Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 995 (2003) 55–66
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

pH dependence of the hydrophobicity of β-blocker amine compounds measured by counter-current chromatography

S. Carda-Broch[1], A. Berthod*

Laboratoire des Sciences Analytiques, CNRS, Université de Lyon 1, Bat CPE-308, 69622 Villeurbanne, France

Received 20 January 2003; received in revised form 10 March 2003; accepted 21 March 2003

Abstract

The octanol–water partition coefficients (P_{oct}) of 17 antiadrenergic β-blocker compounds were determined by counter-current chromatography (CCC). Since CCC uses a biphasic liquid system, the octanol–water liquid system was used with essentially an octanol stationary phase and aqueous buffer mobile phase. The P_{oct} coefficients were obtained directly without any extrapolation. The measured P_{oct} values were in the 0.0015–4070 range ($-2.8 < \log P_{oct} < 3.6$). Since the β-blocking agents are ionizable compounds, the P_{oct} values obtained were strongly dependent on the aqueous-phase pH. The apparent P_{oct} coefficients of the β-blockers were determined at three different pH values (~3, 7 and 11) using 0.01 *M* ammonium phosphate buffers saturated with octanol. A model allowed us to obtain the molecular and ionic P_{oct} value using the solute pK_a with these three experimental octanol–water coefficients. Often, the P_{oct} coefficients of the molecular forms obtained with the CCC method differ significantly from computed literature values and/or experimental values obtained by extrapolation. Relationships between biological properties and hydrophobicity were also examined.

Keywords: Counter-current chromatography; Partition coefficients; Hydrophobicity; Octanol–water partition coefficients; β-Blockers; Ionizable compounds

1. Introduction

Counter-current chromatography (CCC) is a liquid chromatography technique in which the stationary phase is also a liquid. Solute separation is based on partitioning between the two immiscible liquid phases: the mobile phase and the support-free liquid stationary phase [1]. Centrifugal fields are needed to hold the liquid stationary phase when the mobile phase is pushed through it. In CCC, the stationary phase occupies up to 95% of the total volume of the column: this ratio, *Sf*, the retention of the stationary phase ratio, plays a key role in the chromatographic resolution factor.

The advantages of having a liquid stationary phase in chromatography are: (i) a high loading capability—the solutes can access the whole volume of the liquid stationary phase; (ii) a very simple solute retention mechanism; (iii) either phase of the biphasic system can be used as the mobile phase; (iv) no solute can irreversibly adsorb inside the CCC

*Corresponding author. Tel.: +33-4-7243-1434; fax: +33-4-7243-1087.
E-mail address: berthod@univ-lyon1.fr (A. Berthod).
[1]On leave from Area de Quimica Analitica, Universitat Jaume I, 12006 Castello, Spain.

0021-9673/03/$ – see front matter
doi:10.1016/S0021-9673(03)00534-X

''column''; (v) since the CCC machines are made of Teflon tubes, there is no pH problem; and (vi) since aqueous two-phase systems (ATPS) can be used, there is less of a problem from biological solute (protein) denaturation.

The chromatographic selectivity in CCC is only due to solute partitioning between the two immiscible liquid phases. The solute retention mechanism depends on only one physicochemical parameter, the liquid–liquid distribution constant, also called the partition coefficient (P) of the solute. The basic CCC retention equation is similar to that for other partition chromatography techniques:

$$V_R = V_M + DV_S \quad (1)$$

where V_R is the retention volume, V_M the mobile phase volume, V_S the stationary phase volume and D is the stationary phase/mobile phase solute distribution ratio. In CCC, V_M and V_S are not independent. Their sum is the ''column'' volume, V_C. It can be seen that the retention volume, V_R, of a solute allows the determination of its distribution ratio, D, in the biphasic system used in the CCC apparatus.

Two types of CCC machines are available. These are the hydrostatic and hydrodynamic machines. This nomenclature is related to the way the liquid stationary phase is retained inside the CCC ''column'' [1–4]. Hydrostatic machines consist of geometrical interconnected figures placed in a rotor creating a constant centrifugal field [5]. Hydrodynamic machines are made of an open tube coiled on spools rotating in a planetary motion around a central axis [1–4]. The main difference between the two modes is that, in hydrodynamic devices, both phases are in contact throughout the length of the coiled tubes, and in hydrostatic devices, there are zones, the ducts connecting two adjacent geometrical figures or channels, in which only the mobile phase is present. This is why the efficiency of a hydrostatic machine is lower than that of a hydrodynamic unit of comparable volume. Also, it explains why there is little pressure buildup in hydrodynamic CCC machines, and why the maximum stationary phase retention volume can be higher in hydrodynamic machines (up to 96%) than in hydrostatic apparatus (maximum V_S is V_C − duct volume [5]).

CCC is able to work with an octanol stationary phase and an aqueous mobile phase. In this configuration, CCC is a useful and easier alternative to measure the octanol–water distribution ratios, called partition coefficients (P_{oct}), of molecules compared to the classical and tedious shake-flask method. It is also superior to the classical reversed-phase liquid chromatography method, which uses regression equations and approximation. There are no such problems in CCC. The P values obtained with an octanol stationary phase and an aqueous mobile phase are the P_{oct} parameters without any calibration, regression equation and/or approximation. In quantitative structure–retention relationship (QSRR) studies it is necessary to have accurate P_{oct} values to correlate the logarithms of the retention factors ($\log k$) with $\log P_{oct}$ of the substances of pharmacological interest. P_{oct} (or its log) is used to measure the hydrophobicity of molecules and since it is considered to estimate the partitioning over a biomembrane, it is also related to the molecule biological activity [6].

There is an inconsistency in the literature P_{oct} values of pharmaceutical molecules with ionizable character since their P_{oct} is extremely sensitive to the aqueous-phase pH. In a previous work [7] we derived and discussed the equations linking the measured distribution ratio, or partition coefficients (using a hydrodynamic CCC machine), to the true distribution constant, P_{oct} values of the ionic and molecular forms of diuretics with various acid–base properties.

Many drugs of interest in the pharmaceutical industry contain basic nitrogen atoms. β-Blockers are isoprenaline derivatives containing an alkanolamine side-chain terminating with a secondary amino group. They are a class of therapeutic drugs whose optical enantiomers show significant differences in their pharmacological effects and activities and even in their toxic effects [8–11]. These drugs are clinically important and are used in the treatment of neurological, neuropsychiatric and cardiovascular disorders, such as anxiety, coronary insufficiency, glaucoma, cardiac arrhythmia, migraine and hypertension [12–14]. These drugs are also abused in sports due to their blood pressure regulatory and tremor decreasing effects and have been added to the list of forbidden drugs by the International Olympic Committee (IOC) [15,16].

The P_{oct} values of β-blockers found in the literature often show inconsistency. It is suspected that ionization problems may be the reason for the differences. The aim of this work was to determine accurately the P_{oct} of 17 β-blockers at several pH values and their ionization constants using a hydrostatic CCC apparatus. The model and equation derived in our recent work [7] was used with these basic compounds to obtain their true molecular and ionic P_{oct} values.

2. Experimental

2.1. Chemicals

Potassium nitrate, diammonium hydrogenphosphate (Prolabo, Paris, France), methanol (Merck, Darmstadt, Germany), sodium hydroxide, and orthophosphoric acid 85% (Laurylab, Chavanoz, France), were used as received. 1-Octanol and all β-blockers were purchased from Sigma (St. Quentin Fallavier, France and St. Louis, MO, USA).

Water was freshly deionized and distilled before use. Aqueous phases were buffered with 0.01 *M* $(NH_4)_2HPO_4$. The pH was adjusted to the desired value with 85% orthophosphoric acid or NaOH. Octanol was then added to the buffer solution. The mixture was shaken and left standing for at least 1 day. Pure water was added beyond saturation to the octanol phase. The octanol-saturated aqueous phase (4.1% w/w or 34 g/L or 1.9 *M* water in octanol at 20 °C) and the water-saturated octanol phase (0.054% w/w or 0.54 g/L or 4 m*M* octanol in water at 20 °C) were then ready to use in the CCC apparatus.

The structures of the β-blockers are shown in Table 1 with the literature values of their dissociation constants and the octanol–water partition coefficients [17–19]. Stock standard solutions of the β-blockers (5 g/L) were prepared. The β-blockers were dissolved in water at different pH values (depending on the mobile phase pH) or in octanol or methanol depending on their solubility. An ultrasonic bath (Branson, Model 5210, Dansbury, CT, USA) was used to dissolve the most hydrophobic β-blockers.

2.2. Counter-current apparatus

The CCC apparatus was a HPCPC hydrostatic machine built by Sanki Engineering Limited (2-6-10, Imazato, Nagaokakyo, Kyoto, Japan), and purchased from J.M. Science (Grand Island, NY, USA). This hydrostatic machine has a horizontal rotor (radius 7.8 cm) containing 1060 channels and ducts. Two rotary seals are used for the inlet and the outlet of the mobile phase. The total internal volume was 101 mL.

A Shimadzu pump (Model LC6A, Kyoto, Japan) was used to fill the CCC apparatus with the stationary phase and to push the mobile phase. Solute monitoring was performed with a Shimadzu UV detector (Model SPD-6A), usually at 200–225 nm. The signal was recorded by a Shimadzu integrator Model CR5-A.

The flow-rate was usually 1.0 mL/min. The β-blocker solutions were injected using an in-line Rheodyne 7125 valve (Cotati, CA, USA) with a 1.0 mL sample loop.

2.3. CCC procedure

The apparatus was first filled with the octanol water-saturated phase. This takes 101 min at a 1 mL/min flow-rate. A faster flow-rate would have reduced this time but the octanol viscosity precluded it. The rotor was then started and the rotation allowed to stabilize at 900 rpm. The pump was rinsed with the aqueous mobile phase. This phase entered the apparatus in the head-to-tail direction (descending mode) because it is heavier then the octanol stationary phase. As long as more channels equilibrated, the octanol phase was pushed off the apparatus and the pressure rose. The octanol phase displaced by the aqueous phase was collected in a graduated cylinder. Once the aqueous phase appeared at the exit of the apparatus, two liquid layers were seen in the cylinder. The mobile-phase/stationary-phase equilibrium was established in the whole CCC ‘‘column’’ and the pressure stabilized. The displaced octanol phase corresponded 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 used in each injection. This salt was not retained by

Table 1
Structure, octanol–water partition coefficients and dissociation constants of the β-blockers

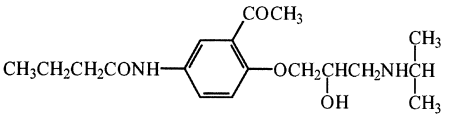
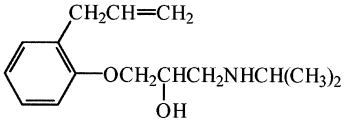
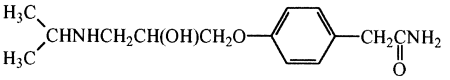
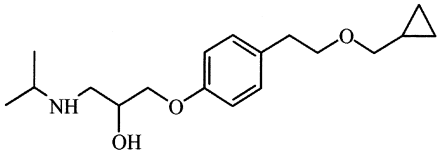
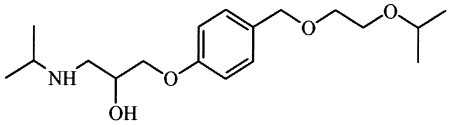
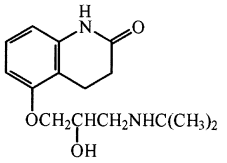
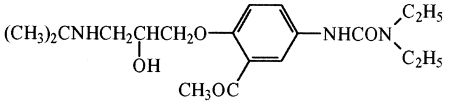
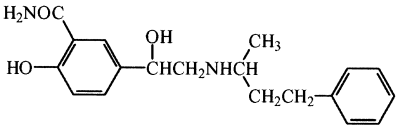
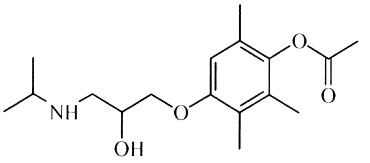
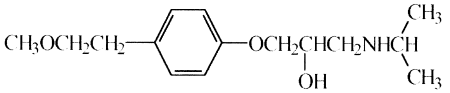
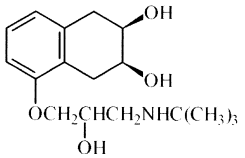
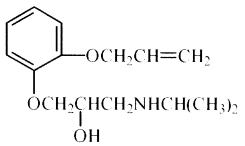
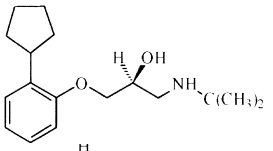
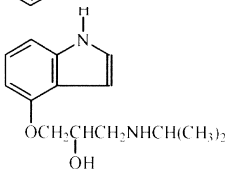
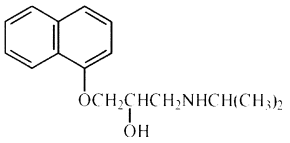
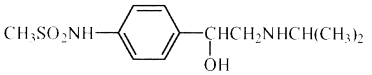
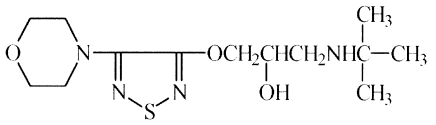
Compound	Structure	Log *P*	Log *K*
Acebutolol		1.19[a]; 1.61 (C), 1.77 (E)[b]; 1.71 (C,E)[c]	9.12[a]; 9.2[b]
Alprenolol		2.81[a]; 2.59 (C), 3.1(E)[b]; 2.65 (C), 2.89 (E)[c]	9.19[a]; 9.65[b]
Atenolol		−0.026[a]; −0.11 (C), 0.16 (E)[b,c]	9.17[a]; 9.6[b]; 8.07[c]
Betaxolol		2.32 (C), 2.81(E)[c]	9.6
Bisoprolol		1.84[a]; 1.69 (C)[b]; 2.12 (C), 1.87 (E)[c]	9.16[a]
Carteolol		1.42[a]; 1.17 (C), −0.46 (E)[b]; 1.29 (C)[c]	9.13[a]
Celiprolol		1.93[a]; 1.66 (C)[b]; 1.86 (C), 1.92 (E)[c]	9.12[a]
Labetalol		2.41[a]; 2.18 (C), 3.09 (E)[b]; 2.5 (C), 1.06 (E)[c]	7.91[a]; 7.4, 8.7[b]
Metipranolol		2.55 (C), 2.28 (E)[c]	ND

Table 1. Continued

Compound	Structure	Log *P*	Log *K*
Metoprolol		1.69[a]; 1.2(C), 1.88(E)[b]; 1.35 (C), 1.88 (E)[c]	9.18[a]; 9.7[b]
Nadolol		1.17[a]; 0.23(C), 0.71(E)[b]; 0.38(C), 0.71 (E)[c]	9.17[a]; 9.4[b]
Oxprenolol		1.83[a]; 1.62(C), 2.18(E)[b]; 2.09 (C), 2.1 (E)[c]	9.13[a]; 9.5[b]
Penbutolol		3.24 (C)[c]	ND
Pindolol		1.48[a]; 1.65 (C), 1.75(E)[b,c]	9.21[a]; 8.8, 9.7[b]; 7.00[c]
Propranolol		2.60[a]; 3.56(E)[b]; 2.75 (C), 2.98 (E)[c]	9.15[a]; 9.5[b]
Sotalol		0.37[a]; 0.23 (C), 0.24 (E)[b]; 0.196 (C)[c]	9.19[a]; 8.15, 9.05[b]
Timolol		1.75[a]; 1.63 (C), 1.91 (E)[b]; 1.58 (C), 1.83 (E)[c]	8.86[a]; 9.21[b]

C, calculated; E, experimental; ND, no data found.

[a] Values from Ref. [16].

[b] Values from Ref. [17].

[c] Values from Ref. [18].

the octanol phase and gave a sharp signal at 210 nm, used as the V_M value.

When octanol is the stationary phase and water the mobile phase the partition coefficient is calculated as:

$$P_{oct} = \frac{V_R - V_M}{V_C - V_M} \quad (2)$$

where V_C is the total internal volume of the CCC ''column'' (apparatus). Since the solute retention volume is used directly, this is the *direct measurement* of P_{oct} [20]. As can be seen from Eq. (2), the solute retention volume increases linearly with the partition coefficient. The maximum practical P_{oct} value that can be obtained by direct measurement is about 25 ($V_R \sim 1.5$ L, experiment duration ~24 h at 1 mL/min).

Solutes with very high P_{oct} values move very slowly in the octanol phase. They need too much time to emerge outside the machine. To force them from the CCC apparatus, the roles of the aqueous and octanol phases and their flowing direction are reversed after some reasonable flowing time in the normal direction. The mode is switched from descending (head to tail) to ascending (tail to head). The solutes are thus eluted by a small volume of octanol (the stationary phase in the first step). Theory shows that the P_{oct} of the solute is simply the ratio of the volume of the aqueous phase (V_{aq}) pumped in the descending mode to the retention volume of the octanol phase (V_{oct}) in the ascending mode [20–22]:

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

This procedure is known as *dual-mode or back-flushing measurement*. It was also used to measure very small P_{oct} values with very polar solutes. 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 of octanol elution, the phase role is inverted. The water becomes the mobile phase in the head-to-tail or descending mode. This forces the polar analyte out of the apparatus. In the dual-mode method the octanol- and the water-phase volumes must not change during phase switching. This is achieved by using a very slow flow-rate (0.1 mL/min) in the back-flushing step since the second volume is usually very small.

3. Results and discussion

3.1. Theoretical model

The ionization change of basic compounds such as amines can be described by:

$$AH^+ \rightarrow A + H^+ \quad (4)$$

The molecular form, A, ionizes into a cationic form, AH^+, as the pH decreases. K_a is the acidity constant or dissociation constant of Eq. (4). Introducing P^0, the P_{oct} value for the A molecular form, and P^+, the P_{oct} value of the AH^+ cationic form, the distribution ratio or experimental partition coefficient, P_{app}, is given by:

$$P_{app} = \frac{[A]_o + [AH^+]_o}{[A]_w + [AH^+]_w} \quad (5)$$

The subscripts ''o'' and ''w'' refer to the octanol phase and to the aqueous phase, respectively. Using the expression for K_a, P_{app} can be formulated as [7]:

$$P_{app} = \frac{P^0 + P^+([H^+]/K_a)}{1 + ([H^+]/K_a)} \quad (6)$$

Eq (6) shows that the measured coefficient increases with the pH.

3.2. Experimental results

3.2.1. General results

Table 2 shows the measured P_{app} coefficients for 17 β-blockers and the calculated molecular and cationic P values. The P_{app} values at different pH values were calculated with Eqs. (2) and (3) using the solute experimental retention volumes. The listed values are the average of duplicate measurements. Molecular and cationic values together with the dissociation constants were fitted with Eq. (6) except for labetalol and sotalol, which do not have the ionization scheme of Eq. (4). As expected, the $\log P_{app}$ values increase with the pH as the solute becomes more and more in its molecular form.

Table 2
Experimental β-blocker octanol–buffer partition coefficients for three pH values

Compound	Experimental log P_{app} [a]			Fitted parameters (Eq. (6))[a]		
	pH 3	pH 7	pH 11	Log P^+ ion	Log P^0 molec.	pK_a
Acebutolol	−2.08	−0.40	1.82	−2.09	1.83	9.24
Alprenolol	−1.39	0.81	3.14	−1.41	3.15	9.34
Atenolol	[b]	−0.83	0.25	−2.77	0.25	8.07
Betaxolol	−1.50	0.34	2.90	−1.50	2.91	9.58
Bisoprolol	−2.29	0.04	2.19	−2.29	2.20	9.2
Carteolol	−2.82	−0.75	1.49	−2.82	1.49	9.24
Celiprolol	−2.34	−0.73	1.97	−2.34	1.98	9.74
Labetalol[c]	−1.16	0.99	0.27	−1.22 (AH^+) 0.250 (A^-)	1.06 ($A^{+/-}$)	6.2, 8.8
Metipranolol	−2.13	0.46	2.59	−2.15	2.60	9.13
Metoprolol	−2.08	−0.39	1.88	−2.08	1.90	9.31
Nadolol	−2.53	−0.995	0.99	−2.57	1.00	9.00
Oxprenolol	−1.82	0.22	2.30	−1.82	2.30	9.08
Penbutolol	−0.29	1.74	3.61	−0.29	3.61	8.88
Pindolol	−2.00	1.62	1.89	−3.29	1.91	6.98
Propranolol	−1.10	1.16	3.41	−1.12	3.41	9.25
Sotalol[c]	−2.92	−1.54	−1.62	−2.92 (AH^+) −1.64 (A^-)	−0.77 ($A^{+/-}$)	7.7, 9.0
Timolol	−1.84	−0.20	1.98	−1.85	1.98	9.19

[a] Accuracy ±0.06 log units.
[b] Out of range, measurement at pH 5 gave log $P_{app} = -2.5$.
[c] The parameters were fitted with Eq. (7) using also measurements at pH 5 and 9 that gave log $P_{app} = -0.13$ and 0.74 for labetalol and log $P_{app} = -2.8$ and −1.03 for sotalol, respectively.

3.2.2. The case of labetalol and sotalol

Labetalol and sotalol both have an apparent coefficient, P_{app}, that decreases at pH 11. These two compounds form anions at elevated pH due to a phenolic OH group and a sulfonamide group, respectively. These results confirm those previously obtained by Gulyaeva et al. [23]. Labetalol and sotalol are cationic in acidic media, non-charged in neutral media, and anionic in basic media. As their behavior is similar to that of amino acids, their P_{app} can be calculated using [7]:

$$P_{app} = \frac{P^0 + P^+([H^+]/K_{a1}) + P^-(K_{a2}/[H^+])}{1 + ([H^+]/K_{a1}) + (K_{a2}/[H^+])} \quad (7)$$

where P^- is the P_{oct} value of the A^- anionic form, and K_{a1} and K_{a2} the dissociation constants of the cationic (AH^+) and anionic (A^-) forms, respectively.

3.2.3. Hydrophobicity versus pH

Fig. 1 shows the evolution of the apparent P_{app} coefficient vs. pH for several β-blockers. This figure

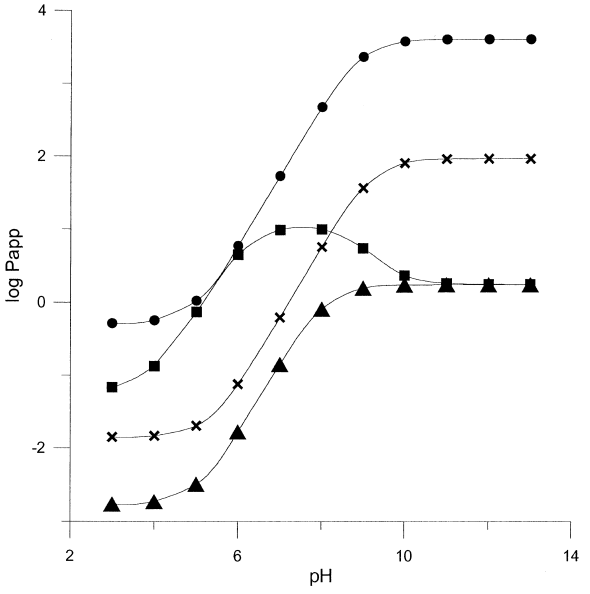


Fig. 1. Relative hydrophobicity, log P_{app}, versus pH for penbutolol (●), timolol (×), labetalol (■), and atenolol (▲).

illustrates different dependencies of the relative hydrophobicity upon the pH for compounds with a single ionic group (e.g., penbutolol, timolol and atenolol), and a compound with two different ionizable groups and two pK_a values such as labetalol. Since the true molecular log P_{oct} of all β-blockers lies between the log P_{oct} value of penbutolol (log $P_{oct} = 3.61$) and atenolol (log $P_{oct} = 0.25$) (Table 2), it may be possible to measure the P_{oct} values of several compounds in a single run. However, the chromatographic efficiency was very low, in the 10 to 100 plate range (Fig. 2), due to the octanol viscosity and the limited volume (101 mL) and channel number (1068 channels) of the hydrostatic machine used. When several compounds were injected together, peak overlapping was observed, which introduced an error in the determination of the maximum position and consequently in the octanol–water partition coefficient measurement. All results listed in Table 2 were obtained by injecting each solute separately.

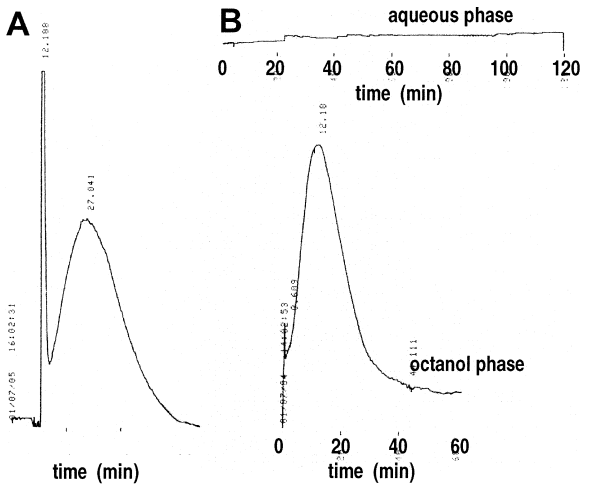


Fig. 2. CCC chromatograms at pH 7 for P_{app} measurements. (A) Direct mode chromatogram of acebutolol: rotor speed, 900 rpm; mobile phase, aqueous with 0.01 *M* ammonium phosphate buffer at pH 7; flow-rate, 2 mL/min in the descending head-to-tail mode; stationary phase, octanol; injection volume, 1 mL (0.5 mg β-blocker + 0.1 mg potassium nitrate); UV detection at 210 nm. (B) Dual-mode chromatogram of labetalol: rotor speed, 900 rpm; top—the aqueous phase 2 h step; flow-rate, 1 mL/min of aqueous mobile phase with 0.01 *M* ammonium phosphate buffer at pH 7 in the descending head-to-tail mode; injection volume, 1 mL (0.5 mg labetalol), no signal; bottom—the octanol phase step; flow-rate, 1 mL/min in the ascending tail-to-head mode; stationary phase, aqueous; UV detection at 225 nm.

At acidic pH, all β-blockers are in the cationic form, AH^+. This ionic form is very hydrophilic but the CCC method allows the estimation of very small log P_{app} values. Trivially, the ionic forms of the solutes are more soluble in the aqueous phase than in the apolar octanol phase. As the pH increases, the ionization degree of the β-blockers decreases, and their P_{oct} value increases following a sigmoidal curve related to their dissociation constant (Fig. 1). It should be noted that the hydrophobicity of the molecular form of a given solute is related to the hydrophobicity of its cationic forms. Plotting log P^+ for the cationic AH^+ forms of the β-blockers versus log P^0 for the corresponding molecular form gave a R^2 regression coefficient of 0.83, indicating a loose but significant correlation. The slope was 0.67 and the intercept −3.34.

The cationic log P^+ values listed in Table 2 correspond to the phosphate salt of the positive form of the β-blockers. These log P^+ values may vary if the anion of the buffer salt is changed. This work shows that it is possible to quantify the hydrophobicity of ions when the P_{oct} values of the ions are commonly neglected, assuming $P_{oct} = 0$ for any ion. Ionic P^+ values are indeed small. The higher P^+ value is 0.5 obtained for the penbutololium cation (log $P^+ = -0.29$). The lower P^+ value is three orders of magnitude lower, 0.0005 (log $P^+ = -3.29$), obtained for the pindololium cation.

3.2.4. Direct and dual-mode CCC measurements

Fig. 2 shows the actual chromatograms of acebutolol and labetalol at pH 7 obtained with direct and dual-mode CCC, respectively. The sharp peak at 12.11 min in Fig. 2a corresponds to potassium nitrate, the dead (aqueous phase) volume marker ($V_M = 24.22$ min at 2 mL/min). The broad peak at 27.04 min (only 10 plates) is the β-blocker peak ($V_R = 54.08$ mL). The calculation of P_{oct} was performed using Eq. (2) with the column volume $V_C =$ 101 mL: $(54.08-24.22)/(101-24.22) = 0.39$ and log $P_{oct} = -0.41$. The experiment was performed in 60 min. Fig. 2b (bottom) shows the actual UV signal after the mode switching for labetalol. The peak at 0.61 min corresponds to the phase change in the detector. From this point, the octanol phase replaced

the aqueous phase. The labetalol peak appeared in the octanol phase at 12.18 min at 1 mL/min (V_{oct} = 12.18 mL). The V_{aq} volume in the head-to-tail direction was 120 mL. Finally, P_{oct} was calculated as 120/12.18 = 9.85 ($\log P_{oct} = 0.99$). The dual mode was used to measure high P_{oct} values of the β-blockers at pH 11. The first step was a long elution with the aqueous phase (Fig. 2b, top), followed by mode switching and octanol-phase elution of the solute (Fig. 2b, bottom). The dual-mode method was also used to measure very small P_{oct} values accurately at pH 3. There, the first step was a long elution with octanol, followed by aqueous-phase elution of the solute.

3.2.5. Hydrostatic versus hydrodynamic CCC machines

All experiments in this work were performed with a hydrostatic machine with channels linked by ducts and a constant centrifugal field retaining the liquid stationary phase [1,5]. It is known that this kind of machine retains the liquid stationary phase better than the hydrodynamic machines with coiled tubes and a periodic centrifugal field [1–4]. However, the chromatographic efficiency is much lower. At best, liquid–liquid exchanges in three channels will be needed to obtain a single theoretical plate. This is fully confirmed by comparing the results obtained in this work with those presented in another work carried out with a hydrodynamic 53 mL machine [7]. Fig. 2 shows nice spike-less chromatograms when compared with Fig. 5 of Ref. [7]. Spikes are produced by microdroplets of stationary phase passing through the detector cell. This does not occur with the hydrostatic machine, which retains the stationary phase very well. A constant 10 μL/min octanol-phase leak was observed with the hydrodynamic machine [7]. A small phase change of ~4 μL/min was observed during long experiments (<1 mL stationary phase volume change over more than 4 h) with the hydrostatic machine without any droplets seen in the detector. It is assumed that this change was due to dissolution of octanol by the aqueous phase at elevated pressure. The two liquid phases were saturated at atmospheric pressure [20].

The solute peaks are broad because the mass transfer between water and octanol is very slow due to octanol viscosity (~7 cP at 25 °C). Potassium nitrate, which does not partition at all with octanol, elutes with a sharp peak. Fig. 2 shows broad peaks with about 10 plate efficiency. Fig. 5 of Ref. [7] also shows broad peaks, but with about 30 plates. Considering the 101 mL hydrostatic machine volume and the 53 mL hydrodynamic machine volume in Ref. [7], it can be estimated that we have one plate per 10 mL of hydrostatic machine compared with one plate per 2 mL of hydrodynamic machine. One hundred channels were merely able to produce one theoretical plate. Our hydrostatic machine is about five times less efficient than the hydrodynamic machine of Ref. [7], as already observed [1–5,20].

3.2.6. CCC measurements and literature values

As can be seen from comparing the values obtained by CCC listed in Table 2 with the literature values listed in Table 1, there is good agreement for most compounds. However, the P_{oct} literature values and dissociation constants (pK_a) of some β-blockers are significantly different from the CCC values depending on the original reference. It is clear that there is inconsistency in some literature values. For example, the $\log P_{oct}$ literature values of carteolol were in the −0.46 to 1.42 range ($0.35 < P_{oct} < 26$), or a two orders of magnitude difference. Clearly, the CCC results confirm that the P_{oct} value of carteolol in its molecular form is 30 ($\log P_{oct} = 1.49$). The $P_{oct} = 0.35$ value was obtained at physiological pH (7.4), a pH value at which the ionic and molecular forms of carteolol both exist [18]. Reliable values are obtained by CCC since there is no assumption and the aqueous phase can be well buffered [1,7,20].

The major difference is observed for the molecular form of labetalol with $\log P_{oct}$ literature values between 2.18 and 3.09 ($150 < P_{oct} < 1200$) and a much lower CCC $\log P_{oct}$ value of 1.06 ($P_{oct} = 11$, Table 2). Labetalol becomes a cation at low pH by protonation of its secondary amine. It becomes an anion at high pH by ionization of its phenol group. The hydrophobicity of its molecular form should be measured at intermediate pH (around pH 7). It is interesting to note that our CCC results are confirmed by the recent work of Gulyaeva et al. [23]. They measured $\log P_{oct}$ values of 0.68, 1.15, 1.14 and 0.21 at pH values of 6.6, 7.4, 8.5 and 11, respectively.

Fig. 3 shows the molecular CCC P_{oct} values fitted

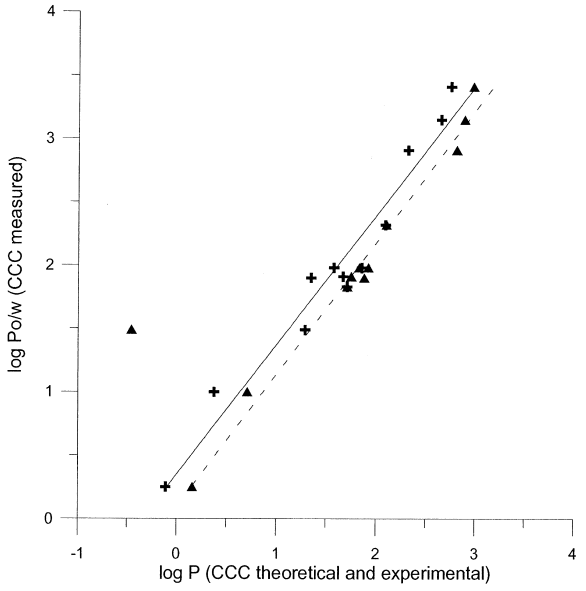


Fig. 3. β-Blocker P_{oct} values obtained in this work compared with experimental literature values (+, full line, $\log P_{o/w} = 1.02 \log P_{CCC} + 0.347$, $r^2 = 0.975$) and theoretical values obtained using the ClogP 4.01 program (▲, dotted line, $\log P_{o/w} = 1.04 \log P_{CCC} + 0.090$, $r^2 = 0.992$).

by Eqs. (6) and (7) (Table 2) plotted versus the theoretical and experimental log P literature values (Table 1). The theoretical values were calculated from the molecule structure using the ClogP 4.01 program [19], which is freely accessible from the website (last connection, Dec. 2002) http://www.daylight.com/daycgi/clogp. The theoretical regression line (full line, Fig. 3) has a slope, intercept and regression coefficient of 1.02, 0.347 and 0.975, respectively. The experimental line (dashed line, Fig. 3) has a similar slope (1.04), a slightly lower intercept (0.090) and a better regression coefficient ($r^2 = 0.992$). Clearly, the slope value, close to unity, indicates that the partition coefficients obtained by CCC and log P_{oct} are identical, even for ionizable compounds. The experimental value found in the literature for carteolol (log $P_{oct} = -0.46$) was excluded [18]. It likely corresponds to its cationic form. It can be noted that the experimental P_{oct} values found in the literature are usually higher than the calculated values. It should be pointed out that the ClogP program is given to produce the order of magnitude of the log P_{oct} value of the molecular form of an ionizable molecule. Some calculated values listed in Table 1 may well be overestimated.

CCC produces reliable P_{oct} values, and also dissociation constant, K_a, values. Most CCC K_a values listed in Table 2 correspond to their respective literature values listed in Table 1. A significant difference is obtained for atenolol (literature K_a value 9.2, CCC K_a value 8.1) and pindolol (literature K_a value 8.8, CCC K_a value 7.0). In both cases, the CCC K_a value is lower than the literature value. No K_a values were found in the literature for betaxolol and penbutolol. Their CCC K_a values were found to be 9.6 and 8.9, respectively (Table 2).

3.3. Hydrophobicity–biology relationships

3.3.1. Literature survey

The log P_{oct} parameter has been successfully applied as a structural descriptor in quantitative structure–activity relationships (QSARs) for structurally related compounds and in some cases even for sets of chemically different compounds [18]. Liquid chromatography is a powerful technique for the measurement of physicochemical parameters and, in order to emulate biological barriers, different reversed stationary phases have been developed such as immobilized artificial membranes [24], or immobilized liposomes [25]. Micellar liquid chromatography (MLC) was also successful in establishing relationships between chromatographic indexes and octanol–water partition data for β-blockers [26–28].

Using MLC with an anionic surfactant, Detroyer et al. [17] studied the correlation of the hydrophobicity parameters of six β-blockers with their cellular permeability coefficients through Caco-2 monolayers (in vitro model for human intestinal membrane) and through rat intestinal segments. Ranta et al. [29] developed a HPLC gradient method to determine the hydrophobicity of eight β-blockers in corneal permeability experiments in vitro. Molero-Monfort et al. [30] studied the possibility of using retention in MLC with Brij 35 as a nonionic surfactant to predict passive drug adsorption of β-blockers by red cells. De Castro et al. [31] developed a non-invasive spectrometric method to quantify the partition coefficients of atenolol and nadolol in aqueous solutions of bile salt/lecithin micelles which

were used as a simple model for the naturally occurring mixed micelles in the gastrointestinal tract. Gulyaeva et al. [22] examined the partitioning of nine β-blockers in aqueous dextran–PEG two-phase systems and octanol–buffer systems at pH from 2 to 12.5, and related their results to biological activity.

The HPLC technique is well known to be able to evaluate chromatographic lipophilicity indexes (expressed as $\log D_{\mathrm{HPLC}}$) even for highly lipophilic compounds out of the range of the shake-flask method. These indexes are usually calculated based on the average retention factors of the analyzed compounds after two runs using linear equations for two adjacent standards relating their $\log D$ and retention time values [23]. The correlation between the $\log P_{\mathrm{app}}$, obtained by CCC, and the $\log D_{\mathrm{HPLC}}$ indexes at pH 11, taken from Ref. [23], for nine β-blockers (acebutolol, alprenolol, atenolol, labetalol, metoprolol, pindolol, propranolol, sotalol and timolol) produced the line:

$$\log D_{\mathrm{HPLC}} = 0.51 \log P_{\mathrm{app}} + 1.43, \quad n = 9, \quad r^2 = 0.981 \tag{8}$$

Clearly, there is a correlation between the data, but the 0.51 value of the slope indicates that the HPLC retention factors of the β-blockers are empirically related to the square root of the octanol–water partition coefficient. The intercept value of 1.43 indicates that even polar β-blockers are retained by the octadecyl-bonded HPLC stationary phase. These parameters would likely be different with another compound family. Clearly, hydrophobicity measurements are possible using HPLC, but a set of standards will always be needed to calibrate the particular set of compounds studied. There is no such need with CCC which uses octanol and water. CCC is a good technique to measure octanol–water partition coefficients for many solutes, including ionizable compounds.

3.3.2. Log P_{oct} and biological activity

β-Blockers have also been used to study the influence of the lipophilicity of drugs on permeation through biological membranes, since various structurally related β-blockers exhibit a wide range of lipophilicity [32,33]. The $\log P_{\mathrm{oct}}$ values for eight β-blockers (acebutolol, alprenolol, atenolol, metoprolol, nadolol, oxprenolol, pindolol, and propranolol) were compared with the permeation coefficient K_s of natural membranes {red cell membrane lipid liposomes (MLs) and egg phospholipids liposomes (EPLs) [34]}. Fig. 4 shows the line for the EPL (top) and ML membranes (bottom). It is confirmed that permeation through these biological membranes is correlated to the drug hydrophobicity.

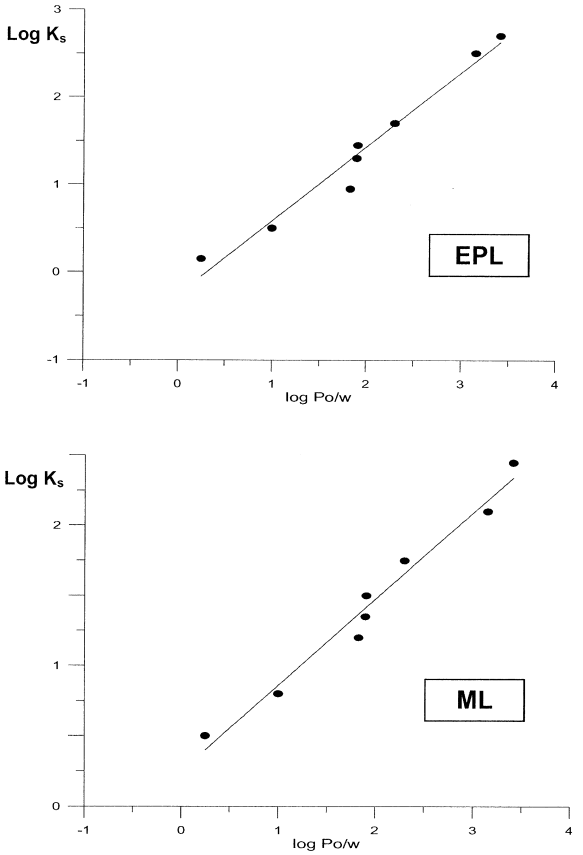


Fig. 4. Correlation between the permeation coefficient K_s of β-blockers through egg phospholipid liposomes (EPLs, top) and red cell membrane lipid liposomes (MLs, bottom) (data from Ref. [34]) and the molecular P_{oct} values obtained in this work:
$\log K_s$ (EPL) = 0.848 $\log P_{\mathrm{oct}}$ − 0.262 n = 8, $r^2 = 0.983$
$\log K^s$ (ML) = 0.614 $\log P_{\mathrm{oct}}$ + 0.247, $n = 8$, $r = 0.986$.

4. Conclusion

The CCC technique uses an octanol stationary

phase and an aqueous buffer mobile phase or vice versa. The retention times of the injected solutes are directly proportional to their P_{oct} coefficients without any assumption. It was demonstrated that CCC was able to determine accurately the P_{oct} value of ionizable compounds, separating the P_{oct} value of the molecular form of the compound from the P_{oct} value of its ionized form. The octanol–water partition coefficients of the molecular and ionic forms of β-blockers were determined by CCC. It was found that the P_{oct} values of the ionic forms were small, but not zero, except for pindolol. Measuring the P_{oct} coefficient by CCC does not require ultra-pure solutes like the shake-flask method. Indeed, the possible impurities would be separated from the solute, forming minor peaks. The dual-mode use of the CCC method extends the measurable P_{oct} range to five orders of magnitude ($-2.5 < \log P_{oct} < +2.5$). This may not be wide enough for very hydrophobic compounds.

Acknowledgements

S.C.B. thanks the European Community for the Marie Curie fellowship HPMF–CT–2000-00440 (Human Potential), which allowed him to stay in Lyon. A.B. thanks the Centre National de la Recherche Scientifique FRE2394 for financial support. This work was also supported by the INTAS research program (grant No. 00-00782).

References

[1] A. Berthod, in: D. Barceló (Ed.), Countercurrent Chromatography, The Support-free Liquid Stationary Phase, Comprehensive Analytical Chemistry, Vol. XXXVIII, Elsevier, Amsterdam, 2002.
[2] Y. Ito, in: J.C. Giddings, E. Grushka, J. Cazes (Eds.), Advances in Chromatography, Vol. 24, Marcel Dekker, New York, 1984, p. 181.
[3] W.D. Conway, Countercurrent Chromatography, Apparatus, Theory & Applications, VCH, New York, 1989.
[4] N.B. Mandava, Y. Ito, Countercurrent Chromatography, Chromatographic Science Series, Vol. 44, Marcel Dekker, New York, 1989.
[5] A.P. Foucault, Centrifugal Partition Chromatography, Chromatographic Science Series, Vol. 68, Marcel Dekker, New York, 1994.
[6] R.N. Smith, C. Hansch, M.M. Ames, J. Pharm. Sci. 64 (1975) 599.
[7] A. Berthod, S. Carda-Broch, M.C. Garcia-Alvarez-Coque, Anal. Chem. 71 (1999) 879.
[8] R. Howe, R.G. Shanks, Nature (London) 210 (1966) 1336.
[9] T. Walle, J.C. Webb, E.E. Begwell, U.K. Walle, H.B. Daniell, T.E. Gaffney, Biochem. Pharmacol. 37 (1988) 115.
[10] G. Egginger, W. Lindner, C. Vandenbosh, D.L. Massart, Biomed. Chromatogr. 7 (1993) 277.
[11] W.A. Clementi, T.Q. Garvey, G.D. Clifton, R.A. McCoy, S. Brandt, S. Schwartz, Chirality 6 (1994) 169.
[12] J.E.F. Reynolds, Martindale, The Extra Pharmacopoeia, 29th ed., Pharmaceutical Press, London, 1989.
[13] A. Scribiane (Ed.), Pharmacology of Antihypertensive Drugs, Raven Press, New York, 1980, p. 179.
[14] J.M. Cruickshank, β-Blockers in Clinical Practice, Churchill-Livingstone, New York, 1994.
[15] International Olympic Committee (IOC), Medical Commission, List of Doping Classes and Methods, 1998.
[16] C. Ceniceros, M.I. Maguregui, R.M. Jimenez, R.M. Alonso, J. Chromatogr. B 705 (1998) 97.
[17] A. Detroyer, Y. Vander Heyden, S. Carda-Broch, M.C. Garcia-Alvarez-Coque, D.L. Massart, J. Chromatogr. A 912 (2001) 211.
[18] C.C. Hansch, in: R.G. Sammes, J.B. Taylor (Eds.), Comprehensive Medicinal Chemistry, Vol 6, Pergamon Press, Oxford, 1990.
[19] ClogP computer program version 4.01, BioByte Corp., Claremont, CA (using the website address).
[20] A. Berthod, in: A. Foucault (Ed.), Centrifugal Partition Chromatography, Chromatographic Science Series, Vol. 68, Marcel Dekker, New York, 1995, p. 167, Chapter 7.
[21] R.A. Menges, G.L. Bertrand, D.W. Armstrong, J. Liq. Chromatogr. 13 (1990) 3061.
[22] S.J. Gluck, E.J. Martin, J. Liq. Chromatogr. 13 (1990) 2529.
[23] N. Gulyaeva, A. Zaslavsky, P. Lechner, M. Chlenov, A. Chait, B. Zaslavsky, Eur. J. Pharm. Sci. 17 (2002) 81.
[24] S. Ong, H. Liu, C. Pingeon, J. Chromatogr. A 728 (1996) 113.
[25] A. Lundqvist, P. Lundhal, J. Chromatogr. B 699 (1997) 209.
[26] J.I. Vila, R. Obach, R. Prieto, J. Moreno, Chromatographia 22 (1986) 48.
[27] F. Barbato, G. Caliendo, M.I. La Rotonda, P. Morrica, C. Silipo, A. Vittoria, Farmaco 45 (1990) 647.
[28] I. Rapado-Martinez, M.C. Garcia-Alvarez-Coque, R.M. Villanueva-Camañas, J. Chromatogr. A 765 (1997) 221.
[29] V.P. Ranta, E. Toropainen, A. Talvitie, S. Auriola, A. Urtti, J. Chromatogr. B 772 (2002) 81.
[30] M. Molero-Monfort, Y. Martin-Biosca, S. Sagrado, R.M. Villanueva-Camañas, M.J. Medina-Hernandez, J. Chromatogr. A 870 (2000) 1.
[31] B. de Castro, P. Gameiro, C. Guimarães, J.L.F.C. Lima, S. Reis, Biophys. Chem. 90 (2001) 31.
[32] R.D. Shoenwald, H.S. Huang, J. Pharm. Sci. 72 (1983) 1266.
[33] W. Wang, H. Sasaki, D.S. Chien, V.H.I. Lee, Curr. Eye Res. 10 (1991) 571.
[34] F. Beigi, I. Gottschalk, C.L. Hägglund, L. Haneskog, E. Brekkan, Y. Zhang, T. Österberg, P. Lundhal, Int. J. Pharm. 164 (1998) 129.