Pergamon

0731-7085(94)00107-3

Journal of Pharmaceutical & Biomedical Analysis, Vol. 12, No. 12, pp. 1475–1481, 1994

0731-7085/94 $7.00 + 0.00

Mechanized determination of *n*-octanol/water partition constants using liquid–liquid segmented flow extraction

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Abstract: A simple and rapid method for the determination of partition constants in the *n*-octanol/water system is presented. The substance under study is dissolved in *n*-octanol or water, and injected into a stream of the corresponding phase. Liquid–liquid segmented flow is utilized for equilibration of the two phases and after equilibration a fraction of the aqueous phase is separated with the aid of a hydrophilic membrane. The amount of substance in the aqueous phase is measured spectrophotometrically. The measurements are repeated at a small number of different phase flow ratios and the results obtained are plotted against these ratios. From slope and intercept for the regression line through these points the conditional partition constant, D', can be calculated. A simplified procedure using only two measurements, one of which is obtained with the flow rate of organic phase set to 0, can also be used. Partition constants in the range $0.48 < D < 873$ ($-0.32 < \log D < 2.94$) have been determined using this system. The precision, measured as relative standard deviation (RSD) is below 15%, corresponding to a standard deviation of ±0.1 log unit or less over the range of measurement. Measured values correspond to literature values within 0.07 log units. 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.

Keywords: *Partition constants; n-octanol/water; flow injection analysis; screening; distribution.*

Introduction

The partition of a substance in the system *n*-octanol/water has for a long time been extensively used when trying to assess the impact of a substance on biota [1]. The reason is that this partition is the best measure found for the ability for a substance to penetrate biological membranes. The partition, measured as the ratio of the concentrations of the substance in the two phases at equilibrium (D) has, therefore, been used in drug and pesticide design as a parameter for Quantitative Structure Activity Relationship (QSAR). It has also been used to predict bioaccumulation factors in aquatic organisms, water solubility and soil adsorption coefficients [2]. The link between biological activity and this D value has made it an essential tool in the development of pharmaceutical and agricultural chemicals. In many cases an accurate determination of D is required for the registration of such products [3]. The traditional method for the determination of D is the manual batch technique [4]. A small amount of the substance under investigation is partitioned between *n*-octanol and water in a separatory funnel by shaking and the concentrations in both phases are determined after separation. The range of D values that can be determined is set by the lowest concentration of the substance that can be reliably measured in either phase. This limitation can often be overcome for protolytes by determining D', the conditional partition constant, and correcting for the protolytic equilibria in the aqueous phase. A major drawback with this procedure is the large amount of manual labour involved. This problem is not serious if only a few samples are to be handled. However, for screening purposes, when a large number of substances are to be tested, the need for a more efficient technique is obvious.

One way to meet this demand has been to use high-performance liquid chromatography (HPLC) [5]. Besides ease of automation, this technique offers insensitivity to impurities in the sample and limited sample consumption. The measurement is based on the partition of the substance under investigation between the

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mobile aqueous phase and a stationary C8-phase immobilized on a silica support. A general difficulty with this approach is that the partitioning observed only mimics the true *n*-octanol/water partitioning. The results, thus, do not accurately reflect the *D* values sought [6]. One further disadvantage with this technique stems from the limited stability of the packing material at high and low pH. This limits the range of *D* values that can be measured by HPLC under isocratic conditions. The countermeasure is to add modifiers to the eluent but this leads to a further loss of accuracy.

Centrifugal partition chromatography (CPC), using *n*-octanol and water as the two phases, has been shown to be feasible for providing *n*-octanol/water partition coefficients [7–9]. Although it offers the advantages of possible automation, small sample size, and insensitivity to impurities of the HPLC procedures with the potential accuracy of the shakeflask method, some disadvantages still remain. For example, for screening purposes, the time consumption of the CPC procedure seems to be too large (run times vary from 25 min to 16 h). Problems with baseline shifts, wide peaks and low signal-to-noise ratios might also necessitate manual surveillance.

Over the years a number of different methods based on flow systems have been devised [10–13]. All these systems seem to work fairly well for solvents easily separated with the aid of hydrophobic membranes, e.g. chloroform, benzene. A largely unsuccessful attempt at applying this type of technique to the *n*-octanol/water system was presented by Gluck [14]. Unfortunately, *n*-octanol performs less well in phase separators due to its high viscosity and low surface tension towards water. This makes conventional phase separation difficult, as witnessed by Gluck.

In this paper, ways to circumvent this problem are presented. An FIA/extraction manifold for determining partition constants, *D*, in the *n*-octanol/water system has been designed. The substance to be tested is dissolved in either phase and subsequently injected into the flow of the corresponding phase. The two streams are segmented with each other and the substance is partitioned between the two phases during passage through an extraction coil. A fraction of the aqueous phase flow is separated and led to a spectrophotometer. The absorbance peak area (a measure of the amount) of the substance is measured. From results so obtained, at different phase flow ratios, a *D* value can be calculated. The primary aim of the work is to create a rapid and reliable method for screening purposes.

Experimental

Apparatus

A schematic diagram of the flow injection system used is shown in Fig. 1. The manifold is similar to those presented earlier [15]. The two HPLC pumps (HPLC 2150, Pharmacia, Sweden) were controlled by a programmable controller unit (LC 2152, Pharmacia, Sweden). In the present application, because of the difficulty in reliably separating a pure *n*-octanol phase, measurements were exclusively performed on the aqueous phase. The spectrophotometric detector (LC-55B, Perkin–Elmer) was, therefore, permanently connected to the path carrying the aqueous phase flowing from the separator. The signal from the detector was fed to an integrator (Spectra–Physics, SP 4270) and printed in the form of peak areas. Sample introduction into aqueous or organic phase could be made by moving the injector to the appropriate flow path. The volume injected was 25 μl.

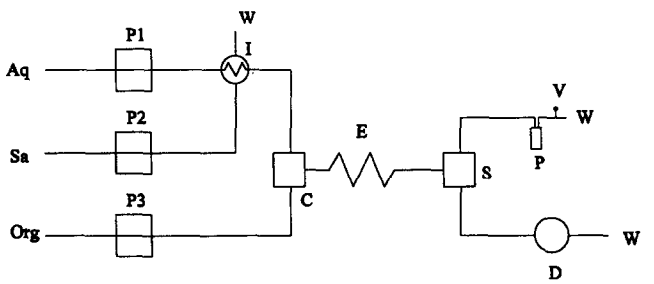

Figure 1
Manifold used for the determination of partition constants. HPLC pumps (P1) and (P3); peristaltic pump (P2); sample (Sa: S_{org} or S_{aq}); injector (I); segmentor (C); extraction coil (E); separator (S); detector (D); pressure bottle (P); check valve (V); waste (W).

Chemicals

All aqueous solutions were prepared using deionized water treated with a Milli-Q polishing system (Millipore). *n*-Octanol, buffer substances and pH adjusting chemicals were all of analytical grade. The two phases used in the flow system were pre-equilibrated with each other and degassed using suction prior to use.

Phase separation

Previous work by Gluck as well as our own experience has shown that it is not feasible to separate *n*-octanol from water by penetration through hydrophobic membranes. We have, therefore, chosen to separate a part of the aqueous phase with the aid of a hydrophilic membrane. A small number of slightly different types of membrane separators were tested and the only one giving satisfactory results was the one made of steel according to the design by Bäckström *et al.* [16, 17]. This separator has a cylindrical cavity for the incoming segmented flow with a volume of 78 μl and an active membrane area of 0.78 cm^2. Three different hydrophilic membranes were tested: a mixed cellulose nitrate/cellulose acetate membrane with 0.45-μm pore size and two membranes made from hydrophilized polyvinylidene difluoride (PVDF) with pore sizes 0.22 μm and 0.1 μm, respectively. The pressure needed to push water through the membrane was obtained with a pressure bottle supplied with an adjustable check valve. A low flow of nitrogen kept the bottle at the pressure set by the check valve. The valve was normally adjusted to give a pressure of below 1 bar in order to avoid pushing *n*-octanol into the hydrophilic membrane.

Procedure

The substances to be tested were dissolved in water (or an appropriate buffer solution) for aqueous phase introduction or in *n*-octanol for introduction into the organic phase. The sample injector was installed in the appropriate flow path, the pumps started and the flow of nitrogen to the pressure bottle initiated. After some time for stabilization, 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_{org}/Q_{aq}. The repetitive actions performed during data collection for one substance, such as injections and changes of phase flow ratios, could all be managed by the LC 2152 controller, thus reducing the need for manual labour even more. The results collected were then evaluated according to the following two equations for introduction of sample into aqueous or organic phase, respectively.

$$\frac{1}{A_{aq} \cdot Q_{aq}} = \frac{K}{M^*_{aq}} + \frac{K \cdot D'}{M^*_{aq}} \cdot \frac{Q_{org}}{Q_{aq}} \quad (1)$$

$$\frac{1}{A_{aq} \cdot Q_{aq}} = \frac{K}{M^*_{org}} + \frac{K \cdot D'}{M^*_{org}} \cdot \frac{Q_{org}}{Q_{aq}} \quad (2)$$

Where M^*_{aq} and M^*_{org} are the total amounts of substance injected into aqueous or organic phase, respectively. K is a constant including factors from Beer's law and also instrumental factors involved in the transformation from absorbance to peak area. A_{aq} is the integrated peak area for measurements on aqueous phase and Q_{aq} and Q_{org} are the flow rates of aqueous phase and organic phase, respectively. The flow rate ratio is changed by varying Q_{org} or Q_{aq}. D' is the conditional partition constant to be evaluated. In most cases the D value, the partition constant for the undissociated compound is sought. This value can be obtained either by performing the measurements under conditions where the compound is not dissociated or by calculation from D' and the acid dissociation constant of the compound. All results presented in this paper are given as D values. With one exception, salicylic acid in Table 4, the measurements were performed under such conditions that the substances were undissociated, hence D values were obtained directly. Further details on the method of evaluation can be found in [15].

Results and Discussion

Membrane phase separation

Although it is possible to separate *n*-octanol from water using the hydrophobic membranes conventionally used in FIA-extraction, this kind of separation cannot be used for practical measurements. The physical properties of *n*-octanol make the separation inefficient and unreliable. This was also found by other workers [14]. In order to circumvent this problem we chose to use hydrophilic membranes aiming at the separation of a pure aqueous phase free from droplets of organic phase.

Three different hydrophilic membranes have been tested. The membranes had different pore sizes but were also manufactured from different materials. The mixed cellulose esters in the 0.45-μm pore size membrane are intrinsically hydrophilic while PVDF is a hydrophobic material that has been given a hydrophilic surface. Good separation was obtainable with all three membranes. The main difference observed was that the smaller the pore size the higher the back pressure generated in the separator. Membranes with smaller pores seemed to be slightly more reliable than the 0.45-μm membrane.

The results obtained for a selection of substances using the three different hydrophilic membranes are shown in Table 1. There are no significant differences between the results obtained using different membranes. The results are all in good agreement with the literature values determined by the shake-flask method. Internal agreement between systems using different membranes and external with the results from shake-flask determinations indicate that the difficulties experienced by Gluck [14] and ascribed to adsorption on the membrane are not present here.

Some guidelines can be given for the attainment of successful operation of membrane phase separators in the *n*-octanol/water system based on experience gained during the preparation of this paper. The separating ability of hydrophilic membranes is easily destroyed by *n*-octanol. Once the first drop of *n*-octanol has been pushed into the membrane the selectivity of the transport through it is gone. Further, contrary to the situation with hydrophobic Teflon membranes, a hydrophilic membrane that has been wetted by the phase to be rejected cannot be easily restored to original condition. In order to safeguard against destroying the membrane, the separation efficiency should not be set to more than 30%. If a higher separation efficiency is attempted the risk of pushing *n*-octanol into the membrane increases dramatically. In a previous paper it has been shown that with the kind of evaluation method used the results will be affected only insignificantly by changes in separation efficiency [15]. With increasing total flow rates the residence time in the separator will decrease and, therefore, the separation efficiencies attainable with trouble free operation will decrease. Some typical values for flow rates and the corresponding phase separation efficiencies are given in Table 2. Another extraordinary feature with the present system, as compared to those previously used with solvents such as chloroform and tetrachloride, is the fact that the material used for

Table 1
Comparison of partition constants measured in *n*-octanol/water using different membranes for phase separation. The partition constants are given for the undissociated molecules

	Mixed cellulose 0.45 μm			PVDF 0.22 μm			PVDF 0.1 μm			
Substance	*D*	S. dev.	log *D*	*D*	S. dev.	log *D*	*D*	S. dev.	log *D*	Lit.* log *D*
Acetic acid†	0.51	0.02	−0.29	0.48	0.01	−0.32	0.58	0.03	−0.24	−0.31
Caffeine	0.80	0.07	−0.10	0.83	0.05	−0.08	0.79	0.01	−0.10	−0.07
p-Toluidine	26.7	4.1	1.43	27.4	0.9	1.44	24.0	0.10	1.38	1.41
2,5-Dinitrophenol†	55.4	2.1	1.74	61.4	1.2	1.79	63.1	4.1	1.80	1.75
Alprenolol†‡	—	—	—	2.27	0.06	0.36	2.12	0.24	0.33	—

*From [18].
†Measured at pH = 1.
‡1-(o-allylphenoxy)-3-(isopropylamino)-2-propanol.

Table 2
Maximal flow of *n*-octanol and phase separation efficiencies obtainable at given aqueous flow rates

Q_{aq} (ml min^{-1})	Q_{org} (ml min^{-1})	Q_{org}/Q_{aq}	Phase separation (%)
0.6	0.4	0.67	30
1.0	0.4	0.4	30
1.5	0.3	0.20	25
2.0	0.3	0.15	20

the phase separator seems to have a decisive effect on the separation. Two separators made from PVDF, one with larger membrane area and cavity volume, and one with smaller dimensions than the steel separator were tested but both failed to work. We believe that this behaviour is caused by a wall effect where the hydrophilic phase prefers to flow along a hydrophilic (steel) wall and on to the membrane. With PVDF the hydrophobic wall material tends to direct the *n*-octanol phase to the membrane leading to separation problems.

Two point evaluation

A normal evaluation was made by fitting a straight line to at least four points measured at different phase flow ratios as illustrated in Fig. 2. The D' value was obtained by dividing the slope of this line with its intercept. However, for substances with high partition constant (e.g. $D' > 200$) this kind of evaluation could not be used. Due to the limitations imposed by the segmentation and the phase separation the range of phase flow ratios is rather narrow. For substances with high D' values and with Q_{org}/Q_{aq} larger than 0.1 most of the substance will be in the organic phase at all phase flow ratios. Thus, very small differences in signal are to be expected between measurements at different ratios. When the difference approaches the random variation of the peak areas the measurements will be imprecise and finally meaningless. In order to extend the range of measurable D' values, a method of two point evaluation, including a measurement taken at $Q_{org} = 0$ (intercept point) for aqueous phase introduction of sample, was used. Figure 3 shows an example of two point evaluations of the partition constant for 1-naphthol between *n*-octanol and water. The results are given numerically in Table 3. The evaluation is straightforward. A direct measurement of the intercept is made at $Q_{org} = 0$ and one further measurement is taken at a suitable Q_{org}. The slope is calculated from the difference in $1/(A_{aq} \cdot Q_{aq})$ for the two points, divided by the flow rate of organic phase. The two determinations of D at different aqueous flow rates give closely similar results using this method of evaluation. It can also be seen from the figure that all four points fall along a line and thus the normal evaluation could be applied provided that the measurements of the intercept is included. In this case a D value of 910 would be obtained which is within the range of results obtained from a series of two point evaluations. Using only the two points measured with the organic phase flowing (a', b') does not give meaningful results, however. The points are closely placed and, therefore, the uncertainty of the evaluations of slope and intercept will be very large. The results from a group of substances with moderate to high partition constants are collected in Table 4.

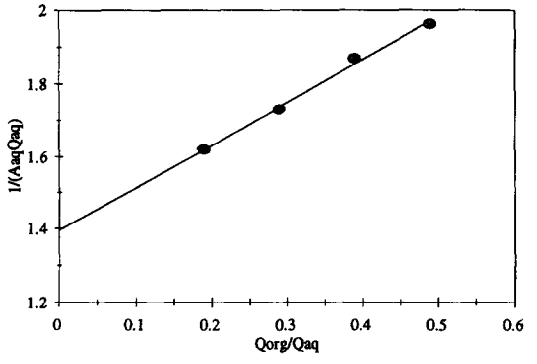

Figure 2
A normal evaluation line for determining partition constants in *n*-octanol/water. Sample: caffeine in water; D evaluated: 0.83 ± 0.05; conditions: measurement on water stream; Q_{aq} = 1.0 ml min^{-1}; membrane, PVDF 0.22 μm.

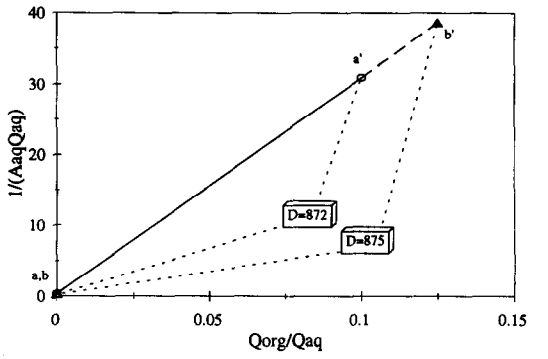

Figure 3
Two sets of two point evaluations for determining a partition constant in *n*-octanol/water system. Sample: 1-naphthol in water; conditions: membrane PVDF 0.22 μm, measurements on aqueous phase; point a, Q_{org}/Q_{aq} = 0/1.0; point a', Q_{org}/Q_{aq} = 0.1/1.0; point b, Q_{org}/Q_{aq} = 0/0.8; point b', Q_{org}/Q_{aq} = 0.1/0.8. Other conditions and the results are given in Table 3.

Table 3
Data for Fig. 3. Determination of partition constant for 1-naphthol between *n*-octanol and water

Point	Q_{org}/Q_{aq}	$1/A_{aq}$	$1/(A_{aq}Q_{aq})$
a	0/1.0	0.350	0.350
a'	0.1/1.0	30.8	30.8
b	0/0.8	0.280	0.350
b'	0.1/0.8	30.9	38.6

Table 4
Partition constants for *n*-octanol/water from two point evaluations; 0.22-μm PVDF membrane used for separation

Substance	Results				Lit.*
	D	S. dev.	No. of meas.	log *D*	log *D*
Oxine	91	2	17	1.96	1.96
p-Nitrophenol	96	5	4	1.98	1.91
Salicylic acid†	210	4	5	2.32	2.26
Ethyl benzoate	441	32	5	2.64	2.64
1-Naphthol	873	75	11	2.94	2.98

*From [18].
†Measured at the condition of pH = pK_a = 2.70, so $D = 2$. $D' = 210$.

The standard deviations given are estimated from repeated measurements based on two point evaluations. Again it can be seen that the results conform nicely to values found in the literature. This method of extending the range of measurable D' values does not complicate the measurements and is reasonably accurate and it is thus suitable for screening purposes.

Estimation of measurable range of D'

For high D' values the two point evaluation described above is used. The upper limit of measurable partition constants is determined by two factors. The noise in the peak area measurements and the minimal phase flow ratio, Q_{org}/Q_{aq}, that can be attained with maintained stability of the flow system.

High D' values result in low concentrations of substance in the aqueous phase after equilibration. In order to obtain a reasonable precision in the peak area (A_{aq}) measured, the weakest signal (absorbance) should not be lower than 0.010. At an aqueous flow rate of 2.0 ml min^{-1} this corresponds to an area of about 0.040 and, therefore, the largest value of $1/(A_{aq}Q_{aq})$ is 12.5. The absorbance signal obtained in a given system is of course also related to the total amount of substance introduced in each injection. Higher concentrations of the substance in the sample will give less steep slopes and lower intercepts (see equations 1 and 2). Limits will be set by the solubility of the substance in the medium of introduction and by the linearity of the detector at the high absorbance values encountered when $Q_{org} = 0$. Assuming linearity up to 1.2 we would obtain an area of about 5 for Q_{aq} = 2.0 ml min^{-1} and thus an intercept of 0.1. All together these considerations give a maximal measurable D' value of 2480, a reasonable value considering the results obtained for 1-naphthol.

The lower limit of measurable partition constants is set by the smallest measurable difference in peak areas at different phase flow ratios and by the maximal phase flow ratio, Q_{org}/Q_{aq}, that can be reliably used. Again the limited variability of the phase flow ratio sets limits that can be widened by the use of two point evaluations including a measurement at $Q_{org} = 0$. With low D' values most of the substance will be present in the aqueous phase at equilibrium and in order to observe a difference a large range of phase flow ratios is desired. The practical limit for reliable phase separation was found to be $Q_{org}/Q_{aq} = 0.67$ at Q_{aq} = 0.6 ml min^{-1}. The relative variation between peak area measurements is normally around 2%. If we set 4% as the lowest measurable difference between peak areas at different phase flow ratios, a lowest measurable D' value can be estimated to 0.06.

Conclusions

This method for the determination of partition constants in the *n*-octanol/water system gives results corresponding to those obtained with the shake-flask method. It is considerably faster, however, and uses less sample and manpower. The method is equally useful for sample introductions in both organic and aqueous phases while measurements are only performed on the aqueous phase. An estimation of time needed for each substance gives about 15 min and it can be reduced further by using the two point evaluation method for sample screening purpose. The sample consumption is, in most cases, less than 1 mg and the consumption of *n*-octanol less than 10 ml

per determination. Using this method, D' values over the estimated range of 0.06–2500 can be measured. Successful measurements have been performed in the range $0.48 < D < 873$ ($-0.32 < \log D < 2.94$). The values obtained correspond very well with literature values and the uncertainties are equal to or smaller than those obtained using counter current chromatography.

Acknowledgements — The authors are indebted to Folke Ingman, Lage Nord and Jörgen Vessman for discussions throughout this work. Financial support from ASTRA-Hässle AB, the Swedish Natural Science Research Council and the Magnus Bergvall Foundation is gratefully acknowledged.

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[*Received for review 18 March 1994; revised manscript received 9 June 1994*]