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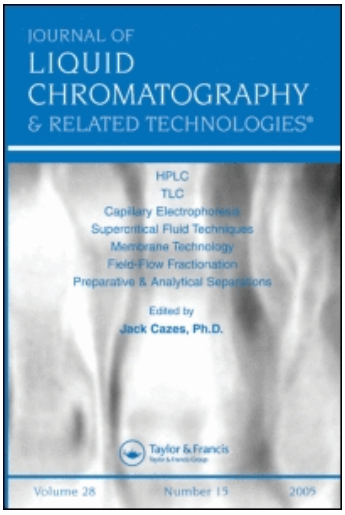

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A Rapid Microscale Method for the Determination of Partition Coefficients by HPLC

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A RAPID MICROSCALE METHOD FOR THE DETERMINATION OF PARTITION COEFFICIENTS BY HPLC

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ABSTRACT

A rapid, reliable and simple microscale method for the determination of octanol-water partition coefficients has been developed and evaluated. Rapid solute partitioning and facile octanol-water phase separation is accomplished in a commercially available mixer-separator device. The relative concentration of solute in each phase is then measured directly by computer-based reverse phase HPLC. The procedure requires only 10 µg of sample, which need not be pure, and uses 1 ml or less of both *n*-octanol and pH 7.0 phosphate buffer. Log P values of 26 compounds, mainly nucleoside analogues with anti-HIV and antitumor activity, have been determined in the range 0.7 to -2.4 with a precision better than ±0.04 log units. For compounds with literature data available, measured log P values are in good agreement (better than ±0.2 log units) with those values obtained by traditional "shake-flask" methodology. The described method is applicable to both single compound analysis and simultaneous multiple compound determinations through use of isocratic or gradient HPLC techniques.

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INTRODUCTION

The octanol-water partition coefficient (P) is widely used to define the lipophilic character of a compound [1]. This physical parameter, which expresses the distribution of a compound between an aqueous phase and immiscible organic solvent, often correlates well with certain pharmacological properties (*e.g.* absorption, tissue distribution, toxicity, activity) and has been used as a parameter in quantitative structure activity relationship (QSAR) studies [2]. However, the shake-flask technique that is traditionally used to measure the partition coefficient is time-consuming and prone to errors and experimental problems [3]. This has motivated the investigation of alternative experimental and theoretical approaches to estimate this important parameter.

Among the chromatographic methods that have been employed for this purpose, reverse phase HPLC has been used the most frequently and the most successfully [4-9]. Good correlations have been found between the chromatographic retention data (log k') and the measured log P within groups of structurally similar compounds [4-6,8,9]. However, problems arise with new compound classes where structurally similar standards are not available to calibrate the correlation [10]. Similar limitations are also inherent in the use of certain theoretical methods. Estimates of log P utilizing the additive properties of the Hansch π substituents require that the log P of the parent structure be accurately known for optimum extrapolation [11] or that the interactions of the various substituents be predictable [12].

The development of novel chemotherapeutics agents often precludes the availability of any structurally similar analogues that are necessary to establish a predictive correlation by the above methods. Furthermore, these new compounds may initially be available in only very small amounts, but the timely evaluation of their potential for central nervous system (CNS) penetration may be of crucial importance to guide continued development or the synthesis of new congeners. Because of these concerns and our own

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laboratory's interest in the development of CNS-active agents for cancer and AIDS [13-15], we have focussed on improving the reliability, sensitivity and timeliness of the conventional shake-flask technique. This report describes a rapid, reliable and simple microscale method for measuring log P. This method combines the advantages of HPLC for compound characterization and quantitation with the low sample requirements and speed of a microscale partitioning apparatus.

MATERIALS

The structure and abbreviation of each compound evaluated in partitioning experiments are depicted in Figures 1 and 2. N^4-benzoyl-2'-deoxycytidine (**1**), N^4-benzoylcytidine (**2**), N^6-benzoyl-2'-deoxyadenosine (**3**), 3'-azido-3'-deoxythymidine (**4**), N^6-benzoyl-adenosine (**6**), 2'-deoxyadenosine (**11**), thymidine (**15**), 2'-deoxy-inosine (**20**), 1-(β-D-arabinofuranosyl)cytosine (**23**), inosine (**24**) and cytidine (**25**) were purchased from Sigma Chemical Company (St. Louis, MO). Adenosine (**14**), 2'-deoxycytidine (**21**), uridine (**22**) and caffeine (**5**) were obtained from Aldrich Chemical Co (Milwaukee, WI). 3'-Deoxythymidine (**10**) was purchased from Calibiochem (San Diego, CA). 2',3'-Dideoxyadenosine (**8**), 2',3'-didehydro-2',3'-dideoxy-adenosine (**9**), 3'-azido-2',3'-dideoxycytidine (**12**), 2',3'-dideoxy-inosine (**17**), 2',3'-dideoxycytidine (**18**), 2',3'-didehydro-2',3'-dideoxycytidine (**19**) and cyclopentenyl cytosine (CPE-C, **26**) were supplied by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, NCI (Bethesda, MD). 9-(2,3-Dideoxy-2-fluoro-β-D-*threo*-pentofuranosyl)adenine (**7**) and 9-(2,3-dideoxy-2-fluoro-β-D-*threo*-pentofuranosyl)hypoxanthine (**16**) were kindly supplied by Dr. Victor E. Marquez of this laboratory [13], while 2',3'-dideoxy-uridine (**13**) was synthesized by chemical deamination of **18** with aqueous sodium bisulfite [16]. All nucleosides and nucleoside analogues were used without further purification, although each was subjected to HPLC analysis (*vide infra*) before study.

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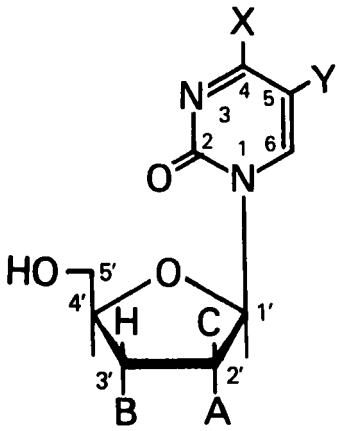

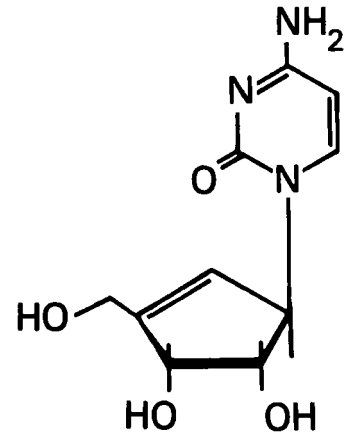

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1	A = H,	B = OH,	C = H,	X = $NHCOC_6H_5$,	Y = H	N^4-Bz-2'-dC
2	A = OH,	B = OH,	C = H,	X = $NHCOC_6H_5$,	Y = H	N^4-Bz-C
4	A = H,	B = N_3,	C = H,	X = OH,	Y = CH_3	AZT
10	A = H,	B = H,	C = H,	X = OH,	Y = CH_3	dT
12	A = H,	B = N_3,	C = H,	X = NH_2	Y = H	3'-N_3-ddC
13	A = H,	B = H,	C = H,	X = OH,	Y = H	ddU
15	A = H,	B = OH,	C = H,	X = OH,	Y = CH_3	Thymidine
18	A = H,	B = H,	C = H,	X = NH_2,	Y = H	ddC
19	A & B = C=C,		C = H,	X = NH_2,	Y = H	ddCytidinene
21	A = H,	B = OH,	C = H,	X = NH_2,	Y = H	2'-dC
22	A = OH,	B = OH,	C = H,	X = OH,	Y = H	Uridine
23	A = H,	B = OH,	C = OH,	X = NH_2	Y = H	Ara-C
25	A = OH,	B = OH,	C = H,	X = NH_2,	Y = H	Cytidine
26						CPE-C

FIGURE 1. Structures of pyrimidine nucleosides and cyclopentenyl cytosine (**26**).

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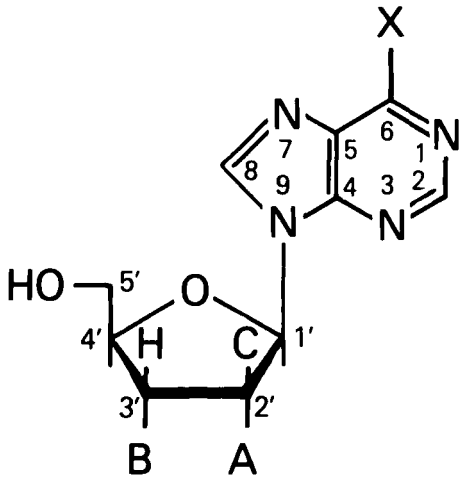

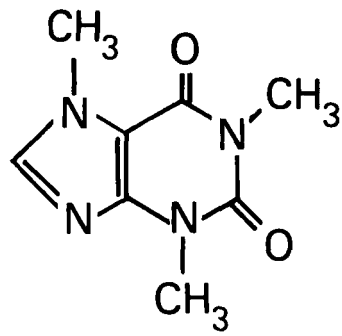

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3	A = H,	B = OH,	C = H,	X = $NHCOC_6H_5$	N^6-Bz-2'-dA
6	A = OH,	B = OH,	C = H,	X = $NHCOC_6H_5$	N^6-Bz-A
7	A = H,	B = H,	C = F,	X = NH_2	2'-F-dd-ara-A
8	A = H,	B = H,	C = H,	X = NH_2	ddA
9	A & B = C=C,		C = H,	X = NH_2	dd-Adenosinene
11	A = H,	B = OH,	C = H,	X = NH_2	2'-dA
14	A = OH,	B = OH,	C = H,	X = NH_2	Adenosine
16	A = H,	B = H,	C = F,	X = OH	2'-F-dd-ara-I
17	A = H,	B = H,	C = H,	X = OH	ddI
20	A = H,	B = OH,	C = H,	X = OH	2'-dI
24	A = OH,	B = OH,	C = H,	X = OH	Inosine
5					Caffeine

FIGURE 2. Structures of purine nucleosides and caffeine (**5**).

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HPLC-grade *n*-octanol and anhydrous dimethyl sulfoxide (DMSO) were purchased from Aldrich, while HPLC-grade acetonitrile, HPLC-grade water and sodium hydroxide were obtained from Fisher Chemical Co. (Fairlawn, NJ). HPLC mobile phases were prepared on a daily basis and mobile phase components were degassed before use or mixing by vacuum filtration through 0.22 μm Fluoropore (organic) or nylon (aqueous) membranes. All other chemical reagents were of the highest grade commercially available.

METHODS

Micro Shake-Flask Experiments. Partitioning experiments were performed according to the outline shown in Scheme I. Each compound (0.5 mg) was dissolved in 1.0 ml DMSO. A 20 μl aliquot of this DMSO solution was dissolved in 1.0 ml octanol-saturated, pH 7.0, 0.01M potassium phosphate buffer. This aqueous phase was then mixed thoroughly with 1.0 ml buffer saturated n-octanol by 30 piston strokes of a 2-ml Mixxor apparatus (Genex Corp., Gaithersburg, MD, Figure 3) at 24-26°C. After standing for 15 min, the octanol and buffer phases were separated and individually transferred to 1.5-ml Eppendorf microcentrifuge tubes which were centrifuged using a Personal Centrifuge (Millipore, Bedford, MA) at 2000 x g for 5 min. The relative concentration of sample in each phase was then determined by HPLC analysis (see below). For very water soluble compounds with log P values $\leq$ -2.0, 0.5 mg of sample was dissolved in only 100 or 200 μl DMSO. Partitioning was then carried out as above, except the buffer phase was diluted 10-fold before HPLC analysis. Three independent partitioning experiments were performed for each sample.

HPLC Analysis. A Model 204W liquid chromatography system (Waters Associates, Milford, MA) consisting of a U6K injector, a Model 6000A solvent delivery system and a LC-235 diode array detector (Perkin-Elmer, Norwalk, CT) set to the wavelength maxima of the nucleoside under investigation was used for isocratic sample analysis. A Model

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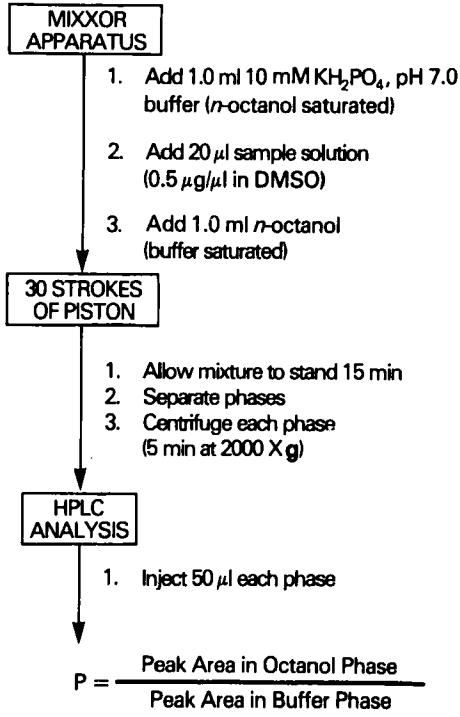
Micro Shake-Flask Method



Scheme I

324 liquid chromatograph (Altex/Beckman, Berkeley, CA) composed of a Model 210 sample injection valve, two Model 100A pumps, a high pressure dual chamber mixer, a Model 421 system controller and a Model 994 programmable photodiode array detector (Waters Associates) was employed for mixture analysis with gradient elution. All separations were carried out on a 4.6 X 250 mm 5-μm Ultrasphere-ODS column (Altex/Beckman). Isocratic separations employed a mobile phase of 0.5 - 20% CH_3CN in 0.01 M pH, 7.0 phosphate buffer at a flow rate of 1.0 ml/min. The percentage of acetonitrile was varied

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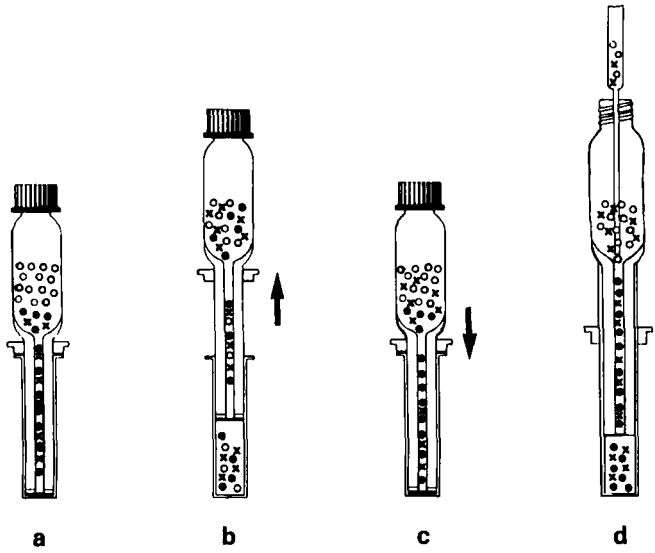

FIGURE 3. Micro shake-flask partitioning with a Mixxor apparatus. Key: *n*-Octanol (O), pH 7 buffer (●), analyte (x). a) Before partitioning with phases still separated and analyte in buffer phase. b) During partitioning with piston extended to mix upper phase with lower phase. c) During partitioning with piston compressed to force components through axial channel to mix in top chamber d) Analyte partitioned between both immiscible phases with piston raised so that phase interface is at the top of the piston capillary to facilitate removal of samples for HPLC analysis.

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to achieve a retention time of 4-8 min for each nucleoside. Gradient separations used 0.01 M, pH 7.0 phosphate buffer as one mobile phase (A) and CH_3CN as the other (B) in a two-step gradient at a flow rate of 1.0 ml/min. Initial conditions of 10% B for 0.5 min were followed by a 2 min linear gradient to 20% B; then after 18 min at these conditions, a 2 min linear gradient to 60% B with a 10 min hold was employed to wash the column. Initial conditions were restored with a 5 min linear reverse gradient to 10% B and a 5 min equilibration period. A 50 µl aliquot of each phase from individual partitioning experiments was injected once. To avoid peak

distortion following cumulative *n*-octanol injections during isocratic analysis, the column was washed with 60% $CH_3CN:H_2O$ to restore an optimum peak elution profile after every three octanol injections. Peak area measurements were made using a SP-4200 computing integrator (Spectra-Physics, Santa Clara, CA) which was interfaced to a WINner/386 chromatography data system (Compaq 386 version, Spectra-Physics) for data reduction and storage. The partition coefficient (P) was calculated by dividing the absolute area of the appropriate integrated peak from the octanol phase by that of the buffer phase. The logarithm of P ($\log_{10}$ P) from each independent determination was used to determine the mean.

RESULTS

The isocratic HPLC conditions used to separate and measure the compounds studied are listed in Table 1. Buffer and *n*-octanol solutions of all nucleosides and nucleoside analogues could be analyzed using a standard C_{18} reverse phase column. Nucleoside retention was adjusted by addition of 1-20% CH_3CN organic modifier to the 0.01 M, pH 7.0 phosphate buffer mobile phase. UV detection at each compound's λ_{max} afforded sufficient sensitivity to measure directly greater than a 1000-fold relative concentration difference in either buffer or *n*-octanol. Detector response, as calculated by absolute area measurement of the appropriate chromatographic peak, was also linear and equivalent for both partitioning phases. Equimolar solutions of AZT (4) in buffer and *n*-octanol at five concentrations over the range 1.0-103 μM were quantitated by HPLC analysis with alternating injections of each phase. The average (n=2) standard curves generated for each phase were linear and equivalent within determined measurement precision (*vide infra*):

Buffer: $Y = 1.97X - 0.42$ ($r = 0.9998$)

n-Octanol: $Y = 2.01X - 0.85$ ($r = 0.9996$)

Similarly, quadruplicate injection of the same 18.8 μM AZT solution for each phase gave equivalent absolute area with a coefficient of variation of 2.6% for the pH 7 phosphate buffer and 3.8% for the *n*-octanol.

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Table 1. Chromatographic Characteristics of Nucleosides Studied.

Compound	Abbreviation	Mobile Phase CH_3CN (%)	λ_{max} (nm)	Capacity Factor (k')
1	N^4-Bz-2'-dC	20	260	2.4
2	N^4-Bz-C	20	260	2.1
3	N^6-Bz-2'-dA	20	281	2.1
4	AZT	20	266	1.3
5	Caffeine	15	274	1.6
6	N^6-Bz-A	20	281	0.9
7	2'-F-dd-ara-A	12	259	1.2
8	ddA	12	260	1.1
9	dd-Adenosinene	10	260	1.4
10	dT	8.5	269	2.3
11	2'-dA	10	260	0.8
12	3'-N_3-ddC	7	270	3.3
13	ddU	5.5	264	2.7
14	Adenosine	10	260	1.0
15	Thymidine	4	267	3.3
16	2'-F-dd-ara-I	10	247	0.8
17	ddI	10	249	0.9
18	ddC	4	271	2.2
19	ddCytidinene	4	270	1.8
20	2'-dI	5.5	249	0.8
21	2'-dC	2	270	1.4
22	Uridine	2	261	1.4
23	Ara-C	2	271	1.3
24	Inosine	4	248	1.3
25	Cytidine	2	270	0.9
26	CPE-C	2	275	0.7

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Log P was determined for twenty-six nucleosides and nucleoside analogues (Table 2) using the micro shake-flask procedure of Scheme I and the isocratic HPLC conditions of Table 1. The partition coefficients of these compounds covered a thousand-fold range or three log P units from -2.4 to 0.7. For nucleosides with a log P in the range -1.2 to 0.7 measurement precision was typically better than ±0.02 log P units. Of the 18 nucleosides previously determined by the classical shake-flask method [9], sixteen had measured values within ±0.1 log P units and none differed by more than 0.2 log P units.

The effect of varying concentrations of dimethyl sulfoxide (DMSO) on octanol-buffer partitioning was investigated for three model compounds of varying lipophilicity (Table 3). DMSO concentration was varied from zero (buffer only) to 5%, which would be equivalent to using 100 µl of the DMSO stock solution of a particular compound. The variation in measured log P under the above conditions was most pronounced for the most lipophilic of the three compounds, N^6-benzoyl-2'-deoxyadenosine (**3**), which exhibited a significant decrease in apparent log P with increasing DMSO concentration. In contrast, all log P values for the hydrophilic nucleoside cytidine were comparable within experimental error under these same conditions. One per cent DMSO, which would be equivalent to carrying out the method of Scheme I using 20 µl of a DMSO stock solution, resulted in log P values that differed only slightly from those obtained using 100% buffer stock solutions.

2',3'-Dideoxyadenosine (**8**) was used as a model compound to determine the effect of centrifugation on octanol-water phase separation. Individually centrifuging both the water and octanol phases for 5 min after partitioning was sufficient for complete phase separation. No difference was observed in the log P of compound **8** after a 5-min or 60-min centrifugation.

Partition coefficient determination for the individual components of mixtures was also investigated. Isocratic HPLC conditions were employed for the separation and quantitation of mixtures of compounds similar in structure and lipophilicity.

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Table 2. Nucleoside Partition Coefficients Determined by a Micro-Shake Flask Method.

Compound	Abbreviation	Measured[a] Log P	Literature Log P
1	N^4-Bz-2'-dC	0.673 ± 0.006	-
2	N^4-Bz-C	0.304 ± 0.022	-
3	N^6-Bz-2'-dA	0.218 ± 0.007	-
4	AZT	0.052 ± 0.009	0.038[b],-0.016[c],0.225[e]
5	Caffeine	-0.100 ± 0.008	-0.070[d]
6	N^6-Bz-A	-0.101 ± 0.009	-
7	2'-F-dd-ara-A	-0.183 ± 0.004	-0.096[b]
8	ddA	-0.287 ± 0.005	-0.220[b],-0.218[c]
9	dd-Adenosinene	-0.526 ± 0.019	-0.507[b],-0.357[e]
10	dT	-0.609 ± 0.009	-0.578[b],-0.633[c]
11	2'-dA	-0.652 ± 0.011	-0.611[b],-0.548[c]
12	3'-N_3-ddC	-0.695 ± 0.007	-0.682[b],-0.636[c]
13	ddU	-0.996 ± 0.012	-0.889[c],-0.409[e]
14	Adenosine	-1.052 ± 0.005	-0.979[b],-1.23[d]
15	Thymidine	-1.145 ± 0.019	-1.102[b],-1.174[c]
16	2'-F-dd-ara-I	-1.210 + 0.017	-
17	ddI	-1.242 + 0.028	-1.167[b]
18	ddC	-1.328 ± 0.009	-1.260[b],-1.301[c]
19	ddCytidinene	-1.570 ± 0.032	-1.553[b],-1.420[c]
20	2'-dI	-1.710 ± 0.016	-
21	2'-dC	-1.891 ± 0.009	-1.886[b],-1.770[c]
22	Uridine	-1.936 ± 0.008	-1.886[b]
23	Ara-C	-2.095 ± 0.037	-2.097[b]
24	Inosine	-2.097 ± 0.009	-2.000[b],-1.81[d]
25	Cytidine	-2.288 ± 0.043	-2.097[b]
26	CPE-C	-2.453 ± 0.038	-2.301[b]

[a]Mean ± standard deviation of three independent determinations.
[b]Reference 9. [c]Reference 8.
[d]Reference 17. [e]Reference 18.

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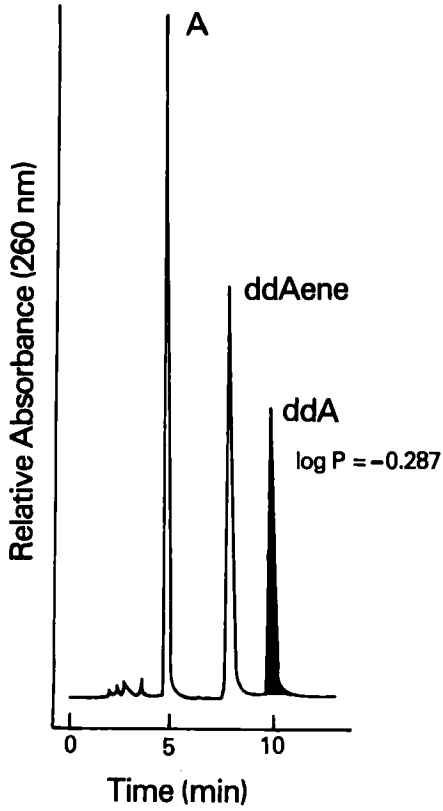

FIGURE 4. Isocratic HPLC analysis of buffer phase from partition coefficient determination of adenine nucleoside mixture. A mobile phase of 8% CH_3CN in pH 7, 0.01 M phosphate buffer was used with other chromatographic conditions as indicated in Methods. ddA (**8**, shaded peak) was employed as an internal standard.

Figure 4 shows the HPLC analysis of the buffer phase of a 1:1:1 mixture of adenosine (**14**), 2',3'-dideoxyadenosinene (**9**) and 2',3'-dideoxyadenosine (**8**). Mixtures with these compounds in a 0.25:1:1 and a 4:1:1 ratio were also studied to evaluate whether mixture concentration had an effect on log P. Mean log P values for the individual mixture components were within ±0.05 log units of the values measured in single component experiments (Table 4).

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Table 3. Effect of DMSO on log P Determinations

	Apparent Log P		
DMSO (%)	N^6-Bz-2'-dA (3)	2'-dA (11)	Cytidine (25)
0	0.256 ± 0.008	-0.609 ± 0.011	-2.246 ± 0.025
1	0.239 ± 0.026	-0.651 ± 0.011	-2.288 ± 0.043
2.5	0.171 ± 0.003	-0.704 ± 0.023	-2.257 ± 0.022
5	0.101 ± 0.003	-0.733 ± 0.023	-2.272 ± 0.011

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Table 4. Determination of Partition Coefficients in Mixtures

	Log P[a]			
Compound	Single Component	Adenosine:ddAdenosinene:ddA[b] 0.25:1:1	1:1:1	4:1:1
ddA	-0.287	-0.322	-0.315	-0.268
ddAdenosinene	-0.526	-0.534[c] (*-0.499*)[d]	-0.535 (*-0.507*)	-0.487 (*-0.506*)
Adenosine	-1.052	-1.094 (*-1.059*)	-1.069 (*-1.041*)	-1.011 (*-1.030*)

[a]Mean of three independent measurements. Standard deviations were ≤ 0.04 log units in all cases.
[b]DMSO concentration of mixture components was such that 20 µl total was always added to 1.0 ml buffer.
[c]Determined directly.
[d]Italicized values in parentheses corrected using ddA as an internal standard.

The log P of the components of the mixture of Figure 4 were also determined using ddA as an internal standard, whose individually determined log P of -0.287 provided a constant reference for each analysis. The apparent log P of ddA in each mixture was measured and compared to its standard or accepted value. This reference compound log P difference ($\Delta\log P_{ref}$) was used to calculate a corrected log P ($\log P_{corr}$) according to the following formula

$$\log P_{corr} = \log P_{meas} + \Delta\log P_{ref}$$

where $\log P_{meas}$ was the measured log P of a mixture component. Using an internal reference to adjust the log P's of adenosine and 2',3'-dideoxyadenosinine measured during mixture analysis increased consistency and improved overall agreement with values measured in single component experiments (Table 4).

Determination of partition coefficients using gradient elution was evaluated with a mixture containing components with a broader range of lipophilicity. Figure 5 shows the chromatographic analysis of both the n-octanol and buffer phases for the simultaneous log P determination of mixture components. Measurement precision and agreement with log P's determined in single component experiments were comparable to the results of isocratic mixture analysis seen in Table 4. Table 5 contains both the directly determined and internal reference corrected log P values measured for the components of the mixture depicted in Figure 5.

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DISCUSSION

Several of the difficulties inherent to the classical shake-flask approach for partition coefficient measurement have been avoided by employing the Lidex mixxor apparatus for solute partitioning and solvent separation. By design, the mixxor apparatus minimizes the amount of solvent (and hence sample) required for efficient partitioning between two phases and simplifies phase separation and sampling once partitioning is complete [19,20]. The up-down action of the piston capillary forces the two phases to pass back and forth as very small droplets and results in a highly

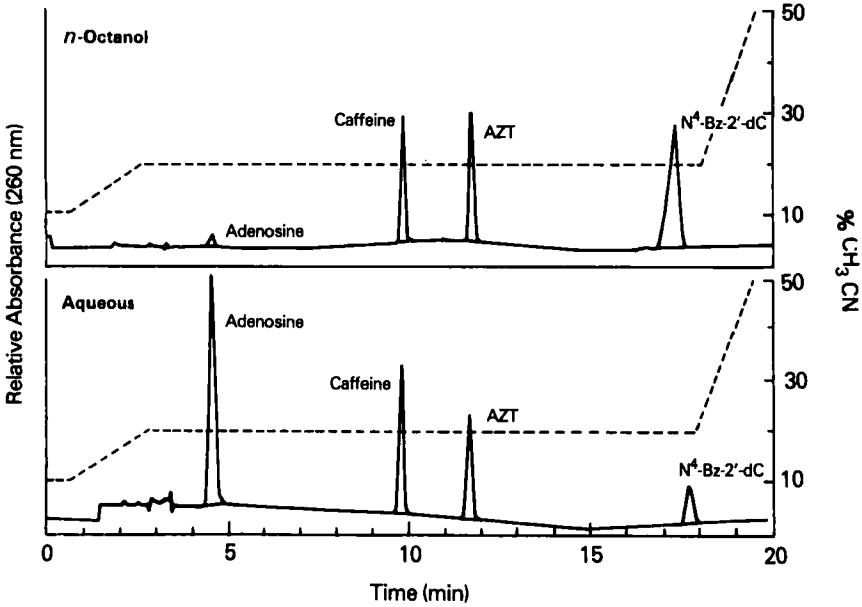

FIGURE 5. Gradient HPLC analysis of *n*-octanol (top) and buffer (bottom) phases from partition coefficient determination of nucleoside mixture. Dashed line indicates CH_3CN composition of mobile phase. Other chromatographic conditions are as indicated in Methods.

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Table 5. Gradient Elution Log P Determination of Mixtures

	Log P[a]		
Compound	Single Component	Direct[b] Mixture	Internal Standard[c] Mixture
N^4-Bz-2'-dC	0.673	0.685 ± 0.002	0.666 ± 0.006
AZT	0.052	0.055 ± 0.002	0.038 ± 0.003
Caffeine	-0.100	-0.082 ± 0.004	-
Adenosine	-1.052	-1.250 ± 0.037	-1.268 ± 0.040

[a]Mean of three independent measurements.
[b]Determined directly.
[c]Adjusted using caffeine as an internal standard.

efficient mass transfer operation (Figure 3). Six piston strokes of this apparatus are equivalent to forty shakes of a separatory funnel [20]. Thus, we have employed 30 piston strokes to ensure consistent results, since 100 shakes of a separatory funnel is generally considered sufficient for complete partitioning in the shake-flask procedure [1]. Indeed, the measured log P for N^6-Bz-2'dC (1) is the same within experimental variation whether 15, 30 or 60 piston strokes are used. Use of this apparatus also allows the partitioning to be done on a 1-2 ml scale, which is more consistent with the 50-100 µl volumes required for replicate HPLC analyses.

Even though the mixxor apparatus allows the piston to be positioned after partitioning so that the phase boundary falls in the capillary, each immiscible solvent is centrifuged after removal to ensure complete phase separation [1,3]. A short 5-min centrifugation time is employed to eliminate microemulsions and complete phase separation. Studies with selected nucleosides show log P measurements are equivalent in magnitude and consistency regardless of longer centrifugation periods. This abbreviated interval also prevents heating of the partitioned samples, which can alter solute distribution.

The effect of DMSO on the octanol-buffer partitioning was also investigated, since solutes are initially dissolved in this solvent before spiking the aqueous phase. DMSO provides a medium in which most solutes are readily soluble and stable, and hence they can be stored for repeat or additional determinations. As one might expect, the hydrophilic nature of DMSO (log P -2.03 [1]) enhances the aqueous solubility of these solutes and results in a decrease in measured log P. The magnitude of this decrease depends on the lipophilicity of the compound and the amount of DMSO employed as a cosolvent (Table 3). When a minimum amount of DMSO is used, as is the case for the method presented here, log P values do not differ by much more than measurement precision (0.01 - 0.04 log units).

The use of HPLC for the measurement of the solute in each phase during partition coefficient determination has been described previously [17, 21]. However, in both cases milligram amounts of

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solute were used for partitioning and the potential of HPLC as a microanalysis technique was not exploited. The microscale method described here routinely uses only 10 μg of compound in a single partitioning experiment. For compounds of limited availability, the volume of DMSO needed to make a stock solution of 0.5 μg/μl can be easily adjusted to the amount of compound available. Since HPLC techniques are often employed to characterize and purify new compounds, separation systems may already exist. Absolute compound purity is also not a requirement, as it is where quantitation is accomplished by classical UV spectroscopy methods [1,3], because of the HPLC separation process that is inherent to the measurement of the solute in each phase. In fact, the ability to determine the partition coefficient of a component in a mixture can be used to good advantage to insure both accuracy and efficiency (see below).

The differences in solvent viscosity, UV spectral characteristics and reverse-phase column interactions of the aqueous and *n*-octanol phases prompted a study to insure that analysis of a solute in either phase was equivalent and linear. This was indeed found to be the case for at least a 100-fold concentration range in either phase for the model dideoxynucleoside AZT (**4**). This concentration range would correspond to measured log P values of 2 to -2, a range which includes almost all the compounds of Table 2. For those compounds which fall outside of this range by being either more hydrophilic or lipophilic, partitioning can be carried out with unequal phase volumes and the more concentrated phase appropriately diluted before analysis [17]. Direct analysis of *n*-octanol did present some problems, since repeated injections of this phase onto the C_{18}-column caused eventual peak distortion and loss of resolution. However, column performance was acceptable for at least four octanol injections to allow the replicate log P determinations of an individual compound. Column performance could be restored by washing the column with 60% acetonitrile:water and re-equilibrating with the appropriate mobile phase of Table 1. Column deterioration from *n*-octanol deposition was not as much a problem in gradient elution analysis, since a column washing step was incorporated into the end of the gradient.

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HPLC analysis also includes the capability of performing multiple log P determinations for several compounds in a single partitioning experiment. This feature is contingent upon good chromatographic separation of all components, including impurities, in the sample. For mixtures of compounds with similar hydrophobicity, such as the adenine nucleosides of Figure 4, isocratic conditions may suffice for separation. More typically, gradient elution may be used to separate and measure mixtures of partitioned compounds in which a wide range of lipophilicity or log P is expected. Figure 5 depicts such an analysis and Table 5 indicates that measured log P values are comparable in magnitude and precision to those obtained from single sample analysis. This procedure for simultaneously determining partition coefficients offers a convenient and rapid means for evaluating a large number of compounds, as would be necessary in QSAR studies of drug analogues [2].

The ability to determine the log P in mixtures means it is possible to employ one of the mixture components as an internal reference to standardize measurements or to correct systematic bias. To be an internal reference, a compound must have its log P accurately known and within the anticipated range of measurements, be readily (preferably commercially) available, have an absorbance similar to the compounds being analyzed (if UV detection is used), and be separable in the HPLC analytical system. Tables 4 and 5 show the results when the respective mixture components ddA (Figure 4, log P -0.287) and caffeine (Figure 5, log P -0.100) are used as internal references. The corrections in these cases are minimal, although they do serve to tie the log P measurements to known log P values and provide a frame of reference to determinations done in other laboratories. This latter function is probably quite useful, since literature log P values can vary widely and an agreement of ±0.1 between measured values is considered exceptional [3].

CONCLUSION

The determination of partition coefficients has been greatly simplified by the synergistic combination of a microscale parti-

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tioning process with the inherent analytical power of high performance liquid chromatography. The microscale method described above offers substantial savings in materials and time as well as the increased certainty resulting from the separation and direct measurement of the compound(s) of interest in both phases. Because of the widespread availability of HPLC instrumentation in analytical and synthetic chemistry laboratories, the individual researcher can now easily, rapidly and reliably obtain log P values for both new compounds and those for which this data does not exist or is questionable.

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