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Hydrophobicity of ionisable compounds studied by countercurrent chromatography

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ABSTRACT

Countercurrent chromatography (CCC) is a liquid chromatography technique in which the stationary phase is also a liquid. The main chemical process involved in solute separation is partitioning between the two immiscible liquid phases: the mobile phase and the support-free liquid stationary phase. The octanol–water partition coefficients ($P_{o/w}$) is the accepted parameter measuring the hydrophobicity of molecules. It is considered to estimate active principle partitioning over a biomembrane. It was related to the substance biological activity. CCC is able to work with an octanol stationary phase and an aqueous mobile phase. In this configuration, CCC is a useful and easy alternative to measure directly the $P_{o/w}$ of the molecules compared to other methods including the classical and tedious shake-flask method. Three ketones are used as model compounds to illustrate the CCC protocol of $P_{o/w}$ measurement. The focus of this work is put on ionisable molecules whose apparent $P_{o/w}$ is completely changed by ionization. β-Blockers, diuretics and sulfonamides are compound classes that were studied. Some of the experimentally determined $P_{o/w}$ coefficients of the molecular forms disagreed with calculated and experimental values available in the literature. The $P_{o/w}$ coefficients of the ionic forms and the acidity constants were also calculated using a theoretical model. Relationships between biological properties and hydrophobicity are also discussed.

1. Introduction

Countercurrent chromatography (CCC) uses a support-free liquid stationary phase [1–4]. To form a biphasic liquid system, the mobile and stationary phases must be immiscible. In CCC there is no solid support; centrifugal fields are used to keep the liquid stationary phase steady while the liquid mobile phase is pushed through it. Strictly speaking, there is not a countercurrent liquid circulation in CCC. The countercurrent chromatography naming and the CCC acronym were coined by Y. Ito, the founder of the technique, to refer the countercurrent distribution method, which also used two liquid phases.

In CCC, the stationary phase occupies up to 95% of the total volume of the column: this ratio, called retention of the stationary phase, plays a key role on the number of theoretical plates and resolution. Due to the liquid nature of the stationary phase, CCC is a liquid chromatography (LC) technique that uses special columns. Indeed, the CCC machines are just "columns". The liquid stationary phase is stable only as long as the centrifugal field exists, i.e., the CCC column exists as long as the machine rotor is running with the corollary that a fresh column is used for every experiment. Memory effect is practically impossible.

Liquid–liquid partition is the only phenomenon responsible for solute retention in CCC. In the chromatographic process, the solutes undergo a number of back-and-forth mobile and stationary phase exchanges. The solutes separate on the basis of their differing affinity for the stationary phase and enter and exit the column in the mobile phase. The solute retention volume, V_R, is given by:

$$V_R = V_M + K_D V_S \quad (1)$$

where V_M and V_S are the mobile and stationary phase volumes, respectively, inside the CCC apparatus. V_M corresponds to the hold-up or "dead" volume in LC; and K_D is the solute distribution ratio or liquid–liquid partition coefficient. K_D is expressed as the ratio of the solute concentration (in all chemical forms) in the stationary phase over the solute concentration (all forms) in the mobile phase. It does not depend on the CCC machine used or the technique itself. Thus, the retention volume of a solute allows the determination of its distribution ratio in the biphasic system used in the CCC apparatus.

Accurate liquid–liquid distribution ratios (also called partition coefficients), especially $P_{o/w}$ values, are critical in quantitative structure–activity relationship (QSAR) studies. Although some-

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times questioned and challenged [5,6], $P_{o/w}$ is still accepted as the pertinent parameter measuring molecule hydrophobicity. The higher inconsistency in the literature $P_{o/w}$ values of pharmaceutical molecules is found with ionisable molecules. This is not surprising since the hydrophobic character of ionisable molecule (hence their $P_{o/w}$) is extremely depending on the aqueous-phase pH. The classical shake-flask method for $P_{o/w}$ measurements needs extremely pure solutes to be reliable [7]. A trace amount of any impurity will not distribute in the two liquid phases the same way as the compound of interest. False results may be obtained. Also the shake-flask method is a tedious procedure including exact weighting, dissolution of the compound in one phase, introduction of the other phase, ~30 min or more shaking time, centrifugation for several hours to separate the two liquid phases, and spectrophotometric analysis of both phases [7,8]. Chromatography is also used to estimate P coefficients [7]. Linear regressions are used to correlate both log retention factors (log k) and log $P_{o/w}$ parameters [9]. Often, for a given compound, the log $P_{o/w}$ value obtained from its log k value can be off by more than 0.3 units.

CCC is able to work with an octanol stationary phase and an aqueous mobile phase. In this configuration, CCC really measures the octanol–water distribution ratio without any assumption, regression or calibration. CCC produces reliable $P_{o/w}$ values with impure samples: the CCC chromatogram will show several peaks.

Ketones were selected as a classical and simple example of non-ionisable solutes. β-Blockers, diuretics, and sulfonamides were ionisable solutes whose CCC results were extracted from our recent works [10–12]. A model previously described for ionisable compounds [10] allows obtaining the molecular and ionic $P_{o/w}$ values using the solute pK_a with the CCC measured octanol/water distribution ratios. Frequently, the $P_{o/w}$ coefficients of the molecular forms obtained with the CCC method differ significantly from computed literature values and/or experimental values obtained by extrapolation. The aim of this work is to compare and to comment experimentally CCC obtained $P_{o/w}$ values of these different classes of compounds with their bibliographic or calculated values.

2. Experimental

2.1. CCC apparatuses

Two different CCC apparatuses were used. For β-blockers, diuretics, and sulfonamides, a hydrostatic machine (CPC, Sanki Engineering, Kyoto, Japan) with 1060 channels and ducts, and a total internal volume of 101 mL was used. For ketones, a hydrodynamic J-type high-speed CCC centrifuge with a four combined coil arranged in two bobbins (Spectrum CCC, Dynamic Extraction Ltd., Slough, UK) was also used. This modern CCC equipment has two analytical ~18 mL coils made of small bore 0.8 mm diameter Teflon® tubing and two larger volume coils made of 1.6 mm diameter Teflon® tubing and ~70 mL volume for semi-preparative purification. The Spectrum has two rotating bobbins each one bearing an analytical coil and a semi-preparative coil all rotating up to 2000 rpm in a thermostated and sound insulated box. Each one of the four coils can be used independently or they can be interconnected at will. A single semi-preparative coil of the Spectrum was used for our experiments. It was connected to our chromatographic equipment and the volume between the injection valve and the detector cell was accurately determined to be 75.1 mL.

2.2. CCC column preparation

As already mentioned, a fresh CCC column is prepared for every experiment. The CCC apparatus was first filled with the octanol–saturated water phase (the aqueous phase being most often buffered to pH 7.3 with 0.05 M ammonium or potassium phosphate). Then the rotor is started and the rotation allowed stabilization at 900–1000 rpm for the hydrostatic Sanki CCC column or at 2000 rpm for the hydrodynamic Spectrum CCC column. The pump was rinsed with plenty of buffered aqueous mobile phase. This phase entered the CCC column in the head-to-tail direction (descending mode) because it is heavier than the octanol stationary phase. Channel after channel (hydrostatic column) or coil after coil (hydrodynamic column), the octanol and aqueous phases equilibrate. As long as the CCC column is not fully equilibrated, the octanol phase is seen exiting the apparatus when the aqueous phase is entering. All this octanol phase displaced by the aqueous phase is collected in a graduated cylinder. Once the aqueous phase appears at the exit of the apparatus, it gathers under the octanol phase and two liquid layers are seen in the cylinder. The mobile phase–stationary phase equilibrium is fully reached in the whole hydrostatic or hydrodynamic CCC column. The displaced and collected octanol phase volume is measured. It corresponds to the aqueous phase volume, V_M, in the CCC machine. Since small amounts of octanol may further be carried out or dissolved by the aqueous mobile phase, potassium nitrate, a dead volume marker, was added to the samples as a hold-up marker. This salt is not retained by the octanol phase and absorbs well at 210 nm.

2.3. Protocol

With octanol as the stationary phase and water as the mobile phase, the injected solute move in the CCC column eluting at different retention volumes, V_R. Their respective octanol/water partition coefficient is calculated using:

$$P_{o/w} = \frac{V_R - V_M}{V_C - V_M} \quad (2)$$

where V_C is the CCC column volume and V_R is the measured solute retention volume. This is the direct measurement of $P_{o/w}$ [1].

Solutes with very high $P_{o/w}$ values move very slowly in the octanol phase. They need a too long time to emerge outside the machine. To force them from the CCC apparatus, the dual-mode method is used. The roles of the aqueous and octanol phases and their flowing direction are simultaneously reversed after some reasonable flowing time with the aqueous phase. The mode is switched from descending (head to tail) to ascending (tail to head) when changing the flowing liquid nature. The high $P_{o/w}$ solutes are thus eluted out of the column head (where they were injected) by a small volume of octanol that was the stationary phase in the first step. The theory shows that $P_{o/w}$ of the solute depends only on the ratio of the volume of the aqueous phase, V_{aq}, pumped in the descending mode and the retention volume of the octanol phase (V_{oct}) in the ascending mode [1,13,14]:

$$P_{o/w} = \frac{V_{aq}}{V_{oct}} \quad (3)$$

The dual-mode or back-flushing measurement was also used to measure very small $P_{o/w}$ values. In this case, the octanol phase is the mobile phase in the tail-to-head or ascending mode. The compounds are strongly retained, since they move very slowly in the aqueous stationary phase. After several hours, the phase role and circulation direction are inverted. The water becomes the mobile phase in the head-to-tail or descending mode. This forces the analytes out of the apparatus. In the dual-mode method, the octanol- and the water-phase volumes must not change during the switching, but actually, this is difficult to achieve.

3. Results and discussion

CCC is well adapted for hydrophobicity measurement of non-ionisable solutes. The problem for ionisable solutes lies in the confusion that may exist between the definition of distribution ratio and distribution constant. This problem was recently tentatively addressed at the CCC2010 international conference in Lyon [15]. In short, the distribution constant corresponds to the partitioning of a single species which is the stable molecular form of the compounds, and, by definition it does not change, it is a constant. Compounds may not exist in a molecular form, e.g. amino-acids. Some compounds change in solution. Ionisable compounds have the property to ionize partially in the aqueous phase depending on the aqueous phase pH. The ionized forms travel with the molecular forms in chemical equilibrium. The distribution ratio corresponds to the ratio of the concentrations all forms of the compound in the stationary phase over the concentrations of all forms in the mobile phase. This ratio can be mathematically expressed as a combination of the distribution constants of every form. It means that it changes if the concentrations of the forms change. As recalled earlier, CCC measures the distribution ratio of the solutes. Distribution ratios and distribution constants coincide only for non-ionisable solute [15].

3.1. Theoretical model for ionisable compounds

Basic or acid ionisable compounds can be described by the following equilibriums:

$$AH \leftrightarrow A^- + H^+ \text{ (acid)} \quad (4)$$

$$AH^+ \leftrightarrow A + H^+ \text{ (basic)} \quad (5)$$

In Eq. (4) the molecular form, AH, ionizes in an anionic form, A^-, as the pH increases, while in Eq. (5) the molecular form A, ionizes in a cationic form, AH^+, as the pH decreases.

For acidic compounds, e.g., carboxylic acids, P^0 can be introduced. P^0 is the $P_{o/w}$ constant value for the AH molecular form, and P^-, the $P_{o/w}$ constant value of the A^- anionic form. The distribution ratio of AH is what CCC measures. It will be called the experimental apparent partition coefficient, P_{app}, and it is expressed by the ratio of AH in all forms in the octanol phase over the ratio of AH, also in all forms in the aqueous phase:

$$P_{app} = \frac{[AH]_o + [A^-]_o}{[AH]_w + [A^-]_w} \quad (6)$$

The subscripts o and w refer to the octanol phase and to the aqueous phase, respectively. Using the expression of K_a, P_{app} can be formulated as:

$$P_{app} = \frac{P^0 + P^-(K_a/[H^+])}{1 + (K_a/[H^+])} \quad (7)$$

Eq. (7) shows that the CCC measured coefficient is not a constant; it decreases with the aqueous phase pH.

For basic compounds, e.g., amines, it can similarly be introduced P^0, the $P_{o/w}$ value for the A molecular form, and P^+, the $P_{o/w}$ value of the AH^+ cationic form, and taking into account the acidity constant, K_a, of Eq. (5), the CCC measured coefficient, P_{app}, is given by:

$$P_{app} = \frac{[A]_o + [AH^+]_o}{[A]_w + [AH^+]_w} \quad (8)$$

Using the expression of K_a, P_{app} can be formulated as:

$$P_{app} = \frac{P^0 + P^+([H^+]/K_a)}{1 + ([H^+]/K_a)} \quad (9)$$

Eq. (9) shows that the measured coefficient increases with the pH.

Diuretics will be typical example of acid ionisable compounds whose behavior is modeled by Eqs. (4), (6) and (7). β-Blockers are basic ionisable compounds and are described by Eqs. (5), (8) and (9). Sulfonamides (SAs) are typical amphoteric compounds and undergo dissociation according by the double equilibrium:

$$AH_2^+ \leftrightarrow AH + H^+ \leftrightarrow A^- + 2H^+ \quad (10)$$

The molecular form AH, ionizes in a cationic form, AH_2^+, as the pH decreases, and ionizes in anionic form A^- as the pH increases.

K_{a1} and K_{a2} are the dissociation constants of the amine and sulfonic groups of SA compounds, respectively. P^0, P^+ and P^- are the constant $P_{o/w}$ values for the AH molecular, AH_2^+ cationic, and A^- anionic forms, respectively. The distribution ratio or CCC experimental partition coefficient, P_{app}, is given by:

$$P_{app} = \frac{[AH]_o + [AH_2^+]_o + [A^-]_o}{[AH]_w + [AH_2^+]_w + [A^-]_w} \quad (11)$$

Using the expression of all constants including K_a, P_{app} can be formulated as [10]:

$$P_{app} = \frac{P^0 + P^+([H^+]/K_{a1}) + P^-(K_{a2}/[H^+])}{1 + ([H^+]/K_{a1}) + (K_{a2}/[H^+])} \quad (12)$$

Eq. (12) shows that the CCC measured coefficient increases with the pH up to the first pK_a value and begin to decrease before the second pK_a value.

3.2. Experimental results

3.2.1. Detailed results for ketones as an example of classical solutes

Ketones were selected as simple molecule easy to detect to show the classical way to routinely measure solute $P_{o/w}$ ratios. Fig. 1 shows the chromatograms obtained using either the hydrodynamic CCC column and aqueous mobile phase (Fig. 1, top) or the hydrostatic CCC column and octanol mobile phase (Fig. 1, bottom and Table 1). The reversed phase experiment was done injecting 0.8 mL of a mixture made with 1 part of acetone, 2 parts of methyl ethyl ketone (MEK) and 5 parts of methyl isobutyl ketone (MIBK). The normal phase experiment was done with the hydrostatic column injecting 2 mL of the same mixture. Table 1 lists the full results of the Fig. 1 experiment calculated using the solute retention volumes and Eq. (2). The measured $P_{o/w}$ values for these simple molecular solutes do not depend neither on the CCC column used nor on the chromatographic mode. The results are well in agreement (within 0.1 log unit) with the literature values [7]. The MIBK peak in reversed phase mode (aqueous mobile phase) shows some distortion (fronting) due to the large amount (400 mg) injected (Fig. 1, top). This experiment was duplicated injecting a much lower amount of MIBK (20 mg). A lower retention time (84 min) with a better Gaussian peak shape was observed giving a retention volume of 672 mL and a $P_{o/w}$ value of 20.8 ($\log P_{o/w} = 1.32$) corresponding exactly to the literature value [7]. The experiment is not shown due to the ugly chromatogram obtained (lower signal to noise ratio). The normal phase ketone elution with the octanol mobile phase shows obviously an inversed elution order (Fig. 1, bottom) compared to the reversed phase mode with aqueous mobile phase.

It was observed that the chromatographic efficiency, measured as the plate number linked to the peak width (Fig. 1), decreased as the compound retention volume increased. In reversed phase mode, the plate number for the first peak of acetone is 240. The plate count drops to 65 for the last MIBK peak (Table 1). It means that the kinetics of the solute exchange between the aqueous phase and the octanol phase decreases as the solute affinity for the octanol phase increases [8]. In normal phase mode, a similar trend is observed

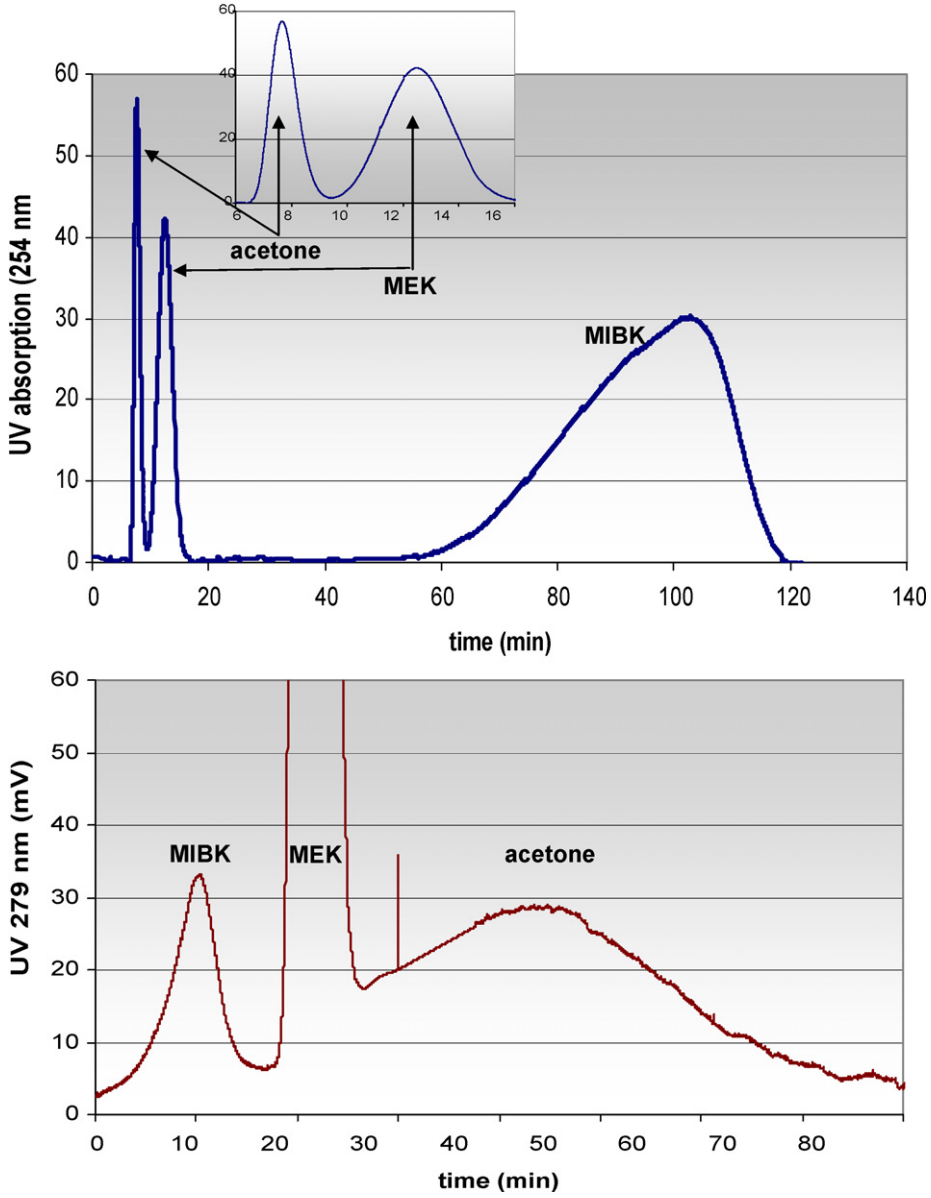

Fig. 1. CCC chromatogram of a mixture of three ketones: acetone (80 mg injected), methyl ethyl ketone (MEK, 160 mg injected) and methyl isobutyl ketone (MIBK, 400 mg). Top: Spectrum® hydrodynamic CCC column and reversed phase mode, 75.1 mL, 2000 rpm. Aqueous mobile phase in the descending (head-to-tail) direction, 8 mL/min. Octanol stationary phase. The inset is an enlargement of time 6–16 min showing the Gaussian shape of the two first peaks. Bottom: Sanki® hydrostatic CCC column and normal phase mode, 103 mL, 700 rpm. Octanol mobile phase in the ascending (tail-to-head) direction, 3 mL/min. Aqueous stationary phase. See Table 1 for detailed experimental data.

Table 1
Experimental results obtained in ketone $P_{o/w}$ determination by CCC.

Solute	t_R (min)	N (plates)	V_R (mL)	$P_{o/w}$	Log $P_{o/w}$	Log $P_{o/w}$ (lit.)
Hydrodynamic CCC column, reversed phase mode						
Acetone	7.7	240	61.8	0.56	−0.253	−0.24
Butanone or methyl ethyl ketone	12.6	110	101	1.85	0.268	0.29
4-Methyl 2-pentanone or methyl isobutyl ketone	100	65	800	25.1	1.40	1.31
Hydrostatic CCC column, normal phase mode						$1/P_{o/w}$
4-Methyl 2-pentanone or methyl isobutyl ketone	10.6	22	31.8	24.7	1.39	0.04
Butanone or methyl ethyl ketone	20.8	350	62.4	2.20	0.342	0.45
Acetone	46.6	11	140	0.66	−0.18	1.49

Data corresponding to the Fig. 2 experiment. Hydrodynamic CCC column volume = 75.1 mL; aqueous mobile phase volume, V_M = 45 mL; octanol stationary phase volume, V_S = 30.1 mL; Sf = 40%; aqueous flow rate = 8 mL/min; rotor rotation speed = 2000 rpm. Peak efficiency measured as the ratio of the peak retention time over its width at 60% height: $w_{0.6h}$ and $N = 4(t_R/w_{0.6h})^2$. Log $P_{o/w}$ literature values taken from Ref. [7]. Hydrostatic CCC column volume = 103 mL; octanol mobile phase volume, V_M = 29 mL; aqueous stationary phase volume, V_S = 74 mL; Sf = 72%; octanol flow rate = 3 mL/min; rotor rotation speed = 700 rpm. The rightmost column lists the normal phase P coefficient obtained that is actually $1/P_{o/w}$.

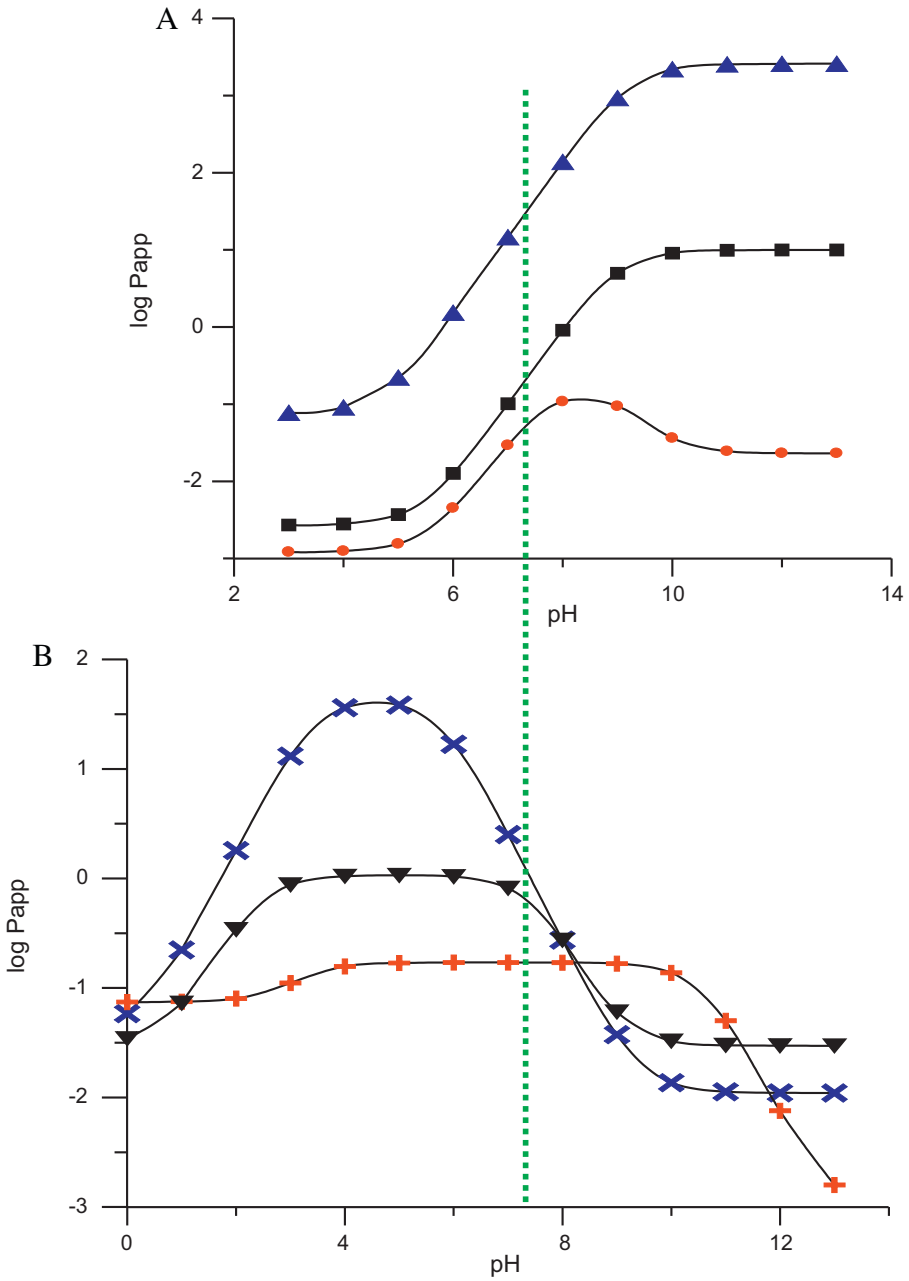

Fig. 2. Relative hydrophobicity, log P_{app}, versus pH for (A) β-blockers: nadolol (■), propranolol (▲), and sotalol (●); and (B) sulfonamides: sulfadimethoxine (×), sulfapyridine (▼) and sulfanilamide (+). The vertical dotted line corresponds to the physiological blood pH.

for the first eluting MIBK (22 plates) and last eluting acetone (11 plates). In between, MEK shows an apparent high efficiency (250 plates) likely due to its very strong UV (279 nm) absorption in the octanol phase. If the Beer law is not followed, peak distortions can be observed. We did not investigate this point further being only interested by peak positions. It is classically observed that hydrostatic CCC columns have lower efficiencies than hydrodynamic CCC columns of similar volumes [1–4].

This example is given to show the ease of the $P_{o/w}$ direct measurement by CCC not involving any assumption or regression analysis and not depending on the column or chromatographic mode used. It also shows its limitations. 120 min were needed to measure the $P_{o/w}$ of MIBK, a solute with intermediate hydrophobicity. The $P_{o/w}$ value of 2,6 dimethyl-3-heptanone 4 or di-isobutyl ketone is close to 1000 [7]. In our experimental reversed phase conditions, this solute would need an aqueous phase volume of 30.2 L (Eq. (1)) meaning a retention time of 63 h or 2 days and 16 h. This corresponds to an experiment duration of three days considering that the peak will likely shows an efficiency of few tens plates. Detection will be a problem, the injected ketone being diluted in a very large volume of aqueous phase. In our normal phase conditions, this solute would elute only 74 μL after the hold-up octanol volume (or 0.0025 min or 0.15 s after hold-up time); these small volumes or times would not be accurately determined leading to an unacceptable uncertainty [1]. This problem was addressed and different solutions were proposed to extend the hydrophobicity window of CCC [16,17].

3.2.2. *Results for ionisable solutes*

Table 2 shows the measured CCC distribution ratios, called P_{app} apparent partition coefficients of some selected β-blockers, diuretics and sulfonamides together with their dissociation constants.

Table 2
Fitted parameters of β-blockers, diuretics and sulfonamides using the experimental octanol-buffer partition coefficients for three pH values.

	Fitted parameters used in Eqs. (7), (9) and (12)				
	log P^+ ion	log P^0 molecule	log P^- ion	pK_{a1}	pK_{a2}
β-Blockers					
Acebutolol	−2.09	1.83		9.24	
Alprenolol	−1.41	3.15		9.34	
Atenolol	−2.77	0.25		8.07	
Betaxolol	−1.50	2.91		9.58	
Bisoprolol	−2.29	2.20		9.2	
Carteolol	−2.82	1.49		9.24	
Celiprolol	−2.34	1.98		9.74	
Labetalol	−1.22 (AH^+)	1.06 ($A^{+/-}$)	0.250 (A^-)	6.2	8.8
Metipranolol	−2.15	2.60		9.13	
Metoprolol	−2.08	1.90		9.31	
Nadolol	−2.57	0.998		9.00	
Oxprenolol	−1.82	2.30		9.08	
Penbutolol	−0.29	3.61		8.88	
Pindolol	−3.29	1.91		6.98	
Propranolol	−1.12	3.41		9.25	
Sotalol	−2.92 (AH^+)	−0.77 ($A^{+/-}$)	−1.64 (A^-)	7.7	
(A^-)Timolol	−1.85	1.98		9.19	
Diuretics					
Acetazolamide		−0.30	−0.96	6.7	
Amiloride		−1.21		6.5	
Bendroflumethiazide	0.057	1.95		6.5	
Benzthiazide		1.73		8.2	
Bumetanide		2.09	−0.70	4.5	
Canrenoic acid		2.40	−0.35	5.2	
Chloraminophenamine		−0.41		7.8	
Chlorothiazide		−0.35		6.75	
Cyclothiazide		1.91		8.1	
Ethacrinic acid		2.20	−0.44	3.9	
Furosemide		1.81	−1.40	3.9	
Hydrochlorothiazide		−0.11	−0.18	6.0	
Hydroflumethiazide		0.32	0.23	6.0	
Piretanide		2.20	−1.70	4.1	
Probenecid		1.40		5.4	
Triamterene		1.22		6.3	
Thrichloromethiazide		1.00	−0.15	6.5	
Xipamide		2.00	−2.00	5.0	
Sulfonamides					
Sulfabenzamide	−1.9	1.32	−2.64	2.16	4.36
Sulfacetamide	−1.1	−0.19	−2.65	1.82	5.85
Sulfachloropyridazine	−1.2	0.71	−2.19	1.72	6.39
Sulfadiazine	−1.6	−0.06	−2.13	1.98	6.01
Sulfadimethoxine	−1.4	1.66	−1.96	3.40	5.76
Sulfaguanidine	−1.7	−1.07	−1.85	2.46	11.15
Sulfamerazine	−1.2	0.11	−2.19	2.16	6.80
Sulfamethazine	−1.3	0.27	−2.09	2.46	7.45
Sulfamethizole	−1.2	0.47	−2.61	2.24	5.30
Sulfamethoxazole	−1.6	0.85	−2.14	1.81	5.46
Sulfamethoxypyridazine	−1.4	0.23	−2.02	1.96	7.16
Sulfanilamide	−1.1	−0.77	−2.89	3.22	10.61
Sulfaphenazole	−1.40	1.36	−1.97	2.57	5.93
Sulfapyridine	−1.5	0.03	−1.53	2.37	7.48
Sulfaquinoxline	−1.3	1.45	−2.15	2.62	6.00
Sulfathiazole	−1.5	−0.04	−2.17	2.06	7.07
Sulfisoxazole	−1.6	0.81	2.51	2.15	5.00

Results obtained using a hydrostatic CCC column of 101 mL. The listed values were obtained after fitting the results (Eqs. (7), (9) and (12)) obtained at 11 different pH values. For each pH value, the P_{app} were the average values of at least two coherent experiments. See Fig. 3 for more experimental information.

The P_{app} values at different pHs (data not shown) for β-blockers, diuretics and sulfonamides [10–12] were calculated with Eqs. (7), (9) and (12) using the solute experimental retention volumes. The listed values are the average of duplicate coherent measurements. Molecular and ionic values together with the dissociation constants were fitted with Eqs. (2) and (3) depending on the acid–base character of the compound. The cationic log P^+ and anionic log P^- values listed in Table 2 correspond to the phosphate salt of the positive form and sodium or ammonium salt of the negative forms of the compounds, respectively. These values may vary if the anion and the cation of the buffer salt are changed. The results of these works show that it is possible to quantify the hydrophobicity of ions when ion's $P_{o/w}$ values are commonly neglected assuming $P_{o/w} = 0$ for any ion. It is acknowledged that all measured ionic P^+ and P^- values were indeed small (Table 2).

For β-blockers, the log P_{app} values increase with the pH range, except for labetalol and sotalol, which decrease at pH 11 (Table 2). This behavior can be explained because labetalol and sotalol are ionized at pH 11 due to the presence of a phenolic OH-group and a sulfonamide group, respectively. For most of the SAs the log P_{app} values increase up to pH 2 (first dissociation constant), and decrease after pH 7 (second dissociation constant). SAs are usually cationic in acidic media (pH < 2), non-charged in neutral media (3 < pH < 7), and anionic in basic media (pH > 7).

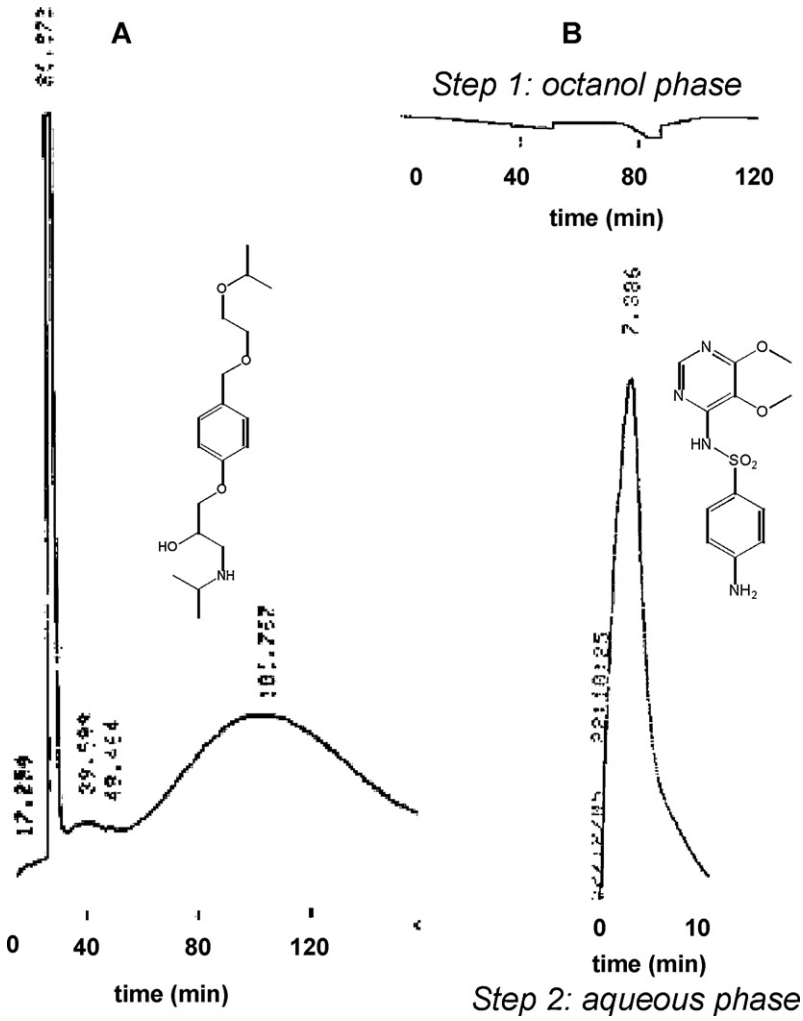

Fig. 3. CCC chromatograms at pH 7.09. (A) Direct mode chromatogram of bisoprolol (0.5 mg + 0.1 mg of potassium nitrate): mobile phase: aqueous with 0.01 M ammonium phosphate buffer at pH 7.09 in the descending head-to-tail mode, V_M = 27 mL; stationary phase: octanol 74 mL; UV detection at 210 nm, V_R = 101.8 mL giving a P_{app} value of 1.01 (Eq. (1)). (B) Dual mode chromatogram of sulfaguanidine at pH 7.09 (0.5 mg): top: Step 1; the octanol mobile phase is flowing in the ascending tail-to-head mode for 2 h, no signal, octanol volume 120 mL; bottom: Step 2: the aqueous phase with 0.01 M ammonium phosphate buffer at pH 7.09 is flown in the descending head-to-tail mode; stationary phase octanol; UV detection at 275 nm. Peak elution at 7.39 mL giving a P_{app} of 7.39/120 = 0.0616 (Eq. (3)) or log P_{app} = −1.21. General conditions: hydrostatic CCC column of 101 mL; rotor speed: 900 rpm; injection volume: 1 mL; flow rate: 1 mL/min.

Fig. 2A and B shows the evolution of calculated apparent P_{app} coefficient versus pH for three β-blockers and three sulfonamides, respectively. 11 different pH were tested to obtain enough results so that the fit can be done for both the hydrophobicity constant ($P_{o/w}$ of molecular and ionized forms) and the acidity constant (pK_a) values. The figure illustrates the dependence of the relative hydrophobicity upon the pH for these compounds, which have a single or two ionisable groups.

At acidic pH, all β-blockers and SAs are in cationic form (AH^+ or AH_2^+, respectively, a nitrogen atom of the aliphatic chain is protonated). This cationic form is very hydrophilic showing very small log P_{app} values (usually <−1). This means that the solutes are more soluble in the aqueous phase than in the apolar octanol phase. Since at increasing pH the ionization degree of the compounds decreases, their affinity for the aqueous phase also decreases and their P_{app} values increase. At basic pH, the β-blockers are in molecular form, except for labetalol and sotalol which are both still ionized. The molecular forms show a hydrophobic behavior with high log P_{app} values (0.25–3.61). This means that the solutes have a higher affinity for the octanol phase. Normally, the SAs in the 3–7 pH range are in the molecular form (AH), showing most of them a hydrophobic behavior and reaching their maximum log P_{app} values in the −1.07 to 1.66 range. In this zone, the solutes have a higher affinity for the octanol phase. In basic media (pH > 8) the sulfonamides are in anionic form (A^-, the sulfonic group has lost a hydrogen), and their hydrophilic character increases accordingly. Their P_{app} value decreases following a parabolic curve (Fig. 2B). Even if this anionic form is very hydrophilic, the CCC method allows the estimation of the very small log P_{app} values.

Fig. 3 shows the pH 7.09 chromatograms of bisoprolol and sulfaguanidine done with direct and dual-mode, respectively. Fig. 3A shows the actual UV signal obtained in the P_{app} measurement of bisoprolol. The sharp peak at 26.87 min retention time corresponds to potassium nitrate. The broad peak at 101.77 min is the β-blocker peak with only 10 plates. This broad peak allows for a quite accurate determination of the bisprolol retention volume of 101.8 mL. This volume and Eq. (2) allow for a computation of P_{app} = 1.01 (log P_{app} = 0.004). Fig. 3B shows the UV signal obtained in the P_{app} measurement of sulfaguanidine. In the later case, the dual-mode was used to measure a very small P_{app} value. Since at pH 7.09 sulfaguanidine eluted with the dead volume in the direct mode with an octanol saturated aqueous mobile phase, the

octanol phase was used first as mobile phase in the tail-to-head or ascending mode (Flat trace Step 1 in Fig. 3B, top), and after 2 h and the large 120 mL volume of octanol phase used to move slightly the sulfaguanidine species inside the hydrostatic CCC column, the phase role was inverted. A small volume of 7.39 mL of aqueous phase in the descending direction was enough to force the analyte out of the apparatus (Trace Step 2 in Fig. 3B). The P_{app} value is simply 7.39/120 = 0.0616 (log P_{app} = −1.21).

In the case of acidic diuretics, the measured octanol–water distribution ratio increases with the aqueous phase pH. For basic diuretics, it is usually the opposite and P_{app} decreases. The diuretic P_{app} values were measured by CCC with three buffered mobile phases. One phase was neutral at pH 7.39. The second was slightly acidic at pH 5.86, and the last one was acidic with a pH of 2.58. As expected, strong P_{app} variations are observed. The larger P_{app} increase was obtained for ethacrynic acid with log P = −0.39 at pH 7.39 which jumped to log P = 2.18 at pH 2.58. The affinity of ethacrynic acid for octanol is greater at pH 2.58 than at pH 7.39 due to the carboxylic acid ionization regression in acidic media. Bumetanide, canrenoic acid, furosemide, piretanide, probenecid, and xipamide all showed a behavior similar to that of ethacrynic acid. Oppositely, bendroflumethiazide and triamterene saw their P_{app} values decreased by more than 3 orders of magnitude in acidic medium. Their molecules bear an amino group that is protonated at pH 2.58. Spironolactone, a nonionizable diuretic, shows little variations within the experimental errors.

3.3. CCC measurements and literature values

The molecular CCC log $P_{o/w}$ values fitted by Eqs. (9) and (12) (Table 2) were plotted versus the experimental literature or computed values for 14 SAs [18] and 12 β-blockers [19,20]. The log P experimental literature data correlated with the CCC measured ones showing a correlation equation:

$$\log P_{o/w\,lit} = 1.13 \log P_{o/w\,CCC} + 0.013, \quad n = 26, \quad R^2 = 0.953 \tag{13}$$

The slope value, close to unity, indicates that the partition coefficients obtained by CCC and the literature log P_{oct} are very similar even for ionisable compounds. Good agreement was also observed between experimental and calculated values of diuretics [10]. Similar conclusions were obtained by Shibusawa et al. when correlating log $P_{o/w}$ values of catechins determined by CCC with those obtained by the traditional shake-flask method [21]. It is interesting to point out that the measured $P_{o/w\,CCC}$ values were almost systematically lower than their corresponding $P_{o/w\,lit}$ values. The very good correspondence between log P values hide the differences between P values. For example the β-blockers have respectively the similar log $P_{o/w\,CCC}$ and log $P_{o/w\,lit}$ of 0.25 and 0.16 for atenolol, 1.0 and 1.11 for nadolol, and 1.91 and 2.15 for pindolol (Table 2 and [10,11]). These values converted in $P_{o/w}$ values become the similar values 1.8 and 1.5 for atenolol, 10 and 13 for nadolol, but the different values 82 and 140 for pindolol. Ammonium and sodium phosphates were used as buffer salts. A small $P_{o/w}$ change may be induced by the added salts, especially for hydrophobic molecules (salting out effect).

It should also be highlighted that an ion should partition with its gegenion. Therefore, the log P^- and log P^+ values listed in Table 2 correspond to the ammonium and sodium salts of the negative diuretic ions and phosphate salts of the positive diuretics. Potassium or chloride salts of the same ions might have different log P^- and log P^+ values, respectively.

CCC produces reliable $P_{o/w}$ values but also dissociation constant, K_a, values. Most CCC K_a values for β-blockers and SAs listed in Table 2 were obtained during the CCC fitting procedure. Each time that a literature value could be found, a good agreement was observed.

3.4. Correlation with HPLC descriptors

Log $P_{o/w}$ has been successfully applied as a structural descriptor in quantitative structure–activity relationship (QSAR) for structurally related compounds, and in some cases, even for sets of chemically different compounds. Liquid chromatography is a powerful technique for the measurement of physicochemical parameters, and in order to emulate the biological barriers different reversed stationary phases have been developed such as the immobilized artificial membranes, or immobilized liposomes. Relationships between octanol–water partition data and chromatographic indexes at a fixed or varying pH values by RPLC or micellar liquid chromatography (MLC) have been studied for β-blockers [22–24].

Since log $P_{o/w}$ is considered to estimate the partitioning over a bio-membrane, it should also be related to biological activity. Good relationships were obtained when the distribution coefficients of the compounds in octanol-buffer (shake-flask method) and the relative lipophilicity measured by HPLC were correlated.

β-Blockers have also been used to study the influence of lipophilicity of drugs on the permeation through biological membranes since various structurally related β-blockers exhibit a wide range of lipophilicity. The HPLC technique can produce chromatographic lipophilicity indexes (expressed as log D_{HPLC}) on octadecyl (C18) bonded silica columns. The solute is injected on the same C18 column and different methanol/water mobile phases are used to elute it. The retention factor, k, of the solute is obtained directly. Log k can be correlated with the methanol percentage in the mobile phase. Extrapolating to pure water (0% methanol), the log k_w also noted log D_{HPLC} is obtained even for highly lipophilic compounds out of the range of the shake-flask method [25]. The published log D_{HPLC} indexes at pH 11 for nine β-blockers (acebutolol, alprenolol, atenolol, labetalol, metoprolol, pindolol, propranolol, sotalol and timolol) [25] were correlated with our CCC log P_{app} value at pH 11 giving a correlation equation:

$$\text{Log}\, D_{HPLC} = 0.51 \log P_{app} + 1.43, \quad n = 9, \quad R^2 = 0.981 \tag{14}$$

In this correlation, the 0.51 slope shows that the mechanisms are different. A two order of magnitude increase in CCC log P_{app} corresponds to only one order of magnitude in C18 HPLC retention factor increase. The bonded silica/methanol–water interaction is obviously different from the liquid–liquid CCC partitioning, both being related to the solute hydrophobicity.

4. Conclusion

The CCC technique is able to determine accurately distribution ratios. When octanol is used as the stationary phase, octanol/water distribution ratios are directly obtained without any assumption or correlation. The solute positions in the chromatogram are only a result of their partitioning with the octanol phase. Distribution ratio and distribution constants or partition coefficients are the same thing for molecular solutes. It is different for ionisable solutes whose peak position in the CCC chromatograms is observed changing according to the aqueous phase pH. The change is due to the change in ionization state and ratio of the molecular and ionic species in equilibrium. Accurate octanol–water distribution ratios of ionisable compounds, such as β-blockers, diuretics and sulfonamides were determined by CCC. The dependence of the hydrophobicity upon the pH is different for acid (decreasing with pH), basic (increasing with pH), or amphoteric compounds (a hydrophobicity maximum is observed on the pH

range). A theoretical model for analytes showing acid–base properties was successfully used to compute the molecular and ionic octanol/water distribution constants as well as the acid dissociation constants. The model represents correctly the change in $P_{o/w}$ when pH changes. Good correlations can be also obtained when log $P_{o/w}$ values are correlated with hydrophobicity indexes obtained with liquid chromatography.

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