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EVALUATION OF OCTANOL–WATER PARTITION COEFFICIENTS BY USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The use of high-performance liquid chromatography (HPLC) for the measurement of octanol–water partition coefficients is demonstrated. Solutes are equilibrated between the two phases by using the conventional "shake flask" approach. An internal reference having a known partition coefficient, which is similar to that of the substance of interest, is added to the system. Samples of both phases are chromatographed by HPLC. The area ratios for the solute and internal reference in both phases are measured and used together with the known partition coefficient of the internal reference to calculate the partition coefficient of the solute in question. The results obtained with a variety of substances having partition coefficients in the range from 10^{-3} to 10^{4} showed excellent agreement with literature data. The technique is rapid and has the advantages that small samples suffice, the substances need not be pure and the exact volume of the phases need not be known. Furthermore, it could readily be developed into a micromethod for measurements with submicrogram quantities.

INTRODUCTION

Partition coefficients in octanol–water systems are frequently used for characterization of hydrophobic properties of drugs. They play a particularly useful role in quantifying substituent effects for linear free energy relationships (LFER) which form the basis of quantitative structure–activity relationships (QSAR) employed widely in drug design. The use of partition coefficients in this field has recently been reviewed by Hansch and Leo[1].

The measurement of partition coefficients is traditionally carried out by the "shake-flask" method which is encumbered by the difficulties in measuring solute concentration in both phases accurately[2–4]. Whereas the use of gas chromatography has greatly facilitated such measurements, the method is confined to volatile solutes and only a few drugs fall into this category.

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In order to overcome technical difficulties in directly measuring octanol–water partition coefficients of complex drug molecules, therefore, a great many attempts were made to correlate chromatographic retention, which is easily measured in thin-layer or paper chromatography, with partition coefficients as well as with biological activity[5]. Upon the advent of high-performance liquid chromatography (HPLC), a particularly convenient and versatile chromatographic technique of high precision, such efforts have gained a great deal of momentum. In all studies reversed-phase chromatography has been employed with or without octanol deposited on alkyl-silica column packing[4,6–14]. The investigation was in all cases aimed at the formulation of a correlation between κ, the logarithm of the retention factor, k, and the logarithm of the octanol–water partition coefficient, P, for various drugs. Computation of log P from appropriate substituent increments, π, may be beset with considerable error in the case of complex molecules despite the extensive library of π values available[1].

We want to report that even if retention values obtained in HPLC may not serve as surrogate partition coefficients, HPLC is eminently suitable as an analytical tool for the measurement of solute concentrations when using the shake-flask method. Thus, the use of HPLC does disencumber and make attractive the traditional direct approach to evaluate octanol–water partition coefficients.

EXPERIMENTAL

Materials

Octadecyl-trimethylammonium bromide was purchased from Tridom Chemicals (Hauppauge, N.Y., U.S.A.); caffeine, hypoxanthine, 8-azaadenine, xanthine, thiouracil, 8-azahypoxanthine and deoxyadenosine from ICN Pharmaceuticals (Cleveland, Ohio, U.S.A.); adenosine and inosine from Sigma (St. Louis, Mo., U.S.A.); uric acid from Aldrich (Milwaukee, Wisc., U.S.A.); and toluene, phenol, resorcinol, hydroquinone, quinone, nitrobenzene, propylbenzene, *tert.*-butylbenzene, anisole and acetophenone from Chem Service (West Chester, Pa., U.S.A.). Methotrexate, N-{*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl}glutamic acid (MTX), was obtained from National Cancer Institute, N.I.H. (Bethesda, Md., U.S.A.). The antineoplastic drugs 2,4-diamino-5-methyl-6-[(3,4,5-trimethoxyanilino)methyl] quinazoline (JB-11, NCS 249008), 2,4-diamino-6-[(3,4,5-trimethoxyanilino)methyl] quinazoline (JB-7, NCS 250422) and α-[2-chloro-4-(4,6-diamino-2,2-dimethyl-*s*-triazine-1(2H)-yl)-phenoxy]-N,N-dimethyl-*m*-toluamide compounded (1:1) with ethanesulfonic acid (TZT, NCS 135105) were a gift of J. R. Bertino (Yale Medical School, New Haven, Conn., U.S.A.). Acetonitrile was "distilled in glass" from Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.) and distilled water was prepared with a Barnstead distilling unit. All other reagents were analytical grade.

A 10-μm Partisil ODS (250 × 4.6 mm) (Whatman, Clifton, N.J., U.S.A.), a 5-μm Supelcolsil LC-18 (150 × 4.6 mm) (Supelco, Bellefonte, Pa., U.S.A.) and a home packed 5-μm Hypersil ODS (150 × 4.6 mm) were used throughout the partition coefficient measurements.

Methods

Shake-flask experiments. The solute and an internal reference having a known log P value were partitioned between *n*-octanol and water in 35-ml glass flasks

equipped with tapered glass stoppers (Fischer, Pittsburgh, Pa., U.S.A.). Distilled water, or with drugs having ionizable groups, 50 m*M* phosphate buffer of pH 7.00, 2.12 or 2.20 was the aqueous phase. The total liquid volume was 30 ml and the volume ratio of *n*-octanol to water was 1:5, 1:1 or 5:1 for the measurement of partition coefficients ranging from 10^{-2} to 10^{4}, respectively. First, *n*-octanol and water were equilibrated, then the solute was dissolved in either water or *n*-octanol to obtain a concentration of 10^{-3}–10^{-4} *M*. Subsequently, the other phase was introduced into the flask which was thereafter inverted 100 times in about 5 min according to Leo *et al.*[19] at ambient temperature ranging from 22 to 25°. The temperature of the shake-flask was maintained at 37° in the partition coefficient measurement of uric acid with perfusion fluid[15] as the aqueous phase using an Eberbach water-bath shaker (Ann Arbor, Mich., U.S.A.). Samples from the upper layer of *n*-octanol were withdrawn directly by a syringe and injected into the column. Before drawing aqueous samples the bottom layer was first isolated from *n*-octanol by using a Pasteur pipette. Then, about 0.5 ml of the aqueous phase was transferred into a test tube from which samples were withdrawn by a syringe for injection into the column.

Chromatography. The liquid chromatograph was assembled from a Model 100 (Altex, Berkeley, Calif., U.S.A.) reciprocating pump, a Model 7010 sample injector (Rheodyne, Berkeley, Calif., U.S.A.) equipped with a 20-μl injection loop, a Model 770 (Kratos-Schoeffel, Westwood, N.J., U.S.A.) variable wavelength UV detector set at 210 nm, and a Model 56 (Perkin-Elmer, Norwalk, Conn., U.S.A.) dual pen recorder with 10 mV input. The linearity of the detector amplifier was checked using a Model 177 digital voltmeter (Keithley, Cleveland, Ohio, U.S.A.). The output signal voltage deviated less than 5% from linearity between the full-scale absorbance settings of 0.01 and 2.00, respectively. The linearity of the detector response to concentration of eluite at 210 nm was determined by the "expodil" method of Lovelock[16]. A Nupro No. 1SA metering valve (Norwalk Valve & Fitting Co., Shelton, Conn., U.S.A.) replaced the injection valve and the column in the liquid chromatograph to give a backpressure of 750 p.s.i. at a flow-rate of 2 ml/min in the calibration experiment. A $5 \cdot 10^{-3}$ *M* solution of MTX in distilled water was prepared and 60 ml were placed into a well stirred reservoir. While the contents of the reservoir were pumped out by the Model 100 Altex pump at a flow-rate of 2 ml/min, the volume of the liquid in the reservoir was maintained constant by introducing distilled water hydrostatically. The absorbance of the liquid pumped through the liquid chromatograph was monitored by the detector and recorded. Fig. 1 illustrates the detector response as a function of time. Since the concentration of MTX in the constant volume reservoir decays exponentially, and in the concentration domain employed Beer's law holds, plots of log *A vs.* time (min) are linear with slope $-F/V$, where A, F and V are the absorbance, the flow-rate (ml/min) and the volume of the solution (ml) in the reservoir, respectively. This relationship is shown in Fig. 1 and we find that for MTX the detector response is linear at concentrations up to $5 \cdot 10^{-3}$ *M*. The slope of the line on the semilogarithmic plot $-F/V = -1/29.5$ is in agreement with the calculated value, $-F/V = -1/30$.

Eluents were made from acetonitrile and 50 m*M* phosphate buffer, pH 7.00 or 2.12. The eluent used in the analysis of TZT contained 10 m*M* octadecyltrimethylammonium bromide. Sample volumes ranged from 1 to 20 μl; the injection volumes of samples from the *n*-octanol phase were kept low in order to avoid deposition of

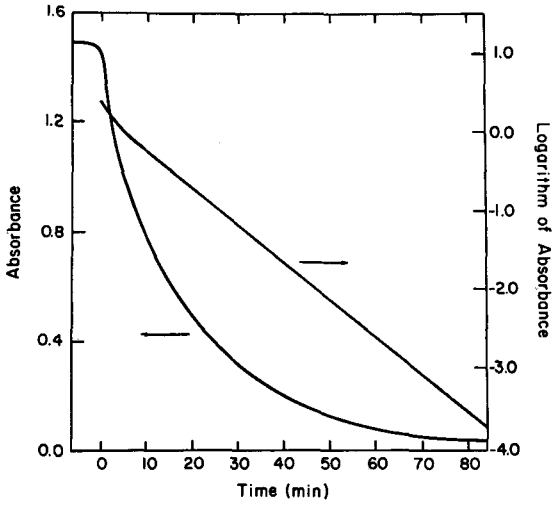

Fig. 1. Graph illustrating the results of the expodil experiment with $5 \cdot 10^{-3}$ *M* MTX solution for testing detector linearity. Both the absorbance of the effluent as monitored by the detector at 210 nm and its logarithm are plotted against time.

excess *n*-octanol in the column. Even so, after four injections of samples from the *n*-octanol phase the column was washed with acetonitrile and subsequently re-equilibrated with the mobile phase.

Data evaluation

Peak areas from the chromatogram were evaluated by triangulation[17]. The partition coefficient of the solute of interest, P_S, was calculated from the relationship

$$P_S = P_R \frac{(A_{S,o}/A_{R,o})}{(A_{S,w}/A_{R,w})} \quad (1)$$

where P_R is the *n*-octanol–water partition coefficient of the internal reference; $A_{S,o}$ and $A_{S,w}$ are the peak areas of the solute from the *n*-octanol and aqueous phases, and $A_{R,0}$ and $A_{R,w}$ are the respective peak areas of the internal reference.

RESULTS AND DISCUSSION

Experimental conditions as well as the partition coefficients measured by HPLC together with the corresponding literature values are listed in Table I. Log *P* values of some antineoplastic drugs, which have been evaluated for the first time, are also given in Table I. For drugs with ionizable groups, the log *P* values were evaluated by using aqueous phases of different pH. The precision of partition coefficient measurements determined successively by using the same internal reference was better than 3%. Comparison of the data in Table I shows an excellent agreement between log *P* values obtained in the range from −2.92 to 4.11 by using HPLC and those reported in the literature when in both cases the same aqueous phase was employed. Certain log *P* values have also been measured by using aqueous phases different from those described in the literature and are listed in Table I.

TABLE I

COMPARISON OF LOG *P* VALUES MEASURED BY HPLC TO THOSE GIVEN IN THE LITERATURE FOR VARIOUS SUBSTANCES

The aqueous phase was neat water except where indicated and the temperature for the shake-flask experiment ranged from 22 to 25°. The name and log *P* value of the internal reference used in the measurements of the partition coefficients by HPLC are stated on the same line as our experimental log *P* value.

Solute of interest			*Internal reference*	
Name	*Log P*		*Name*	*Log P from literature**
	HPLC	*Literature**		
tert.-Butylbenzene	4.07	4.11	Anisole	2.08
Propylbenzene	3.44	3.62	Toluene	2.58
Toluene	2.68	2.58	Nitrobenzene	1.84
Acetophenone	1.58	1.66	*tert.*-Butylbenzene	4.11
Resorcinol	0.88	0.80	Nitrobenzene	1.84
Hydroquinone	0.54	0.56	Nitrobenzene	1.84
Caffeine	−0.05	−0.07	Adenosine	−1.23
Deoxyadenosine	−0.76	–	Inosine	−1.81**
Hypoxanthine	−0.96	−1.11***	Resorcinol	0.88
8-Azaadenine	−0.96	–	Adenosine	−1.23
Riboflavin	−1.46	–	Adenosine	−1.23
Inosine	−1.81	−2.08***	Adenosine	−1.23
8-Azahypoxanthine	−1.97	–	Inosine	−1.81**
Uric Acid	−2.17	–	Adenosine	−1.23
	−2.90***	−2.92***	Inosine	−2.08***
JB-7†††	1.96§	–	Acetophenone	1.66
	0.39§§	–	Acetophenone	1.66
JB-11†††	2.55§	–	Acetophenone	1.66
	0.90§§	–	Phenol	1.47
	−2.43§§§	–	Phenol	1.47
TZT†††	−0.57§§	–	Nitrobenzene	1.84
	−1.84§§§	–	Nitrobenzene	1.84
MTX†††	−2.58†	−1.85††	Phenol	1.47
	−2.54†		Resorcinol	0.80
	−2.65†		Quinone	0.20

* From the recent compilation by Hansch and Leo[1] unless otherwise indicated.
** From this work.
*** Perfusion fluid, pH 7.4, 37° (ref. 15).
§ Aqueous phase was 50 m*M* phosphate buffer, pH 13.00.
§§ Aqueous phase was 50 m*M* phosphate buffer, pH 7.00.
§§§ Aqueous phase was 50 m*M* phosphate buffer, pH 2.12.
† Aqueous phase was 50 m*M* phosphate buffer, pH 2.20.
†† pH 2.20 (ref. 1).
††† The chemical structure of these anticancer drugs is given in the *Materials* section.

Selection of an internal reference

The accuracy of the method greatly depends on choosing the proper internal reference. The substance should meet the following requirements: (i) its partition coefficient should be known accurately; (ii) it should have a high absorbance at the same wavelength where the elution of the substance under investigation is monitored to give an appropriate peak with photometric detectors even at low concentrations;

(iii) its chromatographic retention should be similar to that of the substance investigated, yet it should be completely resolved from the solute of interest and its contaminants; (iv) it should not contain ionogenic groups so that its partition coefficient is independent of the pH of the aqueous phase; (v) it should not be subject to secondary chemical equilibria which would result in concentration dependent partition coefficients, and (vi) it should be readily available in pure form, or at least it should not contain interfering impurities.

The reliability of the partition coefficients can be enhanced by carrying out measurements with more than one internal reference either in a single partition experiment or in subsequent equilibrations as exemplified by the evaluation of log P for the antineoplatic drug methotrexate.

Effect of impurities

The partition coefficient of MTX was measured by using 50 mM phosphate buffer of pH 2.20 as the aqueous phase and three internal references quinone, phenol and resorcinol. The respective log P values, -2.65, -2.58 and -2.54, closely agree with each each other, yet they significantly differ from the value -1.85 reported in the literature[1]. It should be noted, however, that the value is referred to as "unpublished result" and we had no opportunity to find out how the measurement was made. If a spectrophotometric method was used to measure the partition coefficient of commercial MTX, the impurities present in the sample can easily give misleading results. In our method the impurities were completely separated from MTX and the internal reference. For instance, using quinone as the internal reference the impurities were separated from MTX and quinone as shown in the chromatogram of the aqueous phase sample in Fig. 2. Many of the complex drugs are not available in pure form. In contradistinction to spectroscopic techniques, however, the use of HPLC has the advantage that the impurities can be separated and therefore they do not interfere with the evaluation of the partition coefficient as demonstrated in Fig. 2.

Computation of partition coefficients

With the use of appropriate hydrophobic substituent or fragmental constants[1,18] partition coefficients can also be calculated. Despite the large amount of data accumulated in literature, however, such calculations are not yet accurate enough to predict the log P values of complex molecules. Most satisfactory results are obtained by using the additivity rule so that log P is calculated as the sum of the log P value of the "parent" molecule and the π values of the substituents[1]. It is very important, however that special interactions between different groups are taken into account by correct choice of the "parent" molecule. On the other hand, for most "parent" compounds of complex molecular structure log P values are not available in the literature. For similar reasons the fragment method also falls short of predicting accurately log P values of complicated molecules. Consequently there is a definite need to measure experimentally log P values of such substances. In fact, the success of both approaches to calculate with sufficient accuracy log P values of drugs having complex molecular architecture depends on our capability of greatly expanding the present data base concerning the partion coefficients of such compounds. HPLC offers a particularly suitable means for such an endeavor.

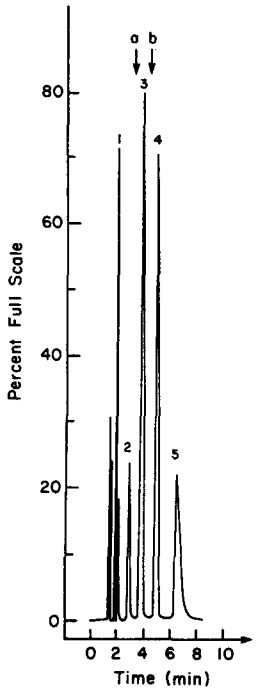

Fig. 2. Typical chromatogram obtained with the aqueous phase of the shake-flask experiment for the determination of the octanol–water partition coefficient of methotrexate with quinone as the internal reference. Peaks: 1 = solvent front; 2 = quinone impurity; 3 = MTX; 4 = quinone; 5 = MTX impurity. The column effluent was monitored at 210 nm at 0.10 a.u.f.s. except between arrows a and b where the sensitivity was 1.00 a.u.f.s. The mobile phase was 10% (v/v) acetonitrile and 90% (v/v) 0.1 *M* phosphate buffer, pH 7.00, and the flow-rate was 1.50 ml/min. The column was 5-μm Supelcosil LC-18 (150 × 4.6 mm) at ambient temperature.

CONCLUSIONS

An advantage of using HPLC for partition coefficient measurements is the small sample size and sample volume required. Due to the high precision of the HPLC equipment available today the present method can be used to obtain reproducible and accurate results rapidly and very conveniently. Moreover, the solute of interest and the internal reference do not have to be pure as chromatographic conditions can be adjusted to separate impurities from the two eluites of interest. Furthermore, the introduction of an internal reference and an analysis of both phases eliminates the need to know the exact volume of each phase, the extinction coefficients of the solutes at the wavelength they are monitored and the size of the injection volume of the sample. If solute adsorption on the walls of the flask[19] causes no error, the method is not only very simple but is eminently suitable for microscale operation.

To extend the calculation methods of log *P* values to complex drug molecules the partition coefficients of numerous compounds which may not be available in pure form and large quantities must be determined. Therefore, there is a need for an expedient method to measure their partition coefficients. We have found HPLC to be a very useful analytical tool for this purpose. The excellent agreement between log *P*

values reported in literature and measured by HPLC as shown in Table I indeed evinces that the method can be used accurately to measure partition coefficients of substances having a broad range of chemical properties. Furthermore the approach can be developed into a micromethod for the evaluation of log P values by using submicrogram quantities. For instance, the partitioning between octanol and water could be carried out in a microsyringe.

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