A Simplified High-Pressure Liquid Chromatography Method for Determining Lipophilicity for Structure-Activity Relationships

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Received July 26, 1982

A highly deactivated octadecyl-bonded silica column and a mobile phase consisting of an water–methanol mixture in the range of blood pH and ionic strength are used to correlate log k' with biological activity for a series of sulfonamides and barbiturates. The results were compared to literature methods by using retention volume (V_R) and retention indexes (I). For the nine sulfonamides tested, log V_R and log k' were used with and without correction for ionization. For each biological end point (protein binding and minimum inhibitor concentration against *Eschericia coli* from two sources) and each independent variable (log k' and log V_R) the residual standard derivation for the regression was determined. The standard derivations were compared in an F test for each of 12 relevant regressions. Log k' was statistically superior in four cases, while log V_R was superior in one case. Overall, the methods were statistically indistinguishable. Log k' values and I values for 15 barbiturates were regressed against three biological end points [hypnotic activity (the minimum effective dose in rabbits), inhibition of Arbacia egg cell division, and inhibition of rat brain respiration]. Standard deviations were compared by an F test, and the two methods were indistinguishable as far as the goodness of biological correlations are concerned. Procedures for controlling the column's activity are presented. Choices for an appropriate mobile phase are discussed, and a method of calculating pH and ionic strength in a methanol–aqueous mobile phase is presented.

Since correlation of organic aqueous partitioning with biological activity was first reported in 1899,[1,2] many organic phases have been used for partition coefficient determinations.[3] Octanol–water partition coefficients (log P) have become an accepted model for lipophilicity in quantitative structure–activity relationships (QSAR).[4,5] The term π was developed as a means of calculating the contribution of substituents to log P.[6,7] For many systems, log P cannot be calculated due to interferences from steric and electronic effects.[8,9] Measurement of log P by the "shake-flask" method is time consuming, wasteful of sample, and subject to errors from impurities, poor detectability, dissociation, decomposition, and stable emulsion formation. Also, compounds of very high or low partition cannot be measured by this method.

Partition chromatography was explored as an alternative means of measuring lipophilicity. Paper chromatography[10] and later thin-layer chromatography[11] (TLC) using supports impregnated with an organic phase (e.g., octanol or silicone oil) were used. The terms R_M and ΔR_M were derived to parallel log P and π[12] and to directly correlate to biological activity.[11] TLC was simple to use, reproducible, and did not require quantitative analysis of the solutes. Samples could be of lower purity, and smaller quantities were required. TLC also expanded the range of log P values that could be determined.[11]

Reverse-phase HPLC has been investigated previously as a means for determining lipophilicity by attempting to correlate log k' (eq 1), a retention-time parameter, with

$$k' = \frac{(t_r - t_0)}{t_0} \quad (1)$$

log P.[13-15] In eq 1, t_r is the retention time of the compound and t_0 is the retention time of an unretained compound. Retention in HPLC has also been correlated directly with biological activity.[13,16,17] The HPLC methods possess all the advantages of the TLC methods plus the added advantages of accuracy, ease of controlling experimental conditions, and potential for automation. A broader range of lipophilicity can be analyzed by HPLC, and the development of chemically bonded reverse phases allows production of stable columns in which the contribution of the support to the chromatography can be minimized.[14] In the choice of an HPLC system for measuring lipophilicity, potential sources of error must be controlled. We describe an HPLC method that incorporates the best principles of reported methods, i.e., the use of a mobile phase with the pH and ionic strength adjusted to that of blood,[13] the use of an organic solvent in the mobile phase so that k' may be adjusted,[16,17] and the use of a heavily silylated reverse-phase C-18 column to reduce undesired eluant interactions with silanol sites.[18] In our system, the pH, ionic strength, and column quality are rigidly controlled. A commercially available column packing is silylated by a procedure that when monitored by the Methyl Red test[35,36] has the least active sites for packing materials analyzed. A method is presented to calculate the pH in the organic solvent modified mobile phase, which eliminates the error caused by direct measurement with a pH electrode. Our system is contrasted with those in the literature, and potential problems are discussed. The HPLC method reported in this paper gives results as good

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0022-2623/83/1826-1014$01.50/0

Table I. Biological Activities and Capacity Factors for Standard Sulfonamides

no.	name	log k' [a,b] obsd	log k' [a,b] corrected	pK_a [c]	−log α (protein binding) [d] obsd	−log α (protein binding) [d] calcd [g]	−log C (bacteriostatic) [e] obsd	−log C (bacteriostatic) [e] calcd [h]	−log C (bacteriostatic) [f] obsd	−log C (bacteriostatic) [f] calcd [i]
1	sulfaguanine	−0.00612	−0.00611	12.00			1.81	−1.93		
2	sulfanilamide	−0.0315	−0.0147	10.08	−2.98	−3.02	−2.11	−1.95	2.824	2.86
3	sulfacetamide	unretained		5.78	−3.15		−0.52		3.248	
4	sulfadiazine	0.223	1.20	6.52	−2.99	−2.52	−0.08	−0.21	4.337	4.32
5	sulfamethoxazole	0.581	2.00	6.05	−2.51	−2.19	0.06	0.15	4.634	4.73
6	sulfisoxazole	0.762	3.21	5.00	−1.73	−1.69	−0.45	−0.49	4.564	4.53
7	sulfamerazine	0.829	1.43	6.98	−2.23	−2.43	0.06	−0.04	4.469	4.48
8	sulfathiazole	0.887	1.30	7.25	−2.45	−2.48	−0.18	−0.13	4.597	4.40
9	sulfamethoxy-pyridazine	1.35	1.79	7.20	−1.75	−2.28	0.00	0.12	4.570	4.67
10	sulfamethazine	1.49	1.69	7.70	−2.28	−2.32	0.11	0.09		

[a] Corrections of log k' data for ionization provide an estimate of the retention of only the neutral molecule form of a compound, rather than that observed for a distribution of neutral and charged species under "real" chromatographic conditions. The variation between log k'(corr) and log k'(obsd) is a function of the pH of the mobile phase and the pK_a of the compound in question. Corrections of this type would be expected to give improved correlation with any variable whose result depends predominantly on lipophilic (neutral molecule) interactions, such as log P, and some biological activities. For further details, see ref 23. [b] Log k'(corr) = log k'(obsd) + log $[(10^{-pK_a} + 10^{-pH})/10^{-pH}]$. [c] Data from ref 17. [d] Negative log of Langmuir's α constant, which is inversely proportional to the effective binding constant. For further discussion, see ref 22. Data from ref 23. [e] Minimum inhibitory concentration against *E. coli* at pH 7.2 (Sauterne's medium). Data from ref 24. [f] Minimum inhibitory concentration against *E. coli* at pH 7.4. Data from ref 21. [g] Calculated by eq 17, Table II. [h] Calculated by eq 3, Table II. [i] Calculated by eq 10, Table II.

as other HPLC methods using the F test.

Results and Discussion

When HPLC is used to determine lipophilicity of a series of compounds, the first consideration is the column packing. It replaces the organic phase of the "shake-flask" method but differs because two components are present, the solid support and the stationary phase. Both can affect the interaction of solutes in the system. It has been argued in the literature[15] that alkyl-bonded phases will produce inferior correlations because they lack the polar character of octanol or because of adsorbtive interactions with the support, and yet these same arguments are used by others[14,18] to explain why superior correlations have been obtained with bonded phases. Due to differences between the column in HPLC and the organic phase in the shake-flask method, several researchers have tried to duplicate shake-flask conditions by using an octanol impregnated stationary phase.[14,17,19] Unger et al.[15] uses an octanol-coated, octadecyl-bonded phase and an octanol-saturated buffer mobile phase. Correlations of the HPLC data with log P and with biological activity were good. However, octanol presents problems in HPLC because of its viscosity, immiscibility, and objectionable odor.

Some researchers have tried a simpler approach, the use of bonded phases alone. These media are stable and convenient. Their major drawback, adsorptive interactions on active silanol sites, can be controlled by proper pretreatment. McCall, for example, used a further deactivation step to remove silanol sites from the reverse-phase medium.[18] However, he found this step was not sufficient to prevent adsorption of basic compounds and that addition of a base to the mobile phase was necessary to compete with basic solutes for active sites.

Another approach, proposed by Baker, uses a retention index as a universal scale for lipophilicity.[16] The intention was to allow direct comparison of retention data between different columns and mobile phases by using 2-ketoalkanes as retention reference compounds. This concept fails because 2-ketoalkane standards cannot account for all interactions with the column and the mobile phase that would be experienced by molecules bearing the full range of possible functional groups. This can be easily seen in Baker's work,[20] where inversion of elution order for various drugs occurred with changing methanol content in the mobile phase.

The method described in this paper was developed to provide a quick and reliable liphophilicity measurement for a broad spectrum of compounds avoiding mechanical and theoretical limitations of earlier approaches. The method should be able to be applied to a series of drug candidates of diverse chemical and polarity characteristics. The method does not require high compound purity.

In developing a lipophilicity constant, our method did not attempt to duplicate log P but rather to develop a new constant. The system chosen consists of a rigorously deactivated reverse-phase packing material and a mobile phase consisting of methanol-modified aqueous phosphate buffer. The pH and ionic strength of the mobile phase are adjusted to parallel blood. The only modifiers used in the mobile phase are methanol and phosphate salts to limit selectivity effects. The activity of the stationary phase is monitored by the Methyl Red test, and the pH of the mobile phase is accurately calculated by methods given under Experimental Section.

Our method was used to measure liphophilicity constants of a series of sulfonamides and barbiturates. The values obtained as log k' for sulfonamides and barbiturates are reported, along with pK_a's and biological activities, in Tables I and V, respectively. In comparing methods, the null hypothesis of equality of variances is tested by $F_0 = s_1^2/s_2^2$, where $s_1 \geq s_2$. F_0 has an F distribution with $n_1 - 1$ and $n_2 - 1$ degrees of freedom. The null hypothesis is rejected if $F_0 > F_{0.95}(V_1,V_2)$, where $F_{0.95}(V_1,V_2)$ is the appropriate critical value; i.e., prob$[F > F_{0.95}(V_1,V_2)] \leq 0.05$.

Table II compares the use of log k' and the log of retention volumes, log V_R, in correlations with sulfonamide bacteriostatic activities and protein binding. Sulfacetamide was eliminated from consideration, since the compound was not retained by our column under experimental conditions. To simplify Table II, no biological activities were corrected for ionization. However, results are reported for both corrected and uncorrected HPLC data. In our method, the calculated pH was used to correct log k' data,

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Table II. Sulfonamides: Correlation of Biological Activity with Log k' and Retention Volume (log V_R)[a]

biol act. (−log C)	correlation equations: physical property (X)	B_2	B_1	B_0	statistical terms: n[b]	r[c]	s[d]	F[e]	eq
bacteriostatic[f]	log k'[i]	−1.51	3.29	−1.63	9	0.871	0.485	9.40	1
	log k'[j]	−0.399	1.84	−1.93	9	0.992	0.123	191.4	2
	log k'[k]	−0.486	2.01	−1.92	9	0.992	0.126	181.3	3
	log V_R[l]	−0.238	1.15	0.125	9	0.485	0.863	0.922	4
	log V_R[m]	−1.93	1.58	0.120	9	0.972	0.233	51.0	5
	log V_R[n]	−2.93	1.07	0.268	9	0.964	0.264	38.9	6
	log V_R[o]	−1.05	1.51	−0.515	9	0.964	0.263	39.3	7
bacteriostatic[g]	log k'[i]	−1.72	3.24	3.20	7	0.903	0.343	8.81	8
	log k'[j]	−0.274	1.44	2.89	7	0.982	0.150	54.7	9
	log k'[k]	−0.341	1.61	2.89	7	0.988	0.126	78.6	10
	log V_R[l]	1.74	2.20	4.85	7	0.310	0.759	0.212	11
	log V_R[m]	−1.51	1.53	4.69	7	0.947	0.257	17.3	12
	log V_R[n]	−1.98	1.17	4.70	7	0.914	0.323	10.21	13
	log V_R[o]	−0.874	1.48	4.00	7	0.996	0.075	221.8	14
protein binding[h,p]	log k'[i]		0.688	−2.89	8	0.736	0.350	7.10	15
	log k'[j]		0.300	−2.90	8	0.658	0.389	4.58	16
	log k'[k]		0.411	−3.0	8	0.774	0.327	8.97	17
	log V_R[l]		0.537	−2.13	8	0.358	0.483	0.884	18
	log V_R[m]		0.675	−2.37	8	0.720	0.359	6.47	19
	log V_R[n]		0.956	−2.26	8	0.824	0.293	12.7	20
	log V_R[o]		0.657	−2.78	8	0.793	0.315	10.17	21

[a] From ref 17. [b] n = number of compound studied. Correlation coefficient. [d] Standard deviation. [e] F test value. [f] Uncorrected bacteriostatic activity (ref 24) determined at pH 7.2. [g] Uncorrected bacteriostatic activity (ref 21) determined at pH 7.4. [h] Uncorrected binding constants determined at pH 7.4 (ref 22). [i] Uncorrected for ionization. [j] Corrected for ionization with calculated pH and pK_a's taken from ref 21. [k] Corrected for ionization with calculated pH and pK_a's from ref 22. [l] Log V_R determined at pH 6.5 and uncorrected. [m] Log V_R determined at pH 6.5 and corrected for ionization with pK_a's from ref 21. [n] Log V_R determined at pH 6.5 and corrected using pK_a's from ref 22. [o] Log V_R determined at pH 4 and uncorrected. [p] Reference 43.

Table III. pK_a's of Sulfonamides

compound	pK_a[a]	pK_a[b]	pK_a[c]
sulfacetamide	5.40	5.78	5.38
sulfamerazine	6.93	6.98	7.01
sulfathiazole	7.10	7.25	7.39
sulfamethoxazole	5.81	6.05	5.80
sulfisoxazole	4.62	5.00	4.89
sulfadiazine	6.15	6.52	6.38
sulfamethazine		7.70	7.66
sulfamethoxypyridazine	7.05	7.20	7.28

[a] Reference 21. [b] Reference 22. [c] This paper.

while the "apparent" pH reported by Henry et al.[17] was used to correct log V_R. The pK_a's of these compounds varied depending on the literature source (Table III). In general, the pK_a's measured by the authors of this paper fall between the values from the literature,[21,22] both sets of literature pK_a values were used for comparison in Table II.

Table IV. Statistical Comparison of Standard Deviations of Log k' and Log V_R Regressions by the F Test

	bacteriostatic:[f] −log C	bacteriostatic:[g] −log C	protein binding:[h,p] −log α
s for log k'[a,j]	0.123 (2)	0.150 (9)	0.389 (16)
s for log V_R[m]	0.233 (5)	0.257 (12)	0.359 (19)
F	3.59 (NS)[b]	2.94 (NS)	1.17 (NS)
s for log k'[k]	0.126 (3)	0.126 (10)	0.327 (17)
s for log V_R[n]	0.264 (6)	0.323 (13)	0.293 (20)
F	4.39 (5%)[c]	6.57 (5%)	1.25 (NS)
s for log k'[e,i]	0.485 (1)	0.343 (8)	0.350 (15)
s for log V_R[o]	0.263 (7)	0.075 (14)	0.315 (21)
F	3.40 (NS)	20.92 (1%)[d]	1.24 (NS)
s for log k'[j]	0.123 (2)	0.150 (9)	0.389 (16)
s for log V_R[o]	0.263 (7)	0.075 (14)	0.315 (21)
F	4.57 (5%)	4.00 (NS)	1.53 (NS)
s for log k'[k]	1.26 (3)	0.126 (10)	0.327 (17)
s for log V_R[o]	0.263 (7)	0.075 (14)	0.327 (17)
F	4.35 (5%)	2.82 (NS)	1.08 (NS)

[a] Standard deviation for the correlation; each s is followed by the equation number given in Table II. [b] Not significant. [c] This result would occur only 5% of the time if the methods were the same. [d] This result would occur only 1% of the time if the methods were the same. [e] Comparison of uncorrected pH 7.4 data with pH 4 data may be an unfair comparison, since one measures primarily ionic species while the other condition measures primarily neutral molecules. A better comparison might be pH 7.4 corrected for ionization vs. pH 4 data; see text. [f-o] See corresponding footnotes in Table II. [p] Reference 43.

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Table V. Biological Activities and Capacity Factors for Standard Barbiturates

no.	name	log k'	pK_a[a]	$-\log C$[b] (hypnotic) obsd	$-\log C$[b] (hypnotic) calcd[c]	$-\log ED_{50}$[d] (*Arbacia* egg division) obsd	$-\log ED_{50}$[d] (*Arbacia* egg division) calcd[e]	$-\log C$[f] (oxygen uptake) obsd	$-\log C$[f] (oxygen uptake) calcd[g]
1	barbital	0.16	7.75	3.09[h]	3.02	1.49	1.30	1.32	1.36
2	allobarbital	0.60	7.62	3.54[i]	3.39	1.79	1.93		
3	phenobarbital	0.56	7.26	3.32[i]	3.36	2.02	1.88	1.88	2.20
4	metharbital	0.71		3.12[j]	3.48				
5	aprobarbital	0.75	7.73	3.60[i]	3.52	2.01	2.15	2.41	2.51
6	butabarbital	0.95	7.89	3.63[h]	3.68				
7	cyclobarbital	0.91	7.36			2.24	2.38		
8	butalbital	1.01	7.54	3.63[h]	3.73			2.80	2.84
9	hexobarbital	1.13	7.36	4.37[j]	3.83				
10	amobarbital	1.33	7.78	3.75[h]	4.00	2.82	2.99	3.12	3.10
11	pentobarbital	1.32	8.02	4.05[k]	3.99	2.92	2.97	3.07	3.09
12	secobarbital	1.52	7.92	4.20[k]	4.16	3.62	3.26	3.19	3.18
13	thiopental	1.52		3.98[l]	4.16				
14	thiamylal	1.72		4.15[l]	4.33				
15	methohexital	1.96	4.74	4.74[l]	4.53				

[a] pK_a from ref 17. [b] Negative log of minimum effective dose (moles per kilogram) in rabbits. [c] Calculated from eq 1, Table VI. [d] Negative log of molar drug concentration required to reduce cell division by 50%. Data from ref 25. [e] Calculated from eq 3, Table VI. [f] Negative log of molar concentration required to produce 50% inhibition of oxygen on rat brain respiration in vitro. Data from ref 26. [g] Calculated from eq 5, Table VI. [h] Data from ref 27. [i] Data from ref 28. [j] Data from ref 29. [k] Data from ref 30. [l] Data from ref 31.

Table VI. Barbiturates: Correlation of Biological Activity with Log k' and Retention Index (I)[a]

biol act. ($-\log C$)	physical property (X)	correlation equations: $-\log C = B_2X^2 + B_1X + B_0$ B_2	B_1	B_0	statistical terms n[b]	r[c]	s[d]	F[e]	eq
hypnotic	log k'		0.835	2.89	14	0.884	0.232	42.9	1
hypnotic	I		3.89×10^{-3}	1.36	14	0.875	0.240	39.2	2
Arbacia egg div	log k'		1.44	1.07	8	0.959	0.215	68.8	3
Arbacia egg div	I		6.44×10^{-3}	−1.42	8	0.961	0.210	72.0	4
O_2 uptake	log k'	−0.796	2.67	0.956	7	0.992	0.0999	128.8	5
O_2 uptake	I	-1.54×10^{-5}	0.0238	−5.97	7	0.997	0.0661	296.4	6

[a] From ref 16. [b] n = number of compounds studied. Only the data for compounds analyzed by both methods (log k' and I) was correlated. [c] r = coefficient of correlation. [d] s = standard deviation of regression. [e] F = F test value.

Data developed by our method are compared to that of Henry et al.[17] in Table IV. The randomness of differences between the methods indicates that they are statistically indistinguishable. However, C_{18}/Corasil (Waters Associates), the column packing material used by Henry et al.,[17] has many more silanol sites (as shown by the Methyl Red test) than the column packing used in our method. Although differences in column performance due to excess silanol sites was not evident in these experiments, it is still considered valuable to block as many of these sites as possible by chemical treatment. A question remains as to whether any amount of silylation will totally eliminate interactions between silanol sites and lipophilic bases. Some researchers choose to approach this problem by adding an amine to the mobile phase.[13,18]

In Table VI, neither the biological activities of barbiturates nor the log k' and retention index, I, values were corrected for ionization prior to regression. There is no statistical difference between the use of either lipophilicity term.

Table VIII contrasts the literature methods with our method. The chief objections to Unger's method are the inconvenience of using octanol and that k' is fixed because no organic modifier is added to the mobile phase. Thus, one has to use a series of column lengths to obtain data on a practical range of lipophilicities. The range of compounds to be studied is also limited to those soluble in an aqueous mobile phase. The method of Henry et al.[17] also suffers the limitations of a totally aqueous mobile phase. Another problem with the method reported by Henry et al.[17] appears to be the use of a reverse-phase column containing many free silanol sites. The principal objection to the method of Baker et al.[16,20] is that 2-ketoalkane standards do not allow for all possible column variabilities. Inversion of I values with changing solvent systems was demonstrated in the Baker et al. paper.[20]

Table VII. Statistical Comparison of Standard Deviations of Log k' and I Regression by the F Test[a]

	hypnotic act.	inhibn of *Arabacia* egg cell division	inhibn of oxygen uptake
s for log k'	0.232 (1)	0.215 (3)	0.099 (5)
s for I	0.240 (2)	0.210 (4)	0.0661 (6)
F	1.07 (NS)[b]	1.05 (NS)	2.28 (NS)

[a] Standard deviations for the correlation; each is followed by the equation number given in Table VI. [b] Not significant.

In general, when using a buffered mobile phase, one should be aware of and account for two effects. First, the buffer species can affect the resulting partition coefficient. This is particularly true for bases, since ion pairing is possible with the buffer anions, but it has also been observed for acids. Phosphate buffer has been reported to cause the least deviation from octanol–water values.[19] Secondly, the use of an organic modifier in a buffered mobile phase affects the pH of the mobile phase. These effects cannot be accurately measured by a pH electrode due to the liquid junction error that exists.

(32) I. T. Harrison, W. Kurz, I. J. Massey, and S. H. Unger, *J. Med. Chem.*, 21, 588 (1978).

Table VIII. A Comparison of Reported Methods Used To Obtain Lipophilicity Data from HPLC

method	Unger[a]	Baker[b]	Henry[c]	method described in text
support treatment (all C-18 bonded phases)	additional silylation step for C_{18}/Corasil used RP18 as received	used as purchased	used as purchased	rigorous silylation step tested for active sites
mobile phase	octanol-saturated buffer of controlled pH and ionic strength	methanol-buffer mobile phase	aqueous buffers of pH 4, 5, and 6.5	methanol-buffer mobile phase of controlled pH and ionic strength
results of correlation	good correlation of log k' with log P and drug activity; in one case, C_{18}/Corasil bonded phase gave better correlation with activity[d]	better correlation with drug activity than log P for propranolols and barbiturates; worse than log P for anthranilic acids	C_{18}/Corasil bonded phase gave better correlations than octanol on silica gel	good correlations with biological activity for sulfonamides and barbiturates
comments	problems of using octanol in an HPLC system; cannot adjust k' in a purely aqueous system; slow eluting drugs are difficult to measure; fast eluting drugs will contain large measuring errors; used an added lipophilic amine to prevent adsorption of lipophilic bases	proposes retention index as universal scale for lipophilicity; many variations in experimental conditions will not be corrected for by this method	same problems with k' as Unger method	advantage of simplicity, can adjust k' accommodating solubility and compounds with long retention time uses a mathematical approach to achieve a more accurate pH for nonaqueous mobile phases than that measured by a pH electrode have not addressed the effect of free silanol sites on bases

[a] Reference 13. [b] Reference 16. [c] Reference 17. [d] Reference 32.

Although many laboratories have adopted the use of HPLC to generate lipophilicity constants for QSAR studies, there are only a few models in the literature to guide one's approach to this problem. We feel that our method is superior due to its simplicity and versatitiliy and because it incorporates the use of calculations to achieve a more precise value for the pH of the mobile phase.

Experimental Section

Materials. Barbiturates were obtained from Analabs and sulfonamides from Sigma Chemical Co. Methanol was MCB reagent "distilled in glass". All other chemicals were reagent grade and used as received.

Chromatographic Conditions. A Waters Associates HPLC system consisting of a M6000A pump, a U6K injector, and a 450 variable-wavelength detector was used. The flow rate was 1 mL/min. Detection was at 254 nm. The void volume was determined by injection of methanol. The capacity factor (k') was measured directly from the chromatograms. Triplicate determinations were made. Correlations were done by using PROPHET statistics.[33]

Preparation of the Mobile Phase. For the sulfonamides, the mobile phase (5% methanol, 95% water) was prepared by combining 3.92 g of $NaH_2PO_4 \cdot H_2O$, 13.06 g of $Na_2H\ PO_4$, 1.9 L of water, and 0.1 L of methanol.

For the barbiturates, the mobile phase was 30% (v/v) methanol. It was prepared by combining 4.00 g of $NaH_2PO_4 \cdot H_2O$, 12.64 g of Na_2HPO_4, 1.4 L of water, and 0.6 L of methanol.

The phosphate salts are dissolved in the aqueous portion of the mobile phase prior to the addition of methanol. The final mixture is suction filtered with a 0.45-μm Millipore filter, the filtrate is degassed, and the "apparent" pH is recorded. For the sulfonamides, "apparent" pH is 7.49, μ(calcd) = 0.15, and pH(calcd) = 7.45. For the barbiturates, "apparent" pH is 7.55, μ(calcd) = 0.15, and pH(calcd) = 7.31. (See pH for aqueous methanol for discussion of calculations.)

Columns were 10 and 50 cm long with 2-mm i.d. These were dry packed by using deactivated C_{18}/Corasil.

Measurement of pK_a. The pK_as for the sulfonamides were determined in our laboratory by computer-controlled spectrophotometric titration (absorbance vs. pH). Samples were prepared in buffered aqueous solutions containing HCl/HOAc/Mops/glycine (0.0005 N each). Manual wavelength scans were performed on each compound to provide the wavelength at which each titration was performed; wavelengths were selected at which the difference in absorptions of the neutral and anionic species is large. A peristaltic pump was used to circulate solutions through a flow cell mounted in a Cary 118 UV–vis spectrophotometer and back to a thermostated beaker (23.0 °C) where the titrations were carried out.

Persilylation Procedure. The C_{18}/Corasil was deactivated via the rigorous procedure described by Elkins et al.[34] The following steps were included in this procedure to assure that the packing material was highly deactivated. After refluxing with hexamethyldisilazane, the packing was washed with toluene (500 mL) and acetone (500 mL); precautions were taken to exclude contact with air during the toluene wash when excess silylating reagent was being removed. Subsequent to the acetone wash in Elkins' procedure, the material was air-dried until powdery and extracted overnight with methanol by using a soxhlet apparatus. The material was rinsed from the soxhlet thimble onto filter paper with methanol and allowed to air-dry until powdery. Residual silanol sites were assayed by the Methyl Red test.[35,36]

pH Calculations for Aqueous Methanol. Upon dissolution of mono- and dibasic phosphate salts in H_2O, the equilibrium

(33) "Prophet Statistics, A User's Guide to Statistical Analysis on the Prophet System", National Institutes of Health, Bethesda, MD, publication no. 80-2169, 1980.
(34) P. D. Elkins, C. E. Cook, C. M. Sparacino, and J. W. Hines, submitted to *J. Pharm. Sci.*
(35) I. Shapiro and I. M. Kolthoff, *J. Am. Chem. Soc.*, **72**, 776 (1950).
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shown in eq 2 is established. The pK_a describing this equilibrium

$$H_2PO_4^- + H_2O \rightleftarrows HPO_4^{2-} + H_3O^+ \quad (2)$$

can be calculated from eq 3, where γ^- is the activity coefficient

$$pK_a = pH - \log \frac{[HPO_4^{2-}]}{[H_2PO_4^-]} - \log \frac{\gamma^{2-}}{\gamma^-} \quad (3)$$

of $H_2PO_4^-$, and γ^{2-} is the activity coefficient of HPO_4^{2-}. The relationship forming the basis of our approach is obtained by rearranging eq 3 to give eq 4, where $pK_a' = pK_a + \log (\gamma^{2-}/\gamma^-)$,

$$pH = pK_a' + \log \frac{\chi}{(1-\chi)} \quad (4)$$

and $\chi = [HPO_4^{2-}]/([HPO_4^{2-}] + [H_2PO_4^-])$. Clark reported pK_a' values for phosphate buffers as functions of ionic strengths (μ) and mole fractions (χ) of dibasic phosphate.[37] Using eq 4, the pH values for phosphate buffers of differing ionic strengths (μ) and mole fractions (χ) can be accurately calculated.

However, when methanol is introduced, the change in the dielectric constant (ϵ) and density (ρ_0) of the solvent effects the activity coefficients (γ) of the ions in solution, and the values reported by Clark can no longer be used to calculate pH values. Furthermore, due to the liquid junction error between the electrode and phosphate buffer solutions containing methanol, the pH cannot be accurately measured potentiometrically, although many authors report an "apparent" pH. The effect of methanol upon potentiometrically measured values of pH was demonstrated by Bates, who observed a dramatic increase in the apparent pH of equimolar solutions of mono- and dibasic phosphates containing increasing amounts of methanol.[38] To prepare methanol-containing buffered mobile phases meeting our criteria, we developed the following procedure.

According to the Debye–Hückel limiting law,[39] the activity coefficients of ions can be expressed as eq 5, where log γ is the

$$\log \gamma = A\left(\frac{\mu\rho_0}{\epsilon^3 T^6}\right)^{1/2} \quad (5)$$

activity coefficients of the individual ions (eq 2), A is a collection of universal constants that are functions of the dissolved ions rather than the solvent; see ref 40 for discussion, μ is the ionic strength, ϵ is the dielectric constant of the solvent, and T is the temperature (in kelvin). With this expression, log γ for ions dissolved in MeOH/H_2O [log γ(mix)] rather than H_2O [log γ(aq)] can be expressed as eq 6, where ρ_0(aq) is 0.9971 at 22 °C,[41] ϵ(aq)

$$\log \gamma(\text{mix}) = \left(\frac{\epsilon^3(\text{aq})\rho_0(\text{mix})}{\epsilon^3(\text{mix})\rho_0(\text{aq})}\right)^{1/2} \log \gamma(\text{aq}) \quad (6)$$

$$= 6.963 \times 10^2 \left(\frac{\rho_0(\text{mix})}{\epsilon^3(\text{mix})}\right)^{1/2} \log \gamma(\text{aq})$$

is 78.48 at 22 °C,[41] and $(\epsilon^3(\text{aq})/\rho_0(\text{aq}))^{1/2}$ is 6.963×10^2. It should be noted that eq 6 is true only for solutions of constant ionic strength and temperature. The relationship between the ratio of the activity coefficients of the mono- and dibasic phosphate anions in MeOH/H_2O (log γ^{2-}/γ^- (mix)) to that ratio in H_2O (log γ^{2-}/γ^- (aq)) follows from eq 6 (eq 7). By definition (eq 4), the

$$\log \frac{\gamma^{2-}}{\gamma^-}(\text{mix}) = 6.963 \times 10^2 \left(\frac{\rho_0(\text{mix})}{\epsilon^3(\text{mix})}\right) \log \frac{\gamma^{2-}}{\gamma^-}(\text{aq}) \quad (7)$$

pK_a' for an aqueous equilibrium is as shown in eq 8. Similarly,

$$pK_a'(\text{aq}) = -\log (K_a[H_2O]) + \log \frac{\gamma^{2-}}{\gamma^-}(\text{aq}) \quad (8)$$

the pK_a' for a mixture of MeOH/H_2O is as shown in eq 9, where

$$pK_a'(\text{mix}) = -\log (K_a f[H_2O]) + \log \frac{\gamma^{2-}}{\gamma^-}(\text{mix}) \quad (9)$$

f is the fraction (by weight percent) of H_2O. By assuming that $K_a(\text{mix}) = K_a(\text{aq})$, eq 8 can be subtracted from eq 9. Substituting for log γ^{2-}/γ^-(mix) from eq 7 into this result yields eq 10, where const = pK_a'(aq) −log f, and ρ_0(mix) and ϵ(mix) are calculated from ref 41.

$$pK_a'(\text{mix}) = \text{const} + \log \frac{\gamma^{2-}}{\gamma^-}(\text{aq}) \left[6.963 \times 10^2 \left(\frac{\rho_0(\text{mix})}{(\epsilon^3(\text{mix})}\right)^{1/2} - 1 \right] \quad (10)$$

While a calculated value for log $(\gamma^{2-}/\gamma^-(\text{aq}))$ in eq 10 can be obtained from the Debye–Huckel law (eq 5), an empirical approach was chosen. For phosphate buffers, $pK_a' = pK_a = 7.221$ at infinite dilution and 18 °C, since log $(\gamma^{2-}/\gamma^-(\text{aq})) = 0$.[42] By substituting 7.221 in the expression for pK_a (see eq 4), log $(\gamma^{2-}/\gamma^-(\text{aq}))$ can be calculated for each entry (pK_a') reported by Clark using eq 11. These values [log $(\gamma^{2-}/\gamma^-(\text{aq}))$] can now be substituted into

$$\log \frac{\gamma^{2-}}{\gamma^-}(\text{aq}) = pK_a' - 7.221 \quad (11)$$

eq 10, and the pK_a'(mix) can be calculated. However, this represents a value of pK_a'(mix) for solutions at 18 °C, and since our research was performed at 22 °C, a temperature correcton must be included. This correction was determined by interpolation, with $pK_a' = 7.221$ at 18 °C and infinite dilution, and $pK_a' = 7.198$ at 25 °C and infinite dilution.[42] Values of pK_a'(mix) can now be calculated from eq 12 for each pK_a'(aq) corresponding to $\mu = 0.15$

$$pK_a''(\text{mix}) = pK_a'(\text{mix}) - 0.013 \quad (12)$$

m, and $0.1 \leq \chi \leq 0.9$, where pK_a'(mix) is the value from eq 10, and pK_a''(mix) is the value corrected to 22 °C. The pH of the solution can be calculated from eq 4. By selecting the two sets of values [χ, pK_a'(mix)] having calculated pH's closest to 7.4 [one set corresponding to pH(calcd) < 7.4 and the other to pH(calcd) > 7.4], values for χ and pK_a'(mix), which yield a pH(calcd) of exactly 7.4, can be obtained by interpolation. With this value of χ and $\mu = 0.15$ m, the concentrations of $H_2PO_4^-$ and HPO_4^{2-} in the mobile phase can be calculated from eq 13 and 14. If the

$$[H_2PO_4^-] = \frac{\mu(1-\chi)}{(2\chi+1)} \quad (13)$$

$$[HPO_4^{2-}] = \frac{\mu\chi}{(2\chi+1)} \quad (14)$$

concentrations of $H_2PO_4^-$ and HPO_4^{2-} are changed from molality to molarity, the weights of salts can be obtained.

Acknowledgment. The authors thank Drs. Hale Sweeny and Gerald Hajian for discussions relating to the statistical analysis used in this manuscript.

Appendix

Calculation of the Weights of Mono- and Dibasic Phosphates. By our method, analysis of a particular drug series begins by first ascertaining what volume percent of methanol will provide good chromatographic results. The

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(42) R. A. Robinson and R. H. Stokes, "Electrolyte Solutions Measurement and Interpretation of Conductance, Chemical Potential and Diffusion in Solutions of Simple Electrolytes", Academic Press, New York 1955.
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volume percent of methanol in the preliminary methanol/water mixture is taken as the volume percent of methanol to be used in the phosphate-buffered mobile phase. Once the volume percent of methanol has been established, the mole fraction (χ) of HPO_4^{2-} required to yield calculation values of μ 0.15 m and pH 7.40 can be accurately determined from eq i, where V is the volume

$$\chi = -4.554 \times 10^{-8}V_3 + 1.673 \times 10^{-6}V_2 - 3.728 \times 10^{-4}V + 0.7971 \quad \text{(i)}$$

$$n = 50,\ r = 0.99996,\ s = 7.265 \times 10^{-5}$$

percent methanol, n is the number of data points, r is the coefficient of correlation, and s is the standard deviation of regression. This expression was obtained for values of V from 1.25 to 62.5%. It has been our experience that precipitation of small amounts of phosphates have been observed in filtered solutions at volume percents greater than 50–55%, and mobile phases with compositions of methanol >55% (v/v) are not recommended.

After substitution of the value of $\chi(HPO_4^{2-})$ calculated from eq i into eq ii and iii, the weights of salts are then

$$[H_2PO_4^-] = \frac{0.15(1-\chi)}{2\chi + 1} - \text{molal concn} \quad \text{(ii)}$$

$$[HPO_4^{2-}] = \frac{0.15\chi}{2\chi + 1} - \text{molal concn} \quad \text{(iii)}$$

determined by multiplying these concentrations first by the calculated total weight of the solvent in kilograms [$\rho(H_2O)$ = 0.9971 g/mL and ρ(MeOH) = 0.7864 g/mL at 18 °C], and secondly by the appropriate molecular weights. A final recommendation is that the salts be dissolved in the appropriate volume of H_2O prior to the addition of MeOH.

Registry No. Sulfaguanidine, 57-67-0; sulfanilamide, 63-74-1; sulfacetamide, 144-80-9; sulfadiazine, 68-35-9; sulfamethoxazole, 723-46-6; sulfisoxazole, 127-69-5; sulfamerazine, 127-79-7; sulfathiazole, 72-14-0; sulfamethoxypyridazine, 80-35-3; sulfamethazine, 57-68-1; barbital, 57-44-3; allobarbital, 52-43-7; phenobarbital, 50-06-6; metharbital, 50-11-3; aprobarbital, 77-02-1; butabarbital, 125-40-6; cyclobarbital, 52-31-3; butalbital, 77-26-9; hexobarbital, 56-29-1; amobarbital, 57-43-2; pentobarbital, 76-74-4; secobarbital, 76-73-3; thiopental, 76-75-5; thiamylal, 77-27-0; methohexital, 151-83-7.

Phenylenebis(oxy)bis[2,2-dimethylpentanoic acid]s: Agents That Elevate High-Density Lipoproteins

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A series of phenylenebis(oxy)bis[2,2-dimethylpentanoic acid]s have been synthesized and evaluated as potential hypolipidemic agents. Compound **18** (CI-924) was found to be the most potent compound in this series. In rats, compound **18** not only reduced low-density lipoprotein cholesterol but also increased high-density lipoprotein (HDL) cholesterol. Comparative studies in rats indicated **18** produced an equal elevation of HDL cholesterol at one-third of the dose required of gemfibrozil. Structure–activity relationships are discussed.

The recent Framingham and other studies[1,2] have pointed out that high-density lipoprotein (HDL) cholesterol levels are inversely correlated with the incidence of atherosclerosis. Clofibrate or other hypolipidemic drugs do raise HDL cholesterol somewhat in patients, but the effect is not significant.[3] The most recent studies on new drugs affecting lipid and lipoprotein levels have therefore been aimed at the development of agents that decrease atherogenic lipoproteins (particularly LDL)[4] or increase high-density lipoproteins. Several hypolipidemic drugs, such as procetofene,[5] gemfibrozil,[6,7] bezafibrate,[8] and BR-931,[9] were recently shown to increase HDL levels in rats, as well as in humans. During our continued search for agents more potent than gemfibrozil, we discovered that phenylenebis(oxy)bis[alkanoic acid]s and their derivatives (Tables I–IV) effectively increase HDL cholesterol in rats treated with high lipid diets. In this paper we report the synthesis and structure–activity relationships of these compounds.

Chemistry. Phenylenebis(oxy)bis[alkanoic acid]s (IV) were prepared from various bis[phenol]s (I) by alkylation with α,ω-dihaloalkanes[10] (II) to give III (Table V), which were condensed with the dianion of isobutyric acid[11] (Scheme I, method A). Alternatively, the bis[phenol]s (I) were alkylated with methyl ω-bromo-2,2-dimethylalkanoate (V) to give the desired esters (VI), which were saponified to give the acids IV (Scheme I, method B).

Esters **54–56** were prepared by treating the corresponding acid chlorides with the respective alcohols or phenols (method C). Acids **3** and **18** on reduction with lithium aluminum hydride in tetrahydrofuran gave the corresponding alcohols **51** and **52** (method D). Acetate **53**

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