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Rapid determination of conditional partition constants in an FIA system

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Abstract: This paper presents a simple and rapid method for the screening of substances for their conditional partition constants in the chloroform–water system. Samples are injected in either aqueous or organic solution into the corresponding phase. After equilibration in segmented flow the amount of sample remaining is measured photometrically. Performing the measurements at a series of phase volume ratios enables calculation of conditional partition constants knowing neither the amount injected nor the absorptivity of the substance under study. The time needed for a determination is about 15 min per substance, in many cases even shorter and the sample consumption is, in most cases, less than 1 mg. These characteristics make the method suitable for screening purposes. A series of compounds has been examined with this method, and acceptable results for the conditional partition constants were obtained in the range $0.2 < D' < 100$ ($-0.7 < \log D' < 2.0$).

Keywords: *Partition constants; chloroform–water; determination; flow injection analysis; screening; distribution.*

Introduction

The determination of partition constants for substances between water and an organic solvent is a common exercise in many areas of chemistry. In the pharmaceutical industry partition constants of drug candidates are measured in order to assess their fate upon ingestion. Similarly, environment control agencies are measuring such constants for substances emitted in order to find possible bioaccumulators. In life sciences the system studied is often *n*-octanol–water, because partition between these two phases are thought to mimic the uptake of various substances by biota. Traditionally, the methods of determination have been based on manual or mechanical shaking of the two phases with a known amount of the substance under study added. After an equilibration period of at least 10 min, often hours, the phases were separated and the concentration of the partitioned substance measured, preferably in both phases. These methods are not only labour intensive and tedious but also demanding in both the amount of substance investigated and of solvent used [1]. In the pharmaceutical industry a method based on reversed-phase liquid chromatography (RPLC) has been extensively used [2]. This method requires less sample but it is too slow to satisfy the users. Specific problems also do occur for some types of substances as, for example, for bases where the limited pH range of RPLC stationary phases complicates application of the method. The transfer from capacity factors to partition constants is also, in some cases, questionable.

The search for more rapid direct methods of the determination of partition constants has concentrated on methods utilizing segmented flow. In such methods very short equilibration times can be used, facilitating studies of substances unstable under the conditions needed for measurement. Flow methods are further relatively simple to mechanize or even automate.

Flow methods using pumping of the sample into the system and measurements at steady state have been presented by Bäckström *et al.* [3] and by Nekimken *et al.* [4]. Measuring at steady state can lead to high measurement precision but at the cost of high sample consumption and low sample throughput.

The first methods using flow injection analysis (FIA) for the determination of partition constants were presented by Johansson *et al.* [5] and by Kinkel and Tomlinson [6]. In both these cases phase separators based on

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dissimilar wetting of organic and aqueous phases were utilized. The first method ([5]) was used for studies of the partition of ion pairs with injection into the aqueous phase and measurements performed on the organic phase after separation. In the second method injection and subsequent measurement took place in the same phase either aqueous or organic. Some years later a system using a dual membrane separator was presented in a series of papers by Fossey and Cantwell [7–9]. The main difference between their method and those presented earlier was that they measured the concentration of the substance under study in both phases after separation making the set up more complicated and less reliable. All these methods seem to work fairly well for solvents easily separated with the aid of hydrophobic membranes, e.g. chloroform and benzene. A largely unsuccessful attempt at a much more difficult system (*n*-octanol–water) was presented by Gluck [10]. Unfortunately, *n*-octanol performs less well in phase separators due to its high viscosity and low surface tension towards water. This makes separation difficult as witnessed by Gluck.

This paper presents the development of a simple and rapid method for the determination of conditional partition constants. The aim of the work was to create a rapid and reliable method for screening purposes, focusing on simplicity and rapidity rather than on high precision or accuracy. The intention was also to be able to use both aqueous and organic solutions of the substances under investigation and avoid separate determinations of, for example, absorptivities.

Theory

In simple extraction, with no side reactions, where a substance is partitioned between two immiscible phases, the process can be described as: $S_{(aq)} = S_{(org)}$. At equilibrium, the distribution constant (D) for an arbitrary substance HL is defined as:

$$D = [HL]_{org}/[HL]_{aq}, \quad (1)$$

where $[HL]_{org}$ and $[HL]_{aq}$ are the concentrations of the substance in organic phase and aqueous phase, respectively. Expressing the concentrations as the quotient between amount of substance and volume of solvent and taking the special case of a flowing system and sample introduction into the organic phase into consideration, equation (1) can be rewritten as follows:

$$D = [M_{org}/(M^*_{org} - M_{org})](Q_{aq}/Q_{org}), \quad (2)$$

where M^*_{org} is the total amount introduced into the organic phase, M_{org} is the amount present in the organic phase and Q_{aq} and Q_{org} are the flow rates of aqueous phase and organic phase, respectively.

If a substance is injected into a flowing stream and the absorbance of the stream is measured continuously with, for example, a flow through detector, then the signal obtained is a measure of the mean value of the concentration over the measuring volume. The integral of this signal is a measure of the amount of substance passing through the measuring volume. Thus, at a constant flow rate of organic phase:

$$M_{org} = KA_{org}, \quad (3)$$

where A is integrated area and K is a constant including factors from Beer's law, organic flow rate and also instrumental factors involved in the transformation from absorbance to area.

Substituting equation (3) in equation (2), we get

$$D = [A_{org}/(A^*_{org} - A_{org})](Q_{aq}/Q_{org}), \quad (4)$$

where A^*_{org} is the area that theoretically should be obtained if the substance was injected in the system with the aqueous flow stopped.

The most common side reaction is the protonation/dissociation of the substance investigated in the aqueous phase. If such reactions occur the amount of substance in the aqueous phase will no longer equal $[HL]_{aq}\,Q_{aq}$ but rather $([HL]_{aq} + [H_2L^+]_{aq})Q_{aq}$ or $([HL]_{aq} + [L^-])Q_{aq}$. In such a case equation (4) will give a value for the conditional partition constant D' instead of the true D value. The relation between these two is as follows:

$$D = D'\alpha, \quad (5)$$

where

$$\alpha = 1 + [H^+]/K_{a_1} \text{ or } \alpha = 1 + K_{a_2}/[H^+], \quad (6)$$

for protonation and dissociation respectively, where K_{a_1} and K_{a_2} are the acid dissociation constants of H_2L^+ and HL, respectively. Thus,

from a measured D' value the true D value can be easily calculated provided that the dissociation constant of the corresponding acid is known. In order to obtain a proper measurement of D a pH at least 2 units away from the pK_a of the acid should be used. On the other hand, for a substance with a D value out of range for the method the D' value can be brought within range by adjusting the pH. Further, measurements of D' at different pH can be used to calculate K_{a_1} or K_{a_2}.

Rearranging the basic equation [equation (4)] gives three possibilities for the evaluation of D' from a series of measurements of A_{org} at different phase flow ratios, Q_{aq}/Q_{org}, obtained by varying Q_{aq} at a constant Q_{org}:

$$A_{org}(Q_{aq}/Q_{org}) = D'A^*_{org} - D'A_{org}, \quad (a)$$

$$1/A_{org} = 1/A^*_{org} + 1/(D'A^*_{org})(Q_{aq}/Q_{org}), \quad (b)$$

$$A_{org} = A^*_{org}/((Q_{aq}/Q_{org})1/D' + 1). \quad (c)$$

The first two of these equations are linear while the last one is non-linear. By plotting either $A_{org}Q_{aq}/Q_{org}$ vs A_{org} or $1/A_{org}$ vs Q_{aq}/Q_{org}, a straight line can be obtained and the conditional partition constant, D', can be obtained from the slope of the first line or from the quotient between slope and intercept of the second. The third equation can be used for the determination of D' by applying non-linear curve fitting on the measured data. For a given system the same D' value should of course be obtained with all three methods. In a test using data from phenol partition at pH 5.00, the D' values found using the three evaluation methods were 2.65, 2.69 and 2.73, respectively. However, the second of the two linear equations opens the possibility to evaluate the results using common statistical methodology if the flow rate ratios can be considered to be known with high enough accuracy. This method was therefore used for all further evaluations.

In the theoretical treatment so far it has been assumed that the sample is dissolved in the organic phase. If the sample is in aqueous solution, which is by far the most common case, the equations have to be rearranged somewhat. Equation (2) then reads:

$$D = [(M^*_{aq} - M_{aq})/M_{aq}](Q_{aq}/Q_{org}). \quad (2^*)$$

Using this, the final equations for evaluation of D' are given by:

$$A_{aq}Q_{org}/Q_{aq} = A^*_{aq}/D' - A_{aq}/D', \quad (a^*)$$

$$1/A_{aq} = 1/A^*_{aq} + D'/A^*_{aq}/(Q_{org}/Q_{aq}), \quad (b^*)$$

$$A_{aq} = A^*_{aq}/(D'Q_{org}/Q_{aq} + 1). \quad (c^*)$$

Experimental

Chemicals

Phosphate buffer solutions (0.15 M) which were prepared from analytical grade Na_2HPO_4 and NaH_2PO_4 and filtered through 0.45 μm Millipore filters, were used as the aqueous phase. The pH of the buffer solutions was determined by means of a Metrohm 632 pH-meter. The organic phase was chloroform, which was shaken with water in order to remove the ethanol and to saturate it with water. Alprenolol, propranolol and metoprolol were obtained from AB Hässle and were all of high purity (>99%). All other chemicals were of analytical grade.

Equipment

The two manifolds used are shown in Fig. 1(a) and (b). As can be seen the manifolds are very similar and a changeover from one system to the other is straightforward. The whole operation is limited to moving the injector and the detector from the aqueous stream to the organic stream or *vice versa*. In order to facilitate start up in the new configuration both items should be given a short rinse with methanol when detached. Two liquid chromatography pumps (ALTEX model 110A; Beckman 110B solvent delivery module), equipped with restrictors and pulse dampers, were used to propel the two phases through the system. This type of pump was chosen for the accuracy of solvent delivery and the ease with which a certain flow rate is achieved. Injections were performed with a Rheodyne 50110 injector driven by a 5701 pneumatic actuator and 7163 solenoids. The injection volume was about 20 μl throughout this work. A FIA-08 peristaltic pump was used to pump sample to the injector. Segmentation was obtained with PVDF T-pieces with 0.7 mm bore. Equilibration took place in a 2 m long, 0.5 mm i.d. Teflon tube wound into a narrow coil. The detector was a Perkin–Elmer LC-55B spectrophotometric detector equipped with an 8-μl

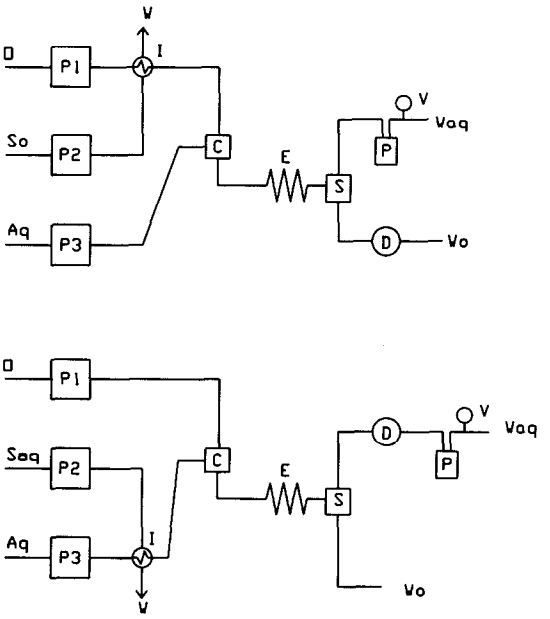

Figure 1
Manifolds used for conditional partition studies. (a) Used for samples in organic phase, (b) used for samples in aqueous phase: sample (S_o) and (S_{aq}), respectively; LC pumps, (P1) and (P3); peristaltic pump, (P2); injector, (I); segmentor, (C); extraction coil, (E); separator, (S); detector, (D); pressure bottle, (P); checkvalve, (V); organic waste, (W_o); aqueous waste, (W_{aq}).

flow cell. A Spectra Physics SP 4270 integrator was used for the measurement of peak areas.

Phase separation was performed with three slightly different types of membrane separators. One with 35 mm long rectangular grooves in two Teflon blocks was described by Nord and Karlberg [11]. The other two both used circular membranes, the design by Bäckström *et al.* [12] with cylindrical cavities and its commercialized version in the Tecator 5105 Extraction module [13] using a spiral shaped grove on the inlet side. Teflon membranes with 0.2 μm pore size were used in the first two separators while the Tecator module was fitted with the 0.45 μm pore size membranes supplied. The pressure needed for separation was in all cases obtained with a pressure bottle supplied with an adjustable checkvalve. A low flow of nitrogen kept the bottle at the pressure set by the checkvalve (1.1 bar). This is convenient since varying flow rates of organic phase complicates the use of restrictors based on lengths of narrow bore tubing. However, the tubing connecting the detector cell with the separator and the pressure bottle and especially the flow cell itself gave rise to a back pressure. This back pressure was larger when measuring in the aqueous phase, due to the higher viscosity, accounting for the partial penetration of aqueous phase in this case.

Procedure

The substance to be tested was either dissolved in an appropriate buffer solution, for aqueous phase introduction or in chloroform. If the substance could not be directly dissolved in chloroform it was dissolved in water and extracted into chloroform after appropriate pH adjustment. The appropriate manifold [Fig. 1(a) or (b)] was assembled, the pumps started and the flow of nitrogen to the pressure bottle initiated. After some time for equilibrating, the flow system injections were made in triplicate. The peak areas were recorded and the procedure repeated at a number (usually 4–5) of different flow rate ratios Q_{aq}/Q_{org} (or Q_{org}/Q_{aq}). The results collected were then evaluated according to one or more of the methods given earlier.

The extraction coil could comprise both Teflon and steel tubing in order to facilitate rapid attainment of distribution equilibria. In order to optimize the extraction conditions, different extraction coils (length, diameter and material), segmentors and separators as well as the 5105 extraction module have been tested.

Results and Discussion

Segmentation

Segmentation is a critical factor in segmented flow extraction affecting both the rate of extraction [14] and the phase separation. In this work two kinds of segmentors, the common T-piece segmentor and the falling drop segmentor [15] have been used. The latter provides the possibility of obtaining segments of a predetermined size through the choice of inlet capillary. Narrow bore capillaries will give shorter segments and a higher rate of extraction. For each capillary size there is, however, a limiting flow that must not be exceeded or else proper segmentation will not be obtained. The effects of segment size was tested using the falling drop segmentor and the results obtained are shown in Table 1. It can be seen that for capillaries with i.d.s 0.15 mm and wider consistent results are obtained while the most narrow capillary gives a higher result indicating that equilibrium has not been reached. This is probably caused by the fact that the flow rate used (0.5 ml min^{-1}) was

Table 1
Limiting flow and measured D' for different capillaries in falling drop segmentor. Sample: alprenolol in chloroform, aqueous phase: 0.2 M phosphate, pH = 6.07, Q_{org} = 0.5 ml min^{-1}

Capillary i.d. (mm)	Limiting flow (ml min^{-1})	D'
0.3	1.4	2.15
0.3	1.4	2.18
0.2	1	2.07
0.15	0.8	2.03
0.1	0.5	2.81

close to the estimated limiting flow for this capillary i.d. giving unstable segmentation occasionally giving 'ripple segmentation' [16]. The results of this process is unstable segmentation with very large segments and severely reduced extraction efficiency.

Phase separation

In order to be able to perform spectrophotometric measurements on a liquid–liquid segmented flow using conventional detectors a separator capable of producing a single phase flow is needed. When measuring in the organic phase this is easily achieved using membrane separators. The excess organic phase is simply transported with the aqueous phase to waste. Keeping the pressure below certain limits will ascertain a flow of organic phase free from water droplets through the membrane. Thus, all the separators tested worked very well in the configuration shown in Fig. 1(a) at Q_{aq}/Q_{org} ratios in the range 0.1–11 and total flow rates in the range 2–4 ml min^{-1}. On the other hand, in order to supply an aqueous phase flow, free from droplets of organic phase, to the detector, 100% of the organic phase must be removed. It was further believed that this had to be achieved without forcing water into the membrane pores as this would block further transport of organic phase. The separator with cylindrical cavities was not capable of producing an aqueous flow suitable for measurements for extended periods of time at these flow rates while the other two worked well. The main differences between the three separators are the lengths and areas of the membranes available for solvent penetration. Both the rectangular separator with a straight linear groove and the commercial separator with its spiral groove have relatively longer pathways for segmented stream in contact with the membrane and larger areas of membranes available for solvent penetration. It is therefore believed that in these separators the first portion of membrane lets only organic phase through while in the later parts, where less organic phase is available, a mixture of organic and aqueous phase penetrates the membrane. In this way successful operation was achieved for aqueous flow rates $0.3 < Q_{aq} < 2.0$ and phase flow ratios $0.03 < Q_{org}/Q_{aq} < 4$. When determinations are made in the organic phase the membranes last very long but in the configuration used for aqueous samples the membrane was exchanged after 2 days of use.

Efficiency of phase separation and its influence on conditional partition measurements

During one run the efficiency of phase separation can vary somewhat with flow rate ratios and with the condition of the membrane. An experiment was therefore made to elucidate whether such variation would affect the determinations. Propranolol dissolved in chloroform was repeatedly injected into the system in Fig. 1(a) using a constant flow rate ratio. Between groups of injections the separation efficiency was varied in the range 33–97% by changing the back pressure. The areas measured were not affected by the changes in separation efficiency, the variation between groups was 1.6% RSD (n = 7). However, within the groups there was a clear trend towards less variation with increased separation efficiency. In another experiment two complete runs were made under the same conditions except that in one run above normal phase separation efficiency (95–98%) was used while in the other conditions were deliberately chosen to give a very low separation efficiency (25–26%). The results obtained for p-nitrophenol for chloroform–0.1 M phosphate (pH = 6.00), $D' = 1.18 \pm 0.07$ and 1.47 ± 0.10 show that there is not much effect on the measured D' values even with such a large difference in phase separation efficiency. This means that the slight variations in phase separation efficiency that will occur between days or during a day will have very little effect on the determinations.

Flow rates and phase flow ratios

The rate of extraction in liquid–liquid segmented flow was found by Nord *et al.* [14] to be very high. A short study was nevertheless undertaken in order to ascertain that equilibrium was established in our system. In this

case the conditions at equilibrium are sought which warrants a special study. The effect of the residence time in the extraction coil can be studied by changing the extraction coil length while the total flow rate is kept constant. In this case matters are complicated by the fact that different total flow rates are used within one run. Comparing results obtained for the same system using different coil lengths and tubing i.d.s will give an indication of lack of equilibrium, should it exist. As the measured quantity is the amount remaining in the phase of injection, one would expect insufficient extraction time to result in high D' values. The results of such an experiment are shown in Table 2. In this experiment, phenol in chloroform (0.05 g l^{-1}) was used as sample, organic flow rate was kept at 0.98 ml min^{-1} and the total flow rate was kept below 4.0 ml min^{-1}. From the results, it can be concluded that a residence time of about 2 s in a 0.35 mm i.d. tubing or 3 s in a 0.5 mm i.d. tubing is sufficient to reach the extraction equilibrium and that there is no difference in measured D' values between different extraction coil lengths or diameters.

Table 2
Effect of extraction coil residence time [t(R)] on partition measurement. Substance: phenol. System: chloroform–0.2 M phosphate, pH = 6.00. Q_{org} = 0.98 ml min^{-1}, total flow rate <4.0 ml min^{-1}

Extraction coil				
Dia. (mm)	Length (m)	Volume (ml)	t(R) (s)	D'
0.35	1	0.096	1.4–3.9	2.14
0.35	2	0.19	2.8–7.8	2.09
0.35	4	0.38	5.6–15.6	2.18
0.35	8	0.76	11.2–31.2	2.10
0.5	1	0.19	2.8–7.8	2.14
0.7	2	0.76	11.2–31.2	2.10
0.35*	1	0.096	1.2–2.6	2.25

*Q_{org} = 2.0 ml min^{-1}, total flow rate up to 5.0 ml min^{-1}.

Using the same chemical system, the distribution of phenol between chloroform and 0.1 M phosphate buffer pH = 6.60, measurements were repeated a number of times with different organic phase flow rates. The data obtained have been collected in Fig. 2 showing also the fitted straight lines. From the results it can be concluded that with organic flow rates in the range 0.27–2.0 ml min^{-1} and flow rate ratios in the range 0.1–11 (total flow rate <4.0 ml min^{-1}) consistent results can be obtained with this method. For several reasons, e.g. stability of segmentation and phase separation, it is advantageous to keep the total flow rate relatively low. This means that in order to reach a wide range of phase flow ratios, a low flow rate of the phase in which measurements are performed should be used. This is clearly illustrated in Fig. 2. However, as can be seen from the standard deviations given in this figure, less precise results are obtained using data covering too large a phase flow ratio range. This is due to increasing relative errors in the intercept with the more extensive extrapolation involved in experiments like run 4 in Fig. 2.

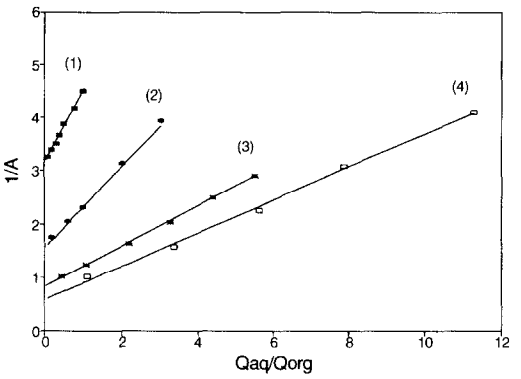

Figure 2
Measurements at different total flow rates: substance: phenol in chloroform (0.05 g l^{-1}); system: chloroform–0.1 M phosphate (pH = 6.60); extraction coil 2.0 m 0.35 mm i.d. (1) Q_{org} = 2.0 ml min^{-1}, Q_{tot} < 4.0 ml min^{-1}, D' = 2.29 ± 0.10; (2) Q_{org} = 0.98 ml min^{-1}, Q_{tot} < 4.0 ml min^{-1}, D' = 2.02 ± 0.07; (3) Q_{org} = 0.45 ml min^{-1}, Q_{tot} < 3.0 ml min^{-1}, D' = 2.21 ± 0.08; (4) Q_{org} = 0.266 ml min^{-1}, Q_{tot} < 2.3 ml min^{-1}, D' = 1.92 ± 0.28.

The need for a certain range of flow rate ratios in order to obtain a useful difference in signals together with the instability of the segmented flow at high flows or high flow rate ratios sets the limit for the D' values that can be determined with this technique. Attempts to determine D' values outside the range tested in this work, $0.2 < D' < 100$, are not recommended at the present stage.

Sample consumption

With readings taken in triplicate at five different flow rate ratios using 20 µl injections, the total amount of sample solution injected would be 300 µl. If the sample solution is to be pumped to the injector, a volume of about five times this will be needed. The concentration of sample in the injected solution varied in this

work from 0.17 g l^{-1} for phenol to 0.045 g l^{-1} for *p*-nitrophenol. Thus, the amount needed for a determination is in all cases tested below 1 mg. This value could be reduced even further for example by using manual injection.

Evaluation

In most cases during the course of this work each run has comprised a set of five to seven triplicate injections at different flow rate ratios giving plots of the type shown in Fig. 2. This is a way to increase precision, the higher the number of points used for the linear regression the lower the standard deviation of the result obtained. However, the cost of this is an increase in time, solvent and sample spent. Another problem can be seen in the results given in Table 3 for measurements of high D' values performed on samples in aqueous solution. In such cases, extrapolation of a steep line with limited variation in Q_{org}/Q_{aq} ratios gives rise to large errors in the intercept. The problem can be circumvented by making a measurement at $Q_{org} = 0$ giving a direct determination of the intercept.

In many cases, for example, when a set of substances is screened for their conditional partition constants, measurements at two different flow ratios for each substance would be enough. Results from such two point measurements are shown in Fig. 3.

The standard deviations given were obtained from the $s_{y/x}$ of the fitted straight line using an equation for the standard deviation of the extrapolated result of a standard addition [17], this extrapolation being equivalent to dividing the intercept with the slope.

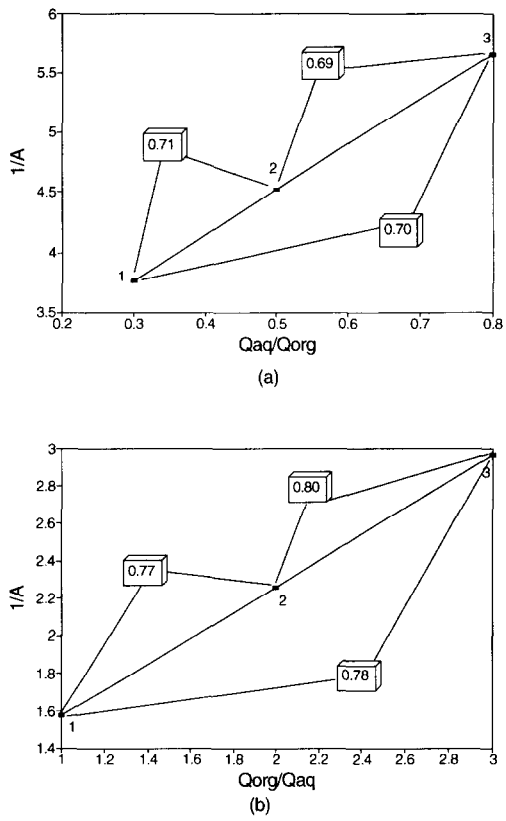

Figure 3
Two point evaluations of conditional partition constant. (a) Measurements on organic phase: *p*-hydroxybenzaldehyde in chloroform–0.2 M phosphate (pH = 5.40), $D' = 0.70 \pm 0.006$ (three points); (b) measurements on aqueous phase: *p*-hydroxybenzaldehyde in 1.0 N H_2SO_4–chloroform, $D' = 0.79 \pm 0.03$ (three points).

Table 3
Partition constants measured*

Substance	pH	Measured Org. Phase D'	SD‡	Measured Aq. Phase D'	SD‡	Literature† D'
p-Hydroxybenzaldehyde	0–6.00	0.73	0.01	0.79	0.02	0.76
p-Nitrophenol	5.00	1.69	0.03	1.32	0.18	1.86
Phenol	5.00–7.90	2.3	0.12	2.1	0.21	2.24
Caffeine	7.04	22	1	22	5.3	20.4
p-Toluidine	5.40	83	7	92§	3	97.7
	5.00	43	2.6	46	8.7	
Alprenolol	6.00	2.20	0.12	1.79	0.08	1.41‖
Propranolol	6.00	3.60	0.30	3.52	0.08	2.75‖
Metoprolol	6.00	0.21	0.02	0.30	0.02	0.21‖
	5.00	0.025	0.012			

* All measurements performed with 0.2 M phosphate in aqueous phase.
† From ref. 18.
‡ Calculated from regression according to equations (b) and (b*).
§ Value obtained utilizing measurements at 0 organic flow.
‖ Batch values.

Validation of system

Measurements of conditional partition constants of a series of organic compounds between chloroform and phosphate buffers have been made utilizing measurements on both the organic and the aqueous phase. The results are collected in Table 3 together with the literature data we were able to find. The agreement between the three sets is satisfactory.

Conclusions

A simple and rapid method for the determination of conditional partition constants has been developed. The method is equally useful for samples presented in organic or aqueous solution. An estimation of the time needed for a determination gives a time per substance of about 15 min. For screening purposes, when measurements at two different flow rate ratios for each substance is sufficient, this time can be reduced further. The sample consumption depends on the absorptivity of the substance but is, in most cases, less than 1 mg. Using this method D' values in the range 0.2–100 can be measured with a precision of 2.5% RSD for samples injected dissolved in organic solution. If aqueous samples are used the precision will be worse by a factor of two.

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