$pK_a values determined in aqueous–organic solvents by Yasuda–Shedlovsky extrapolation [64]. The pH of an organic buffer is measured with electrodes calibrated with organic standards prepared by mixing equimolar quantities of acids and their conjugates with known ${}^{s}_{s}$pK_a values. This leads to the relative ${}^{s}_{s}$pH scale (i.e., pH measured in solvent s, relating to the solvent pH scale). The ${}^{w}_{w}$pK_a values can be estimated from nonaqueous solvents by means of linear extrapolation equations derived for similar types of compounds [41].

The UV detector is the preferred one for pK_a determination by CZE, as it is sensitive enough for the majority of pharmaceutical compounds. Moreover, it enables a simultaneous spectrophotometric determination of pK_a values (i.e., from UV spectra measured at the maxima of the electrophoresis peaks), as described by several authors [51, 65–68]. The pK_a values of non-UV-absorbing compounds can be determined by indirect UV detection [69, 70], amperometric detection [71] or contactless conductivity detection (CCD) [30, 36, 37]. The latter is particularly interesting, as it can be easily implemented in CZE and may provide interesting complementary information to photometric detection. Finally, mass spectrometry (MS) can be coupled to CZE [72]. This detector presents several advantages in terms of sensitivity and universality. Furthermore, several compounds can be analyzed simultaneously due to its high selectivity, increasing the throughput of pK_a determination. However, the big challenge of pK_a determination by CZE-MS is to find volatile and stable enough buffers for the whole pH range.

Towards high-throughput methods

Numerous efforts have been recently made to increase the throughput of pK_a determination by CE. A major challenge with conventional CE methods is the long migration times at low pH due to the limited EOF. An interesting way to overcome this problem is to use dynamic coatings. For anions, a suitable strategy consists of using a positively charged coating, generally polybren, in negative mode [69, 73–75]. Neutral polyacrylamide coatings eliminating the EOF can be utilized for cations in positive mode and anions in negative mode [32, 43, 76]. Finally, the most generic strategy is to employ a double coating (polybren and poly (vinylsulfonate) (PVS) or CEofix®), thus creating a high EOF whatever the buffer pH [14, 30, 77].

The use of short capillaries is not recommended due to the high electric fields, which generate significant Joule heating. On the other hand, short-end injection is a good strategy as it reduces the effective length of the capillary without enhancing Joule heating [14, 30, 55, 72, 78, 79]. The latter strategy coupled with a dynamic coating procedure enabled pK_a determination in about 2 h per compound [30].

To increase the throughput, pressure-assisted capillary electrophoresis (PACE) was developed for pK_a determination [67, 79–84]. The application of an external pressure of up to 2 psi does not seem to affect pK_a determination but it does increase peak broadening. Therefore, it may decrease the precision of t_m and t_{EOF} determination, and μ_{eff} measurement. The throughput of such a PACE method was reported to be about 20 compounds per day [81, 82], but it can be further increased to 50 compounds in less than 3 h using MS detection due to sample pooling [72].

The throughput obtained by instruments with a single capillary is limited by the number of experimental points needed to fit the curves of μ_{eff} versus pH. More recently, several applications using vacuum-assisted multiplexed 96-channel capillary electrophoresis with UV detection have been reported for high-throughput pK_a screening (24 compounds/h) [23, 56, 85–87]. The ability to adapt existing single-capillary electrophoresis methods to a multiplexed instrument proves that CE has its place in drug discovery and development.

Lipophilicity

Lipophilicity is expressed as the logarithm of the partition coefficient (log P) for partitioning between two immiscible solvent phases, and is valid for a single electrical species [88]. The distribution coefficient, expressed as log D^{pH}, refers to the weighted contributions of all electrical forms of an ionizable compound present at a given pH [22]. The 1-octanol/water system is the widely accepted reference system for the determination of lipophilicity.

Traditional methods

The classical shake-flask technique has little evolved over the decades but remains the reference method for lipophilicity measurement [89]. Briefly, the compound is mixed with two immiscible solvents, generally water and 1-octanol, until equilibrium is reached. Then the two phases are separated and the solute concentration is usually measured in both of them. However, this procedure is tedious, time-consuming and sensitive to impurities. It is also prone to emulsion problems and requires large amounts of sample. Therefore,

in the last decade, different strategies have been developed to speed up, automate and miniaturize the shake-flask approach. An automated shake-flask system for high-throughput log *D* measurement (48 samples/day, in duplicate) was described by Hitzel et al. [90]. The entire liquid handling is performed by a robotic system, and the partitioning process takes place on a 96-well plate. After shaking, each phase is directly injected from the plate into the LC-UV system with an auto-injector. A fast generic gradient allows the concentration of the solute of interest to be measured. An alternative is to use an LC-MS system [91]. A commercially available instrument using an automated and miniaturized shake-flask method is manufactured by Analiza (Cleveland, OH, USA) [92, 93]. More recently, a high-speed log D (HSLogD) measurement system based on automated sampling was developed to reduce contaminations from the 1-octanol phase when sampling the water phase [94]. This contamination was prevented by aspirating a plug of water before sampling the water phase. Despite all of these improvements, this technique still requires some time-consuming steps that multiply the manipulations involved, like the mutual saturation and decantation of both phases. In addition, the measurable log P_{oct} range remains limited (from −3 to 4).

When dealing with ionizable compounds, dual phase-potentiometric titration is possible. This method is based on the pK_a shift that occurs in the presence of a partitioning solvent, for instance 1-octanol. A large difference between the true and apparent pK_a values indicates a large log *P* value. A log *P* determination requires two titrations and directly provides a distribution profile rather than single points [22]. This method presents the same features as pK_a determination in terms of sample consumption and concentration, and the same automated instrument (Sirius GLpKa from Sirius Analytical Instruments) can be used. However, this technique is only appropriate to ionizable compounds, and it is difficult to handle weak lipophilic bases with low pK_a or weak acids with high pK_a values.

Cyclic voltammetry at the interface between two immiscible electrolyte solutions (ITIES) is the method of choice for studying the log *P* of ions (log P^I) [22, 95]. The distribution of a particular ion at the ITIES is determined by how similar its electrochemical potentials in the two phases are, and log P^I can be deduced from the Nernst equation at the ITIES.

Capillary electrophoresis

Theory and principles

As reported in several reviews [23, 25, 26, 29, 96–102], capillary electrophoresis is emerging as an interesting separative method for log P_{oct} determination due to its inherent advantages, as enumerated in the relevant subsection of "Ionization constants" (also, cf. Table 3). For this

Table 3 Experimental techniques for log P_{oct} determination

Method	Shake flask	Potentiometry	Cyclic voltammetry	MEEKC-UV	LC-UV
Measurement	Direct single points	Direct distribution profile	Indirect single points	Indirect single points	Indirect single points
log P_{oct} range	−3 to 4	0 to 8	−8 to 0	−1 to 7	−1 to 8
Quantity	2–10 mg	2–10 mg	1–10 mg	<<<1 mg	< 1 mg
High purity	Necessary	Necessary	Necessary	Not necessary	Not necessary
Low/medium-throughput instrumentation	Shaking system and detector	Automatic titrator: GLpKa (Sirius Analytical Instruments, Forest Row, UK)	Electrochemical system	Capillary electrophoresis unit (various vendors)	Liquid chromatography unit (various vendors)
High-throughput instrumentation with 96-well plate technology	Automated shake-flask system ADW (Analiza, Cleveland, OH, USA)	-	-	Multiplexed capillary electrophoresis: cePRO 9600 (Advanced Analytical Technologies, Ames, IA, USA) or MCE 2000 (Pfizer Laboratory, Groton, CT, USA)	Ultrahigh-pressure liquid chromatography (various vendors)
Miscellaneous	Tedious procedure	Only applicable to ionizable compounds	Only for ionic species	Microemulsion stability might be an issue	Secondary interactions can happen

purpose, a CE mode that is able to separate neutral analytes (i.e., one combining the features of conventional CE and LC) is needed. Therefore, different CE modes (including a pseudo-stationary phase) have been tested, namely MEKC, MEEKC and VEKC/LEKC (micellar, microemulsion and vesicle/liposome electrokinetic chromatography, respectively).

MEKC is an electrokinetic chromatography technique that uses buffers containing micelles as a pseudo-stationary phase. The separation of neutral compounds is due to differential partitioning between aqueous and micellar phases. Micellar pseudo-stationary phases, commonly sodium dodecyl sulfate (SDS), are considered to mimic biological membranes better than 1-octanol or RPLC stationary phases [25]. Practically speaking, log P_{oct} determination by MEKC is based on the measurement of the retention factor k:

$$k = \frac{t_R - t_{EOF}}{\left(1 - \frac{t_R}{t_{MC}}\right) \cdot t_{EOF}} \quad (3)$$

where t_R, t_{EOF} and t_{MC} are the migration times of the analyte, neutral marker and micelle marker, respectively. Highly hydrophilic neutral compounds such as acetone are used as EOF markers, whereas highly lipophilic compounds such as dodecaphenone are selected as micelle markers. Good linear correlations were found between log k (measured by MEKC) and log P_{oct} for a wide variety of compounds and conditions [103–122]. However, congeneric behavior was observed [108, 116] and confirmed by LSER analyses, which demonstrated differences in H-bond basicity and dipolarity/polarizability for partitioning into SDS micelles or the 1-octanol/water system [98].

LEKC and VEKC are also electrokinetic chromatography techniques that use liposomes or vesicles (i.e., bilayer structures enclosing an aqueous core region) as a pseudo-stationary phase [102]. Linear correlations between log k measured by LEKC/VEKC (Eq. 3) and log P_{oct} were reported [123–126], and these techniques appear to mimic physiological membranes more closely than ME(E)KC [127]. However, vesicles and liposomes are unstable and difficult to prepare reproducibly. Therefore, their use remains limited for log P_{oct} determination [99].

Another electrokinetic chromatography technique using buffers containing nanometer-sized oil droplets (MEEKC) has been widely used, as it is the most appropriate CE mode for log P_{oct} determination. MEEKC separations are based on the same principles as MEKC, LEKC and VEKC. Numerous linear correlations between log k measured by MEEKC (Eq. 3) and log P_{oct} were reported in the literature [86, 128–145], and calibration curves with standard compounds generally allowed accurate log P_{oct} determinations over a large log P_{oct} range (from −1 to 7) [133–136, 140, 142, 144]. Indeed, microemulsion systems are known to be more stable and reliable than MEKC for log P_{oct} estimation [129], as they more closely resemble phospholipidic vesicles than SDS micellar systems do [128]. LSERs analyses also suggest that microemulsion systems are good models for the 1-octanol/water partitioning process [142, 146]. However, it should be noted that the investigated acidic or basic compounds must be in their neutral forms, since their ionized forms may generate ion pairs with surfactants and additional electrophoretic migration. This additional electrophoretic migration can be corrected for when ion-pair interactions are not involved or are very weak [147].

Experimental conditions

The most critical experimental conditions for log P_{oct} measurement by MEEKC, like the composition of the microemulsion system, need to be pointed out.

Microemulsions are complex ternary mixtures of water, oil and surfactant. Therefore, the microemulsion buffers used in MEEKC are made up of many different components. Variations in the nature and concentrations of these components can affect the microemulsion stability and migration. Microemulsions are stabilized by a surfactant and a cosurfactant, which both reduce the interfacial tension between the oil–water interface. The cosurfactant molecules lie between the surfactant ones at the surface of the microemulsion, thus reducing the repulsion between the surfactant head groups. The most widely used surfactant, oil and cosurfactant in MEEKC are SDS (anionic), heptane and 1-butanol, respectively [99, 148–151]. A typical microemulsion system used for log P_{oct} determination consists of 6.5% w/w 1-butanol, 0.8% w/w heptane and 1.4% w/w SDS. However, some authors have used concentrations of up to 3.3% w/w SDS, both to increase the microemulsion stability and to widen the separation window, especially for compounds with high log P_{oct} values [142].

Although phosphate and phosphate/borate buffered systems have mainly been used for log P_{oct} measurements, zwitterionic buffers such as CAPS are being used more and more due to their lower conductivities [142]. Indeed, even if temperature has less of an effect on log P_{oct} than pK_a measurements, accurate and reproducible results should be obtained in a thermostated environment [89].

Most applications were performed with UV detection. To the best of our knowledge, MS detection has not been used to increase the throughput of log P_{oct} determination by MEEKC. Indeed, microemulsion buffers contain nonvolatile components such as SDS, which could generate ion suppression as well as ionization source contamination and clogging. Therefore, the sensitivity decreases dramatically when using ESI-MS detection [26]. To overcome this problem, it is now possible to switch from ESI (electrospray ionization) to APPI (atmospheric pressure photoionization) in CE. Indeed, the latter offers a higher

compatibility with nonvolatile buffers and has a similar sensitivity to CE-ESI-MS [152–154].

Towards high-throughput methods

As for pK_a determination by CZE, several efforts have recently been made to increase the throughput of log P_{oct} determination by MEEKC.

A major problem is the long migration time at low pH due to the limited EOF. Microemulsion systems usually contain an anionic surfactant (SDS) and are used under alkaline conditions. However, it is sometimes necessary to work at low pH where the EOF is limited or even nonexistent, for example when determining log P_{oct} values of acidic compounds. To overcome this problem, the polarities of the electrodes can be reversed. However, separate experiments with positive polarity are needed to determine the EOF mobility. A more elegant solution to this problem is to use sulfonic acid-coated [132, 133] or dynamically coated capillaries with adsorbed bilayer structures such as poly(diallyldimethylammonium chloride) (PDMAC) and PVS [138] or polybren and PVS [139], which provides a sufficient EOF at low pH.

Other rapid approaches for log P_{oct} determination have been developed. For instance, pressure-assisted MEEKC can improve the throughput to up to 48 samples per day using a single-capillary system [135], while an instrument and software dedicated to this experiment in an automated format with 96 parallel capillary channels allow high-throughput log P_{oct} determinations (46 compounds/h) [86, 140, 142]. The latter is probably the most interesting strategy for drug discovery and development.

Liquid chromatography

Theory and principles

The general methodology of lipophilicity determination by LC has been the subject of several reviews [89, 96–98, 100–102, 155–159]. Indeed, the indirect reversed-phase liquid chromatography (RPLC) approach is considered to be the best alternative to the direct shake-flask method for lipophilicity assessment, since it offers numerous advantages (cf. Table 3). (i) The sample consumption is small: 100 µl of sample at a concentration of 10–500 µM. (ii) The instrumentation is fully automated, compatible with the 96-well plate format, and the sample throughput is high. (iii) There is no requirement for highly pure samples, as it is a separation method. (iv) This technique is universal, since different detection systems can be coupled to LC. (v) The measurable log P_{oct} range is large (from −1 to 8).

The ability of RPLC techniques to assess lipophilicity relies on the similarity between the retention mechanism on RPLC stationary phases and octanol–water partitioning. The determination of log P_{oct} by RPLC is based on the measurement of the retention factor k of the investigated compound between the mobile and stationary phase:

$$\log\ k = \log\left(\frac{t_R}{t_0} - 1\right) \tag{4}$$

where t_R is the retention time of the investigated compound and t_0 is the column dead time. The extrapolation of log k values to 100% water, which leads to log k_w values, is a convenient means of standardizing chromatographic lipophilicity parameters.

Lipophilicity measurements can be performed in either isocratic or gradient mode. Under isocratic conditions, log k values are calculated from t_R measurements at various concentrations of the organic solvent in the mobile phase (Eq. 4), as described in Fig. 3A. When using methanol, isocratic log k values are linearly correlated with the organic modifier percentage φ in the mobile phase (as exhibited by Fig. 3B):

$$\log\ k = \log\ k_w - S \cdot \varphi \tag{5}$$

where S is a constant that depends on the nature of the compound and the organic modifier. With acetonitrile, the correlation between log k and the organic modifier percentage in the mobile phase is quadratic [160]:

$$\log\ k = \log\ k_w + B \cdot \varphi + A \cdot \varphi^2 \tag{6}$$

where A and B are constant for a given compound and organic modifier.

Numerous linear correlations between log P_{oct} and log k_w obtained in isocratic mode have been reported in the literature for different classes of compounds [161, 162], as also shown in Fig. 3C. Valko et al. also observed linear relationships between log P_{oct} and φ_0, which correspond to the organic modifier concentration (from 0 to 100%) that produces an equimolar distribution between the stationary and mobile phases (i.e., when log k=0) [163, 164]. Finally, it should be noted that several linear correlations between log P_{oct} and isocratic log k values at different organic modifier percentages have been described [165–173]. However, all these linear relationships are generally better with structurally related compounds (cf., congeneric behavior) or a low number of analytes.

Gradient approaches were proposed to speed up lipophilicity determination. They are generally based on the linear solvent strength (LSS) theory elaborated by Snyder and Dolan, where the analyte retention time (t_R) in a linear-gradient separation can be expressed as follows [174]:

$$t_R = \frac{t_0}{b} \cdot \log\ (2.3 \cdot k_i \cdot b + 1) + t_0 + t_D \tag{7}$$

where k_i is the k value at the initial isocratic composition of the gradient, t_0 is the column dead time (min), and t_D is the

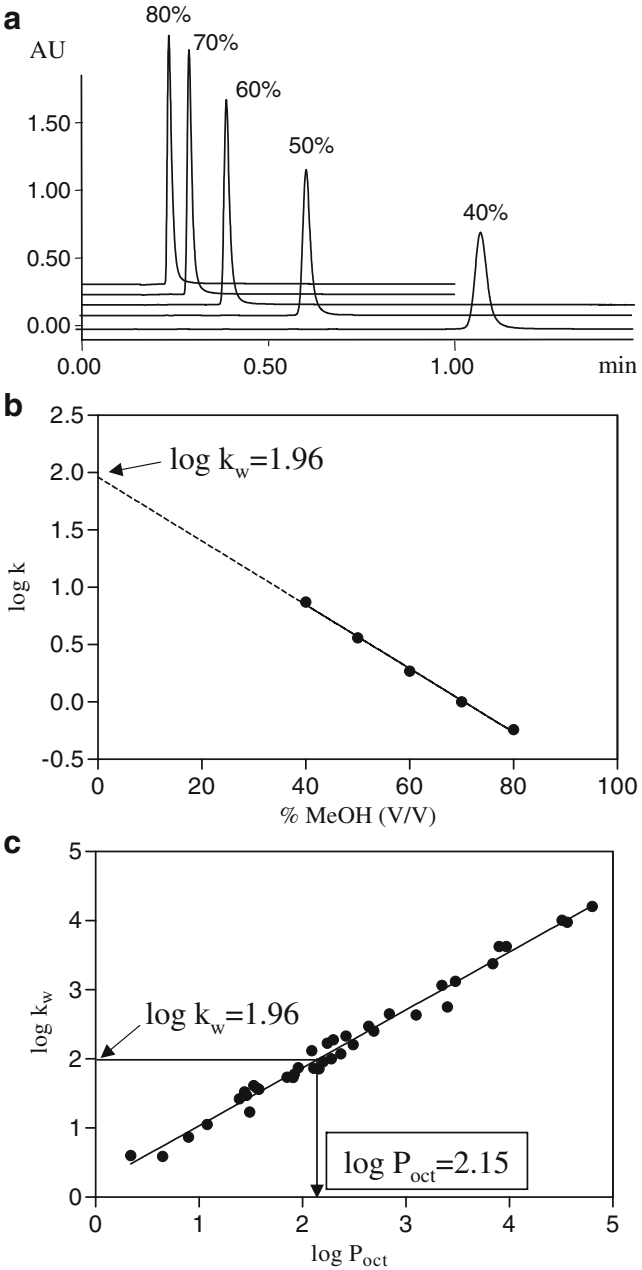

Fig. 3 **A** Chromatograms of propiophenone at different percentages of methanol. **B** Plot of log k obtained for propiophenone as a function of the percentage of methanol, and extrapolation of the log k_w value to 100% water by linear regression. **C** Calibration line allowing the calculation of the log P_{oct} value of propiophenone from the obtained log k_w value

system dwell time for gradient elution (min). The gradient steepness parameter b can be described by the following relationship:

$$b = \frac{t_0 \cdot \Delta\varphi \cdot S}{t_G} \quad (8)$$

where t_G is the gradient time from the beginning to the end of the gradient (min), and $\Delta\varphi$ is the change in composition during the gradient (from 0 to 1). The two unknown parameters k_i and S in Eqs. 7 and 8 can be obtained from two gradient runs differing only in gradient time. These two values then allow estimation log k_w directly with Eq. 5 when using methanol or Eq. 6 when using acetonitrile. Appropriate calculation procedures for log k_w and S are included in available modeling software, such as Drylab (Rheodyne, Rohnert Park, CA, USA) or Osiris (Datalys, Grenoble, France) [175–177]. An alternative strategy which does not require complex calculation procedure involves assuming that S is constant for structurally similar compounds. Although this approach is evidently less accurate than the former, it allows estimation log k_w from a single gradient run [92, 178–182]. Based on this assumption, Valko and coworkers introduced the chromatographic hydrophobicity index (CHI), derived from the φ_0 scale [183–188]. Numerous linear relationships between a retention parameter obtained in gradient mode and log P_{oct} or log D values have been reported in the literature. In conclusion, it can be stated that the gradient mode is a more generic and faster strategy than the isocratic mode. However, data treatment remains complex and it is not as accurate as the isocratic mode [175, 189, 190].

Experimental conditions

The choice of several experimental conditions, such as the organic modifier and the stationary phase, is crucial for log P_{oct} determination by RPLC.

Mobile phase Methanol and acetonitrile are the two organic modifiers most commonly employed for log P_{oct} determination. In the literature, methanol was generally preferred to acetonitrile [89, 101, 160]. Indeed, quadratic extrapolation (Eq. 6) is less accurate than linear extrapolation (Eq. 5) [191]. Moreover, as a protic solvent, methanol can interact with free silanol groups, reducing secondary interactions between analytes and silanol groups. Additionally, during equilibration, MeOH molecules form a monolayer at the surface of the stationary phase, which provides a hydrogen-bonding capacity in better agreement with 1-octanol/water partition [157].

In the last few years, many authors have shown that the addition of a small amount of 1-octanol to the methanol fraction of the mobile phase (usually 0.25% V/V) and the use of 1-octanol-saturated buffers improves the correlation between log P_{oct} or log D and log k_w values, especially in the case of basic compounds [157, 192–201]. Octanol seems to form a coating on the silica support of the column during equilibration, conferring octanol-like properties to the stationary phase. Additionally, it may act as a weak masking agent, reducing secondary interactions caused by

residual silanol groups [201]. However, the precise mechanism through which 1-octanol influences the retention is still not fully established. It should also be noted that the addition of 1-octanol to the mobile phase is a tedious and time-consuming strategy due to the saturation of buffers and the long equilibration times, which are crucial for the repeatability of the measurements.

Stationary phases Octadecyl-bonded silica (ODS) stationary phases have been used extensively for lipophilicity measurements for over 30 years. However, silanophilic interactions due to the free silanol groups (including hydrogen bonding as well as electrostatic interactions) interfere in the partitioning mechanism of these columns. To overcome this problem, apolar and polar end-capped stationary phases were developed. However, the latter had no real advantage over classical ODS columns for log P_{oct} determination [202]. On the contrary, polar embedded stationary phases with an amide, carbamate, ether or sulfonamide group are considered better than endcapped ones for lipophilicity estimation due to the electrostatic shielding provided by the polar group, thus reducing the access to the free silanol groups [202]. However, other interactions between polar embedded groups and analytes may occur. More recently, high pH-resistant stationary phases allowing log P_{oct} determination of highly basic compounds have been launched, such as bidentate stationary phases and organic/inorganic hybrid stationary phases [201]. Hybrid stationary phases with embedded polar groups are the best choice for lipophilicity determination by RPLC [202]. Using such columns, there is no need to add a masking agent such as triethylamine and *n*-decylamine because of the lower amount of free silanols. Finally, monolithic silica stationary phases were also tested for fast lipophilicity determination, but their use remains limited [182].

Immobilized artificial membrane (IAM) columns [156, 158, 203] were widely investigated for lipophilicity determination. Briefly, IAM columns contain different types of phospholipidic monolayers covalently bonded to silica particles and are generally less retentive than ODS columns due to their polar head groups. They allow direct measurement of log k_w values for compounds with moderate lipophilicity, while up to 30% acetonitrile is used in the case of highly lipophilic compounds. There is not always a clear relationship between retention properties measured by IAM chromatography (log k_{IAMw} or log k_{IAM}) and log P_{oct} values. For neutral and acidic compounds, log k_{IAMw} values are generally linearly correlated with log P_{oct} values despite a less hydrophobic environment. However, there is a systematic deviation in the case of basic and zwitterionic compounds. Indeed, the retention mechanism on IAM columns is based not only on lipophilic interactions but also on specific ionic interactions, which are particularly important with positively charged compounds that interact with the phosphate anions of the stationary phase [200]. Consequently, retention on IAM stationary phases is only moderately affected by the presence of positive charges. The distribution coefficients measured in a 1-octanol/water system may thus underestimate the potential of positively charged compounds to interact with biomembranes [204–207]. Practically speaking, IAM chromatography raises several issues, since its retention properties are dependent on the experimental conditions and a column-aging phenomenon was reported by several authors [156, 208].

Recently, a new approach using hydrophilic interaction chromatography (HILIC) has been applied to lipophilicity determination. This separation mode is based on polar stationary phases (underivatized silica or silica bonded with polar groups like diols, amide, cyanopropyl, aminopropyl) with partly aqueous eluents containing a relatively hydrophobic miscible solvent, generally acetonitrile [209–212]. The retention mechanism is considered to be a complex mix of partitioning and adsorption of the compounds between the eluent and a water-rich layer partially immobilized at the surface of the polar stationary phase. There are a number of different interactions depending on the mobile and stationary phases involved: hydrophilic retention when high proportions of organic modifiers are used in the mobile phase, and a reversed-phase-type retention when the mobile phase contains high proportions of water. An additional ion exchange mechanism may occur on ionic HILIC stationary phases [213, 214]. Bard et al. measured retention factors of highly basic compounds in their cationic forms on a ZIC-pHILIC stationary phase based on a grafted polymeric layer with sulfoalkylbetaine zwitterioinic moieties on a wide-pore silica. As lipophilicity expresses the difference between hydrophobicity and polarity, the difference between the isocratic log *k* values measured at 0% and 95% acetonitrile ($\Delta\log k_{0\text{-}95}$) was linearly correlated to their log P_{oct} values (r^2=0.93) [213]. Therefore, this method offers a new and promising way to measure log P_{oct} values of highly basic compounds without working under extreme pH conditions. From a practical point of view, it should be noted that it is necessary to strictly control the nature of the buffer, the pH and the ionic strength, as the retention mechanism implies ion exchange (ionic competition).

Detection methods UV detection is commonly used for log P_{oct} determination by LC. However, some alternative detectors have also been used to deal with UV-inactive compounds. For instance, refractive index (RI) and evaporating light scattering detectors (ELSD) were used in several studies because of their quasi-universality [165, 171, 213]. In the last few years, several other aerosol-based detectors have been launched. Corona aerosol discharge

(CAD) and nano-quantity analyte detectors (NQAD), which are also able to deal with nonchromophoric compounds, are potentially more interesting than RI and ELSD due to their higher sensitivity. Finally, if the cost per analysis is not an issue, the detector of choice is MS, due to its high selectivity and quasi-universality. Indeed, the different atmospheric pressure ionization (API) sources available (electrospray ionization, atmospheric pressure chemical ionization and atmospheric pressure photoionization, ESI, APCI and APPI, respectively) allow a wide variety of compounds to be dealt with [168, 181, 185, 190, 215–218].

Towards high-throughput strategies

Several strategies were applied to speed up lipophilicity determination by LC, such as the use of short columns and high flow-rates. Both approaches decrease the analysis time and extend the range of measurable log P_{oct}. However, they cause a significant reduction in the chromatographic performance and the accuracy of the results [179, 180, 184, 193, 195].

Recently, the development of columns packed with small porous particles (sub-2μm) has enabled a substantial improvement of chromatographic performance in LC. Indeed, this technology enables a significant time reduction without compromising efficiency. However, such columns generate high backpressures (<1000 bar) and require dedicated instrumentation [219–221]. An ultrahigh-pressure liquid chromatography (UHPLC) system was used for log P_{oct} measurements, offering a significant increase in the throughput (by at least a factor of 12 compared to HPLC) [190, 222]. Moreover, due to the stability of Acquity BEH Shield RP18 hybrid columns at high pH (up to 11), log P_{oct} values of basic compounds with pK_a values ranging from 6.3 to 10.3 could be determined. However, these basic compounds displayed a different behavior from the optimal set of calibration compounds selected by cluster analysis. Nevertheless, accurate log P_{oct} values of basic compounds could be obtained in isocratic mode with 1-octanol addition or when using isocratic log k values at 50% methanol [190, 222]. The latter strategy is very interesting in terms of average log P_{oct} determination time, since a single run is required (ca. 5 min per compound). Finally, MS coupling allowed the throughput to be further increased (by an additional factor of 10) due to sample pooling. It should be noted that precise knowledge of the column dead time, extra-column volume and delay time is mandatory and extremely critical in UHPLC configuration for accurate retention factor determination.

Simultaneous determination of pK_a and log P_{oct}

Kaliszan et al. developed methods allowing the simultaneous determination of pK_a and log P_{oct} values from a set of experiments carried out on the same HPLC instrument. Apparent pK_a and log P_{oct} values could be determined in 3–4 gradient runs. Two organic modifier gradient runs allowed log P_{oct} values to be determined (see the "Theory and principles" subsection of the "Liquid chromatography" section) and the organic modifier percentage to be estimated, providing an appropriate retention coefficient for the nonionized analyte. Then, one pH-gradient run carried out with the previously determined eluent composition and one isocratic run to get the retention factor of the ionized form allowed the apparent pK_a values to be determined [175, 176, 189, 223–225]. The obtained log k_w values correlated well with log P_{oct} values. However, there was only a poor agreement with literature pK_a values. Therefore, this generic approach was further improved and a second method requiring 9 or 18 pH/organic modifier double-gradient runs was evaluated. Its success was limited, as the correlation with log P_{oct} values was lower than that seen for the previous method, and there was only a moderate agreement with literature pK_a values [226, 227]. Moreover, this strategy is time-consuming (9–18 runs) and requires complex data treatment.

Solubility

Solubility measurements are fundamental in drug absorption, as insufficient solubility can interfere with both pharmacokinetic and pharmacodynamic properties.

Two solubility values can be measured depending on the experimental method used, namely thermodynamic or kinetic solubility. Solubility assays in discovery and development must be adapted to phase properties and requirements (Table 4). In drug development, high-quality solubility data are needed by chemists to facilitate appropriate decisions to be made so as to overcome potential solubility liabilities. Additionally, solid-state properties (purity, degree of crystallinity, particle size and polymorphism) of the tested compounds are studied and characterized in detail. Therefore, an evaluation of thermodynamic solubility is required and is often considered the gold standard. Nevertheless, it is becoming increasingly common to measure it earlier in the discovery process. Solubility measurements are also performed in biorelevant media to identify in vitro/in vivo correlations in accordance with the Biopharmaceutical Classification System (BCS) [228, 229]. This classification has been developed to identify the fundamental rate-limiting biopharmaceutical factors of intestinal drug absorption, and classifies drug compounds into four classes based on their solubility and intestinal permeability (Fig. 4). This system typically takes aqueous solubilities measured in buffer media across the whole pH range 1–7.5 as input.

Table 4 Differences between the methods used for solubility measurement in drug discovery and drug development processes

	Discovery	Development
Compounds tested		
Number	100–1000	10
Quantity available	A few mg	> g
Purity	Limited	Improved
Solid state	Amorphous or partially crystalline (not characterized)	Stable, crystalline material (characterized)
Distribution	Generally in DMSO stock solutions	Generally in solid form
Methods		
Type of solubility measured	Kinetic solubility (fully dependent on experimental conditions)	Thermodynamic solubility
Throughput	High	Low 25–50 compounds a week
Automation	Fully	Only partially automated
Format	96-well or 384-well microplates	Small scale (single tube)
Incubation time	Minutes	Hours or days
Detection	UV, turbidity	HPLC-UV, HPLC-MS
Media	Aqueous	>20 (aqueous, organic, biorelevant media, formulations, excipients…)
Data generated and intended purpose	Solubility in screening bioassay media to avoid misinterpretation	Solubility and dissolution in biorelevant media
	Rank-order hits	Evaluation of formulations
		Characterization and optimization of solid state
		Selection of promising compounds
		Development of adequate strategies to overcome solubility problems

In drug discovery, kinetic solubility is the most relevant parameter for rapidly evaluating whether the compound, which is generally predissolved in dimethyl sulfoxide (DMSO), stays in solution after dilution in specific screening media. Incomplete solubility could indeed lead to unreliable results, underestimated activity, reduced HTS hit rates and an inaccurate structure–activity relationship (SAR) when not identified [230]. For these reasons, experimental conditions are adapted to the conditions of the bioassay.

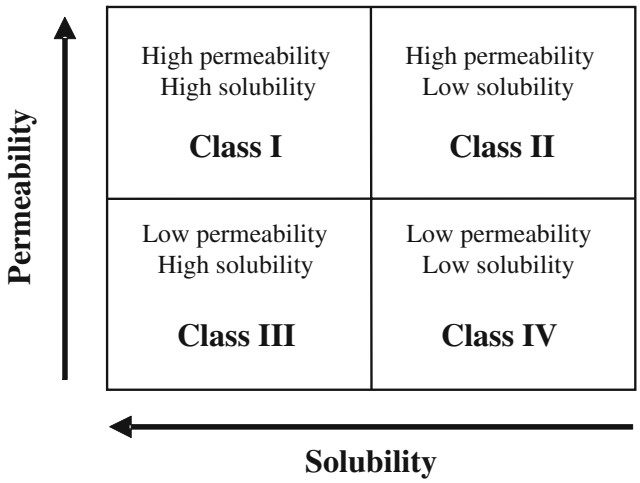

Fig. 4 Schematic representation of the Biopharmaceutical Classification System

Reference methods

Thermodynamic solubility

The shake-flask method is considered the method of reference for solubility measurement. An excess of solid sample is added to the medium in a flask, and the resulting suspension is shaken until equilibrium solubility (i.e., thermodynamic solubility) is reached, generally after 24 h. Undissolved sample is then removed by filtration or centrifugation, and the concentration of the compound in the filtrate is determined. Effects of crystal lattice and polymorphic forms are considered in this process, which is important, as they can significantly influence the solubility [231]. Therefore, thermodynamic solubility is often considered the "true" solubility of a compound. Nevertheless, this method suffers from several weaknesses: (i) relatively large amounts of compounds (several mg) are required to perform the measurements; (ii) the individual weighing step is time-consuming and difficult to automate [232]; and (iii) long incubation times are required to reach equilibrium solubility. Therefore, this method is mainly used in advanced studies of lead compounds, which are often

different from the crystal form used during early discovery stages [231].

Kinetic solubility

In contrast to thermodynamic solubility, kinetic solubility measurements are based on the precipitation of a predissolved compound (in a cosolvent or, for ionizable compounds, in aqueous media by pH adjustment) after dilution in a given medium. Kinetic solubility measurement is not a substitute for thermodynamic solubility evaluation, because crystal lattice disruption is neglected in this process. Moreover, the solubility is not measured at equilibrium, and the appearance of a precipitate is strongly time-dependent.

The potentiometric titration approach first introduced by Avdeef [233–235] is the reference method for kinetic solubility measurements. With this method, solubility measurements are based on the difference between the aqueous drug pK_a measured in the absence of a solid phase and the apparent pK_a $\left(pK_a^{app}\right)$ determined in the presence of an excess of solid compounds. The shift observed is proportional to the loss of compound, and thus to the solubility. Two commercial systems, pSol and CheqSol, are currently available. As the potentiometric method allows reliable measurements of kinetic solubility without cosolvent, this technique is often used to calibrate high-throughput solubility methods and computational procedures [2]. This approach requires only small amounts of compounds [236] and provides a complete solubility pH profile with a limited number of experiments. Therefore, it allows a better understanding of solubility behavior throughout the gastrointestinal tract [235]. However, this technique, suitable for ionizable compounds, needs an accurate knowledge of the pK_a of the molecule to obtain reliable solubility values, and is too slow for screening applications.

A simplified version of this methodology, named "chasing equilibrium," has recently been proposed [237] and validated [238]. This technique, which is based on the same principle as the traditional potentiometric titration method, is faster since the intrinsic solubility of the compound (i.e., the solubility of the neutral form) is determined instead of its entire pH/solubility profile. With this method, it is possible to measure the intrinsic solubility of a drug within 20–80 min (medium throughput). The approximate pH/solubility profile can then be calculated using Henderson–Hasselbach relationships.

Towards high-throughput methods

Considering the large number of compounds and the small amount of sample available at this stage of discovery, small-scale (generally 96-well format), compound-sparing and fully automated methods are necessary. Several HTS methods, in which compounds are introduced as dimethyl sulfoxide (DMSO) stock solutions into aqueous media, have been described. These HTS methods that perform kinetic measurements were designed to evaluate if the compounds dissolved in DMSO remain soluble after dilution in the aqueous screening media. Indeed, incomplete solubility could lead to unreliable results [230]. For this purpose, experimental conditions are adapted as much as possible to bioassay conditions, and final DMSO percentages ranging from 0.33% [239] to 5% [240, 241] have been reported in the literature.

Two different approaches are commonly used. In the first one, the formation of a precipitate, which is an indicator of the solubility limit, is detected (turbidimetric methods). In the second strategy, the precipitates are removed and the concentration of the solubilized fraction of the compound is determined.

In the turbidimetric method first introduced by Lipinski [231], small aliquots of DMSO stock solutions are added to buffer media each minute. The formation of precipitate is determined by measuring light scattering using a nephelometric turbidity detector in the 620–820 nm range. Further adaptations of this methodology were then proposed to keep the DMSO concentration in the buffer constant during addition and to scale down the method to a 96-well plate format [240–243]. The main drawbacks of these approaches are (i) the relatively low sensitivity of the detection, which does not allow compounds with very low aqueous solubilities (<20 μM) to be ranked [244], and (ii) their sensitivity to impurities.

In the second approach, small aliquots of DMSO stock solutions are diluted with aqueous buffer in a filter microplate to obtain a final DMSO percentage varying from 1 to 5% (v/v). When compounds present a poor aqueous solubility, precipitation occurs and precipitates are removed by filtration or centrifugation. Solubilities are then determined by measuring the concentrations of the solubilized fractions of the compounds using UV detection [241, 244, 245], HPLC [241, 244] or more recently UHPLC-MS [246]. This technique has been shown to be more sensitive and less subject to erroneous results caused by impurities than the turbidimetric method [244]. Undissolved material can also be removed by centrifugation instead of filtration. According to Alsenz et al. [5], both methods give comparable results. Nevertheless, divergent results can be observed for hydrophobic compounds due either to nonspecific adsorption on filter material or to the sample floating on the surface of the solvent in the case of centrifugation. However, filtration through filter microplates is faster than centrifugation [5].

Even if these HTS methods are very useful for evaluating solubility in screening bioassay media, solubility values are generally overestimated in comparison with thermodynamic solubility [247]. This phenomenon is attributed to the

cosolvent effect [244, 247] and the formation of supersaturated solutions [247, 248]. Moreover, solubility data obtained from different kinetic methods involving different experimental conditions are not intended to be comparable [237], as the appearance of a precipitate from a supersaturated solution is a slow process. Therefore, kinetic solubilities should not be taken as input data for ADMET prediction tools [5], and HTS methods allowing solubility measurements in good agreement with thermodynamic solubility thus remain highly desired [228, 249].

These limitations have encouraged researchers to find solutions for measuring thermodynamic solubility during the drug discovery process in order to obtain high-quality solubility data [5, 250]. One solution was to evaporate the solvent before starting the assay, thus allowing the thermodynamic solubility to be measured. This procedure was named the lyophilized solubility assay (LYSA) by some authors [5], and various protocols using DMSO [251], ACN [252], or MeOH/DME [253] as solvents for stock solutions have been described. This method gave solubility values in acceptable agreement with the thermodynamic solubilities [5].

Adapting standard protocols of kinetic solubility measurements is another approach used to minimize the effect of predissolution in DMSO. A recent study [247] showed that the increase in solubility registered using kinetic solubility methods was mainly due to the formation of supersaturated solutions with slow precipitation kinetics [247]. Supersaturation is a metastable state that is minimized by increasing the shaking rate and enhancing the incubation time to 24 h. These simple adaptations of experimental conditions were sufficient to give results in good agreement with shake-flask solubility without DMSO [247].

As the discrepancy between thermodynamic and kinetic solubility values may also be due to the difference from the solid state, Sugano et al. [254] proposed a modified version of kinetic solubility methods where the solid form of the precipitant was analyzed by polarized light microscopy analysis. It was reported that the solubility of the crystalline form could be lowered by up to 100-fold compared to that of the amorphous state [255], or 2–5-fold among crystal polymorphs [256]. Therefore, this technique provides a breakthrough in the measurement of high-quality data in early drug discovery.

Pharmaceutical companies are also looking for a HTS version of the shake-flask method. Miniaturized shake-flask methods requiring a minimal amount of sample (<1 mg) for the determination of equilibrium solubility have recently been proposed [236, 257]. However, the throughput of these methods remains relatively low (up to 30 compounds a week), essentially because the accurate distribution of solid compounds into vials or wells of a microplate is still a time-consuming and difficult to automate process. Even if new developments appear in the field of automated powder weighing systems [258], only a limited weighing accuracy (±15% for 2 mg, ±10% for more than 10 mg) can be obtained at the moment for a set of compounds with diverse properties [5].

Biorelevant solubility

The determination of biorelevant solubility is also necessary to evaluate whether the solubility could be a factor that limits absorption (e.g., in the gastrointestinal tract). It has been shown that drug solubility is enhanced in the intestine in comparison with aqueous buffers due to the presence of bile salts and lecithin. These amphiphilic components can self-assemble to form micelles, increasing the solubility of lipophilic compounds [259, 260]. They also act as wetting agents, enhancing the dissolution rate of drugs [261]. Moreover, after food intake, the levels of bile salts increase considerably, which could enhance the absorption of poorly soluble drugs. Therefore, two different in vitro intestino-mimetic media with biorelevant pH values [262] and bile salt concentrations [263] have been developed to evaluate dissolution and solubility in the small intestine: FeSSIF and FaSSIF, which simulate the fed and fasted state conditions, respectively. In these media, sodium taurocholate was chosen as a model bile salt, while lecithin was added to better mimic in vivo conditions, as it is always cosecreted with bile salts in a 4:1 ratio [260]. The compositions of these media have recently been updated to better represent the compositions and the characteristics of gastrointestinal fluids [264]. As in vitro biorelevant media are quite expensive, these solubility studies are generally performed in the advanced stages of drug development.

A HTS UV method was successfully adapted to measure biorelevant solubility in two different in vitro intestino-mimetic media (FaSSIF and FeSSIF), and excellent correlations were obtained with FaSSIF and FeSSIF solubility data measured by the shake-flask method (without DMSO) [247]. Thus, this method provides a reliable high-throughput method for measuring intestino-mimetic solubility during the early stages of drug discovery. Moreover, this method requires low amounts of sample, and the expensive components of these intestino-mimetic media (taurocholic acid and lecithin) are added as small volumes of a separated solution, thus decreasing the cost of these experiments.

Permeability

Permeation properties of drugs are an important challenge in pharmaceutical research. A large number of models (from in silico to in vivo) exist to mimic the main biological

barriers (in particular intestinal, percutaneous and blood–brain barriers). The different models have some inherent advantages and drawbacks, and each of them can be useful at a given step of the discovery and development process of drug candidates.

In this review, emphasis is placed on the most important methods developed to evaluate membrane permeation at the early stages of drug discovery. These methods are based on lipophilicity measurements in the liposomes/water system (providing better information on membrane permeation than the lipophilicity in the octanol/water system) and on artificial membranes.

Partitioning into liposomes as a predictor of passive transport

Lipophilicity measured in isotropic systems such as octanol/water systems accounts only for the partitioning of the neutral form, since ionic interactions are not expressed. Therefore, these systems are not the most appropriate model for in vivo drug distribution. In contrast, an organized phospholipid bilayer accounts for both hydrophobic and ionic interactions, like a biological phospholipidic membrane. For this reason, partitioning in a liposomes/water system is believed to be a better model for biological membrane permeability assessment. Lipophilicity measurements of neutral compounds are known to be comparable in octanol/water and liposomes/water systems, but ionic compounds partition better in a liposomes/water system than in an octanol/water system [265].

The determination of log P in a liposomes/water system (log P_{lip}) can be achieved via two types of methods: direct methods (potentiometry, equilibrium dialysis and NMR) or indirect methods (capillary electrophoresis, HPLC). Since direct approaches have been recently reviewed [266, 267], only highlights are exposed here.

As with isotropic systems (such as the octanol/water system), the determination of partition coefficients of ionizable molecules in a liposomes/water system by potentiometry is based on the shift observed between the ionization constant measured in aqueous solution (pK_a) and the ionization constant obtained in the biphasic liposome/water system $\left(pK_a^{app}\right)$. Indeed, log P is directly related to the difference between pK_a and pK_a^{app}. This method has the great advantage of being continuous. Therefore, it is possible to obtain a complete lipophilicity profile (log D vs pH). Experimentally, several solvents or anisotropic media can be used for potentiometric titrations. However, this method remains relatively time- and compound-consuming in comparison with new approaches based on 96-well technology for the evaluation of membrane permeability. Furthermore, in addition to the usual limitations arising from the determination of log P in isotropic systems, the use of liposomes presents some additional issues in terms of pH range, which should not exceed 3.5–10.5 pH units due to the ionization of phospholipids at lower pH values and hydrolysis at higher pH values [266].

To overcome the fastidious separation of the liposomes and aqueous phases in the traditional shake-flask method, a dialysis technique was proposed to avoid centrifugation and/or filtration steps. In the dialysis experiments, a chamber containing a liposomal suspension in a buffer solution is separated from a second chamber containing the analyte in a buffer solution by a semipermeable membrane. When the equilibrium between the two phases is reached, the solute concentrations in the two compartments can be determined, and a distribution coefficient is then calculated [266, 268, 269]. Radiolabeled solutes (if available) can be used [268–271] to limit the amount of compound needed and thus to be able to work with solute concentrations below the saturation level for the liposomes. This method is commonly reported for the determination of liposome/water partition coefficients, but suffers from several drawbacks. The determination of a complete lipophilicity profile is tedious and expensive as it is a single-point technique. Furthermore, generic methods are required in the early stages of drug discovery. With the dialysis technique, many parameters must be optimized for each tested compound. Therefore, the use of this method for high-throughput screening is impossible. Nevertheless, a dialysis technique coupled to HPLC and LC-MS has recently been applied to bring out the permeant components of a natural product extract [272]. The dialysis technique allows compounds of interest to be "filtered" and ranked in terms of potential permeation through biological membranes, while chromatographic separation and UV (DAD) and/or MS data allow the identification of each "filtered" compound. It is evident that optimal experimental conditions cannot be applied for each component of the extract, and it still takes a long time to reach equilibrium. However, the number of compounds screened simultaneously and the acceptable differentiation of their affinities to liposome membranes make this approach useful.

NMR spectroscopy has also been used to evaluate liposomes/water partition coefficients. The affinity of solutes for liposomes is evaluated using the relaxation rate of hydrogen and carbon nuclei. When the solute interacts with phospholipids, its relaxation time changes. A series of half-height line width measurements for different phospholipid concentrations are performed to evaluate the degree of interaction between solute and liposomes (since the signal broadening is a linear function of phospholipid concentration) [273]. One of the main advantages of this method is that it provides informative data on the interactions between liposomes and solutes. However, it cannot be considered a direct technique for obtaining true log P liposomes/water

values. Furthermore, even if this technique is faster than equilibrium dialysis, it remains slower than potentiometric titration [266].

The direct methods described above can be considered medium-throughput methods. Liquid chromatography is largely used to enhance the throughput of physicochemical profiling, particularly for lipophilicity determination [25, 100, 102]. In terms of permeability predictions, liposome electrokinetic chromatography (LEKC) provides an interesting way to obtain log P_{lip} values or direct correlations with in vivo permeability data. LEKC is a capillary electrophoresis mode with buffers that contain liposomes. In this case, the liposomes act as a pseudo-stationary phase. Lipophilicity in the liposomes/water system was obtained using different types and concentrations of phospholipids for a series of β-blockers [124], and recently permeabilities across the skin barrier [274], the blood–brain barrier [275] as well as intestinal absorption [276] have been targeted. Finally, LEKC possesses some of the advantages of chromatographic techniques but also the limitations associated with the use of liposomes [277] (see above for theory, principles and experimental conditions).

Towards high-throughput methods

An alternative way to evaluate partitioning in liposomes based on the use of TRANSIL® was also proposed [278, 279]. TRANSIL® is a solid (silica beads)-supported lipid bilayer consisting of different lipids or lipid mixtures. The analyte is added along with the TRANSIL® material to a vial or a well containing buffer. After two minutes of incubation, the beads are separated by filtration or low-speed centrifugation, and the supernatant is collected for quantification. The concentration of the analyte remaining in solution is then compared to a control vial or well containing compounds without TRANSIL® [279–281]. This technique was recently adapted for HTS application, using 96 or 384 wells, and is now available as “ready-to-go” plates (Sovicell, Leipzig, Germany), where the only pipeting operation required is to add the analytes [282]. Detection can be achieved using a UV reader plate, HPLC or LC/MS, depending on the compounds. UV detection remains the most rapid technique and is appropriate for almost 60–70% of the tested compounds. Even though the method is based on biological materials, the reproducibility of the assay on three “ready-to-go” plates shows good results.

Artificial membranes as HTS methods

PAMPA technique

PAMPA (parallel artificial membrane permeability assay) is a recent technique developed for the early evaluation of passive transport permeability. In PAMPA, a donor and an acceptor compartment are separated by a 96-well filter plate supporting a liquid artificial membrane, which mimics a biological barrier. The 96-well donor plate receives a buffer solution containing the analytes, and the acceptor plate initially contains a fresh buffer solution. The two compartments are assembled into a "sandwich-like" configuration and incubated for a determined time. After the incubation time, the sandwich is disassembled and the concentrations of compounds in both donor and acceptor compartments are determined and compared to the initial concentration in the donor compartment (the reference concentration).

In PAMPA, the effective (log P_e) or apparent (log P_a) permeability coefficients are determined by single-time-point sampling, thus allowing high-throughput measurements. The equations used to calculate the permeability coefficients can be deduced in several ways according to the experimental conditions and to the design of the in vitro assay, as well described by Avdeef et al. [7]. Therefore, different equations must be used to obtain permeability coefficients in different cases. For example, when the retention of compounds in the membrane cannot be neglected, sink conditions are applied using different pH buffers in the acceptor and donor compartments (single sink conditions), and a protein serum is added to the acceptor compartment (double sink conditions) (for the equations, see [7]).

Depending on the nature of the artificial membrane, different biological barriers can be targeted. The first model published in 1998 by Kansy and coworkers consisted of an egg lecithin membrane immobilized on a PVDF (polyvinylidene fluoride) filter [283]. The results showed a hyperbolic relation between the quantity of compounds that reached the acceptor compartment after the incubation time and human absorption data. However, the steep slope obtained complicates the evaluation of absorption for compounds presenting lower passive transport. Various membrane compositions were proposed, and the use of a mixture of phospholipids also improved the permeability predictions [284]. Later, Faller and coworkers proposed an adaptation of this technique for the prediction of intestinal absorption with hexadecane immobilized on polycarbonate (*PC*) filters [285]. The main advantage of this membrane is that it avoids the variability inherent to biological materials. A good correlation between effective permeability coefficients and percentage of intestinal absorption in humans was obtained for a series of structurally diverse compounds. Since then, the PAMPA technique has been applied to blood–brain barrier (BBB) penetration studies using porcine brain lipids in dodecane [286]. In this case, it has been shown that dodecane plays a crucial role, whereas lipids only slightly influence permeability, except for medium permeants, for which the dodecane/lipids ratio must be controlled [287]. However, the latter artificial membrane allows a ranking of

compounds according to the in vivo classification CNS+ (compounds crossing the BBB) and CNS− (compounds not transported into the brain).

An adaptation of the PAMPA technique has recently been proposed in order to mimic a percutaneous barrier [288]. This artificial membrane is composed of a mixture of isopropyl myristate (30%) and silicone (70%), and allows human skin permeability to be predicted, as well as the affinities of tested compounds for the stratum corneum, in a simple and rapid way.

Liquid chromatography coupled to MS detection has proven its capacity to increase the sensitivity of PAMPA experiments, particularly for poorly soluble compounds or compounds with no or low UV chromophores. Results have shown that MS detection provides greater sensitivity and better selectivity. Therefore, it can advantageously replace the more commonly used UV detection method. However, MS detection considerably decreases the throughput of the assay and should be used only for problematic compounds [289]. More recently, the use of UHPLC coupled to MS detection has been proposed [287, 290]. This method enables the concentrations of the compound to be determined in the reference, donor and acceptor compartments in triplicate in ~40 min at pH 2 (i.e., fourfold faster than with LC/MS(/MS) procedures) [291]. Furthermore, the throughput of the PAMPA assay with UHPLC/MS(/MS) was enhanced by injecting more than one compound at a time (sample pooling). However, this approach failed when the compounds were similar (in terms of retention factors, *m/z* ratio and/or fragmentation pathways) or when the compounds exhibit very low permeation [290]. Cassette incubation was also performed in brain penetration studies using PAMPA [287]. Standards were tested in four sets of six compounds, and permeability results were not significantly different from the permeability coefficients obtained for compounds incubated separately. Finally, the time required to determine the permeability coefficients dropped to 4.5 min for six compounds when both multiple-incubation and UHPLC/MS detection strategies were combined. As with the multiple-injection approach, the similarity of compounds remains potentially problematic, but this multiple-incubation method avoids the dilution step. Therefore, LOQ remains equal to the one expected in single-incubation experiments. Even though the throughput of permeability studies can be greatly enhanced with multiple-injection/incubation, interactions between compounds can occur during separation and/or incubation and should be controlled.

Immobilized artificial membrane chromatography (IAMc)

Immobilized artificial membrane chromatography has been developed to better mimic biomembranes. IAMs are based on phosphatidylcholine (PC) linked to a silica propylamine surface. Three typical phases have been widely used: IAM.PC.DD (a single acyl chain PC lipid phase), IAM.PC.DD2 and IAM.PC.MG (double acyl chain PC lipid phases with an ester linkage). IAM.PC.DD2 and IAM.PC.MG differ in terms of the end-capping of the residual amine [96]. However, only two of them are still commercialized (IAM.PC.DD2 and IAM.PC.MG), since IAM.PC.DD suffers from premature aging [292, 293]. The use of these stationary phases for lipophilicity determination has been widely reviewed [156, 158], in particular their ability to predict passive transport through diverse biological membranes [156]. In summary, the retention mechanism involved in the IAM stationary phase differs from the one involved in C18 columns or in the partitioning in an octanol/water system [294], particularly for basic compounds, which are more strongly retained on IAM columns due to polar effects of the linked phospholipids. Furthermore, ionic interactions between the anionic phosphate groups of the stationary phase and positively charged compounds have been demonstrated [200], as also reported for partitioning in phospholipids [295]. Therefore, IAM columns were tested for liposome/water partitioning, but it was noted that the correlation between the chromatographic index obtained and log P_{lip} depends on the nature of the solute and the phospholipids [296].

Studies on series of compounds showed that IAM retention factors obtained when using IAM.PC.DD, IAM.PC.DD2 and IAM.PC.MG were potential predictors of human intestinal absorption and brain penetration [156, 297]. All of these studies revealed that even though k_{IAM} describes permeation better than other physicochemical parameters, such as log P_{oct}, it is still not sufficient and must be corrected for the molecular weight or using a combination of calculated molecular descriptors and a solubility parameter.

Conclusion

In this review, experimental methods for the determination of solubility, ionization constants, lipophilicity and permeability applied early in drug discovery process were discussed, and important enhancements in terms of throughput were highlighted.

Although potentiometric and spectrophotmetric methods are still employed for pK_a determination, capillary electrophoresis is a valuable alternative in drug discovery and development, especially when multiplexed instruments are used.

The RPLC approach presents some obvious advantages for lipophilicity determination, particularly when using columns packed with small particles under ultrahigh-pressure

conditions (UHPLC). Indeed, it offers a significant increase in the throughput compared to the shake-flask method, which remains tedious. Nevertheless, the potential of MEEKC for log P_{oct} measurements should be further investigated to take better advantage of the similarity of microemulsion systems to phospholipidic membranes.

HTS methods leading to kinetic solubility values are convenient in the early discovery process, as compounds are usually predissolved in DMSO. The HTS UV method is a good compromise for solubility measurements due to its versatility. Additionally, different solubility information can be obtained by adapting the experimental conditions. The solubility under particular conditions or in particular media can thus be obtained prior to screening bioassays. Moreover, controlled experimental conditions can lead to solubility values that are very close to thermodynamic "true" solubility, which can be used as input for ADME prediction tools.

The TRANSIL® and PAMPA approaches can be considered the most potent methods for rapid passive transport studies. In the TRANSIL® method, the lipophilicity in a liposomes/water system can be measured with good accuracy and reproducibility due to the commercialization of "ready-to-go" plates, even though the method is based on biological material. In the PAMPA method, the nature of the artificial membrane allows the variability associated with biological materials to be avoided. Furthermore, the coupling of UHPLC with MS/(MS) detection permits the injection of more than one compound (sample pooling), which drastically increases the throughput and also opens up a new avenue for the analysis of more complex mixtures.

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