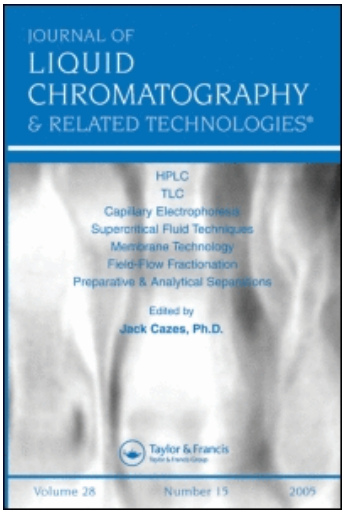

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A COMPARISON OF METHODS FOR THE DETERMINATION OF DEAD TIME IN A REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY SYSTEM USED FOR THE MEASUREMENT OF LIPOPHILICITY

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ABSTRACT

The dead time (void volume) of a reversed-phase system utilizing a column containing the divinylbenzene-styrene copolymer packing (PRP-1) was determined using candidate dead time markers, and two series of homologues, n-alkanols and n-alkyl benzenes. The retention data from the homologous series were subjected to several mathematical treatments. The retention data for n-alkanols gave consistent values for the dead time (1.24-1.27 minutes) provided that data from MeOH and EtOH were not included. Data from the n-alkyl benzene series did not provide a reliable value for system dead time. Of the candidate void volume markers examined, sodium nitrate and water (1.26 minutes) had retention times which were consistent with dead times calculated from the n-alkanol series. As nitrate is readily detected by both UV and RI at low concentrations, it is the preferred dead time marker for this HPLC system. The homologous series of compounds were used to calibrate the system for the determination of lipophilicity. The alkyl benzenes and alkanols provided two different calibration lines on plotting log k' vs log P (octanol). The calibration lines were not statistically different when plotting log k' vs log P (hexadecane).

INTRODUCTION

The determination of dead-time of HPLC systems has been the subject of numerous publications [1]. Generally, the most convenient method involves the measurement of

retention time of a solute which is believed to be unretained; D_2O [2], formamide [3,4], uracil [5], urea [6], methanol [7-9], inorganic salts [10-12] or the solvent disturbance peak [13] are the most commonly used dead time markers in reversed-phase systems. Alternatively, the measurement of retention times of an homologous series of compounds has been used in the determination of dead time [14-17], based on the assumption that there is a linear relationship between the logarithm of the net retention time (i.e. measured retention time minus system dead-time) and homologue number.

One application where knowledge of system dead time is essential is in the use of HPLC for the determination of lipophilicity. A study in our Institute [18] compared three reversed-phase HPLC systems for the determination of lipophilicity of technetium radiopharmaceuticals [19,20]. This resulted in the selection of a system based on the polymer PRP-1 packing material, eluted with an acetonitrile/buffer mixture. In conjunction with this study, it was necessary to investigate the most appropriate method for the determination of dead time on this HPLC system. The PRP-1 packing material is susceptible to swelling [21], so frequent dead time determinations are necessary using a reliable method. This report describes a comparison of methods for dead time determination on the PRP-1/acetonitrile-buffer HPLC system, involving conventional dead time markers, and two homologous series; n-alkyl alcohols and n-alkyl benzenes. The use of homologous series can serve a secondary purpose: calibration of the HPLC system with standard compounds of known lipophilicity.

THEORY

It is well known that, in liquid chromatography, the retention times of a homologous series are governed by the following relationship:

$$R_t = R_o + \exp(an + b) \qquad [1]$$

where R_t is the observed retention time of the n^{th} homologue, R_o is the dead time of the system, and a and b are series constants. Rearranging this equation gives:

$$\mathrm{Log}_e(R_t - R_o) = an + b \qquad [2]$$

As R_t, but not R_o, can be determined directly, a common approach in solving this equation for the determination of R_o is to plot $\mathrm{Log}_e(R_t)$ against n, extrapolate to n = 0 to provide a first estimate of R_o, which is used to plot $(\log_e(R_t - R'_o)$ vs n. The procedure is repeated several times to obtain the best-fit linear plot, from which the 'true' value of R_o

is obtained [22]. This repetitive technique should be amenable to rapid analysis using a personal computer. Alternatively, R_0 can be estimated from retention times of three sequential members of a homologous series [22] from the relationships:

$$\frac{^{n}\Delta R_t}{^{n-1}\Delta R_t} = e^{a} \qquad [3]$$

(where $^{n}\Delta R_t = {}^{n}R_t - {}^{n-1}R_t$) to solve for 'a' and the relationship:

$$^{n}\Delta R_t = (1-e^{a})\exp[a(n-1)+b] \qquad [4]$$

to solve for 'b'. A more involved solution for a, b and R_0 requires retention data on at least four sequential members of an homologous series [23]. In a repetitive linearization technique, candidate values of R_0 can be applied to equation 2, and plots of $\log_e(R_t - R_0)$ vs n subjected to linear regression analysis. The most appropriate value of R_0 is that which provides the highest value of the correlation coefficient [14,24].

Alternatively, Berendsen et al [25] noted that, plotting $^{n+1}R_t$ vs $^{n}R_t$ (where $^{n}R_t$ is the retention time of the nth homologue in the series), a linear plot was obtained, for which the following equation was derived:

$$^{n+1}R_t = A.{}^{n}R_t - (A-1).R_o \qquad [5]$$

where A is the slope of the graph. Thus, following linear regression analysis of the $^{n+1}R_t$ vs $^{n}R_t$, the value of A can be obtained, and from the intercept, the value of R_0 calculated.

EXPERIMENTAL

The HPLC system used for this study consisted of two Rainin Rabbit HPX pumps, controlled by a personal computer operating Gilson 712 software and a Knauer 198.00 refractive index detector. With this system, a Hamilton PRP-1 150x4.1mm, 5 μ resin (Anachem), was eluted with acetonitrile/pH 4.6 0.1M ammonium acetate buffer 65/35 [18], at 1.0 mL/min. The system allowed the moment of sample injection to be detected by the software, and sample retention times were provided automatically by the software on data analysis. Several possible dead time markers was also used. The observed retention times of these compounds are listed in tables 1 and 2. All values are the mean of three determinations.

Table 1. Observed retention times in two homologous series

Alkyl (R) =	ROH t_R (min)	RPh t_R (min)
methyl	1.39	7.55
ethyl	1.47	10.22
n-propyl	1.60	14.64
n-butyl	1.74	20.84
n-pentyl	1.97	29.96
n-hexyl	2.30	43.78
n-heptyl	2.76	62.90
n-octyl	3.44	91.55

Table 2. Determination of dead-time: Observed retention times of candidate dead time markers.

Candidate void marker	t_R (min)	Candidate void marker	t_R (min)
Water	1.26	urea	1.28
sodium nitrate	1.26	formamide	1.32
uracil	1.30	acetone	1.76

The n-alkanols, n-alkyl benzenes, and candidate dead time markers were purchased from Aldrich Chemical Co. HPLC grade acetonitrile was purchased from J.T. Baker, and water was obtained from a Millipore MilliQ water purification system.

Three methods were used in the determination of column dead-time from the observed retention times of the homologous series of alkyl alcohols and alkyl benzenes; the methods of Al-Thamir et al [22] and Berendsen et al [25] were used independently, and in combination. The linearization technique [14,24] was adapted to utilize a programmed Excel spreadsheet (with slight modification) recently described for the use in the determination of absolute charge of anionic technetium complexes from HPLC data and a linearization technique [26].

Log P_{oct} (oct = octanol) values were obtained from the Medchem database [27] and log P_{hex} (hex = hexadecane) were obtained from a published listing in [28].

RESULTS AND DISCUSSION

Several studies have shown that the measurement of the retention times of members of one homologous series and non-linear analysis of the data provides a value of the system

Table 3. Estimation of dead time from retention data on homologous series by the method of Al-Thamir et al [22]

Table 3a. Alkyl alcohols

	Mean Rt	a	b	Corr Rt*	R_0 *	Corr Rt**	R_0 **
methyl alcohol	1.39			0.20	1.19	0.15	1.23
ethyl alcohol	1.47			0.29	1.18	0.22	1.24
n-propyl alcohol	1.60	0.49	-2.54	0.41	1.19	0.33	1.27
n-butyl alcohol	1.74	0.12	-0.23	0.58	1.16	0.48	1.27
n-pentyl alcohol	1.97	0.45	-2.70	0.83	1.14	0.70	1.28
n-hexyl alcohol	2.30	0.35	-2.01	1.19	1.11	1.02	1.28
n-heptyl alcohol	2.76	0.34	-1.93	1.70	1.06	1.49	1.27
n-octyl alcohol	3.44	0.39	-2.38	2.43	1.01	2.18	1.26
Mean		0.36	-1.97		1.11		1.27
standard deviation		0.1284	0.9		0.066		0.0074

Corr R_t is (R_t - R_0)

* Result based on average values of 'a' and 'b', using all available data

** Result based on average values of 'a' and 'b' (0.38 ± 0.0491 and -2.26 ± 0.3573, respectively) using all data except for MeOH and EtOH

Table 3b. Alkyl benzenes

	Mean Rt	a	b	Corr Rt**	R_0 *	Corr	R_0 **
toluene	7.55			6.05	1.50	6.23	1.32
ethylbenzene	10.22			8.99	1.23	9.12	1.10
n-propylbenzene	14.64	0.51	0.89	13.35	1.29	13.33	1.31
n-butylbenzene	20.84	0.34	1.73	19.84	1.00	19.49	1.34
n-pentylbenzene	29.96	0.39	1.41	29.47	0.49	28.50	1.46
n-hexylbenzene	43.78	0.42	1.21	43.79	-0.01	41.68	2.10
n-heptylbenzene	62.90	0.32	1.96	65.06	-2.16	60.95	1.96
n-octylbenzene	91.55	0.40	1.22	96.67	-5.12	89.12	2.43
Mean		0.40	1.40		-0.75		1.77
standard deviation		0.0655	0.39		2.4625		0.4622

Corr R_t is (R_t - R_0)

* Result based on average values of 'a' and 'b', using all available data

** Result based on average values of 'a' and 'b' (0.38 ± 0.0405 and 1.45 ± 0.3274, respectively) using all data except for toluene

dead-time [14-17]. The majority of techniques are based upon equation 2, and assume that a plot of log (R_t - R_0) vs n is linear for the correct value of R_0. One of the simplest approaches involves the method of Al-Thamir et al [22] and requires retention data on only three sequential homologues (or three which are evenly spaced in the series), using equations 3 and 4 to determine the linearity constants 'a' and 'b'. The results of applying these equations to the retention data reported in this study are given in table 3.

As the method of Al-Thamir et al [22] requires data from just three sequential homologues, it is possible to derive 6 values of 'a' and 'b' for each series of eight homologues. The range of values given for these constants provides an indication of the precision of the method. The mean values for 'a' and 'b' are then substituted into equation 1 to obtain the "corrected" retention time (R_t - R_0) for each compound in the series. A value of R_0 is then determined for each data point, and the mean taken as the actual value of dead time

The values for a and b in the alkanol series (Table 3a) are reasonably consistent except those highlighted as italics. This may be the result of a deviation from linearity of the lowest member(s) of the homologous series, a phenomenon noted by others [23,25]. By taking the mean of all determined values of 'a' and 'b', the mean dead time is 1.11 minutes. The standard deviation of the mean of both 'a' and 'b' is much reduced when ignoring values which use data from methanol and ethanol, and the mean dead time derived from these values is 1.27 minutes.

While the values for 'a' and 'b' for the alkyl benzene series are also reasonably consistent (Table 3b), the dead-times calculated from the mean values of these two constants are not. Values range from +1.5 to -5.12! It was noted by others [23,25] that the retention time for toluene deviates from linearity when in the alkyl benzene series is evaluated on alkylsilanol-based reversed-phase columns. By excluding the data for toluene in determining mean values for 'a' and 'b', a strikingly different range of dead-times was obtained (Table 3b), with a range of values from 1.10 to 2.43 minutes. It appears that, while data from the alkanol series can be used to determine R_0 (provided that MeOH and EtOH are not used) using the method described by Al-Thamir et al [22], this method cannot be applied to the alkylbenzene series with this HPLC system.

The first step in the mathematical approach described by Berendsen et al [25] involved plotting $^{n+1}R_t$ vs $^{n}R_t$. Figure 1 displays the graphs obtained on subjecting the data presented in this study to that approach, while table 4 lists the results of linear regression analysis of that data, then using the slope (A) and intercept values to calculate the dead time according to equation 5. Visual (and mathematical) examination of the

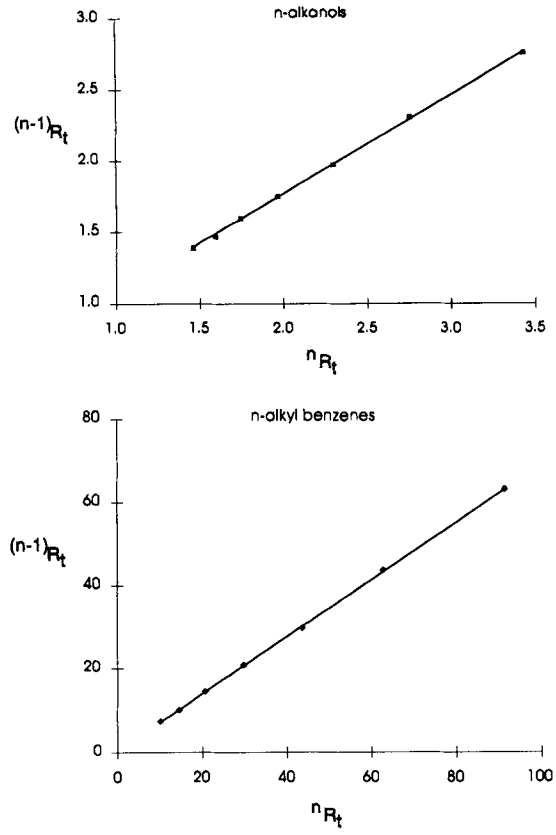

Figure 1. Plots of $^{n-1}R_t$ vs ^nR_t

Table 4. Dead time values determined using equation 5

	Alkanols		Alkylbenzenes	
	all data	partial data *	all data	partial data **
A	0.698	0.690	0.684	0.686
intercept	0.366	0.389	0.355	0.247
R	0.999830	0.999924	0.999928	0.999928
R_0 (min)	1.21	1.25	1.12	0.79

* excluding data from MeOH and EtOH

** excluding data from toluene

graphs shown in figure 1 indicate that, in the alkanol series, the data for MeOH and EtOH deviate slightly from the straight line generated by the other data points, while the data for toluene does this in the alkyl benzene series. Therefore, these data were analyzed including all data, and with these 'deviant' data points excluded. Again, the alkanol data set provided reasonably consistent values for R_0, while the alkyl benzene series did not.

An alternate approach, which involved mixing the Berendsen et al and Al-Thamir et al methods with the alkanol series, was also examined. In the ${}^{n+1}R_t$ vs ${}^{n}R_t$ plot, extrapolation of the graph to the point where n = 0 (i.e. ${}^{1}R_t$ on the y-axis and ${}^{0}R_t$ on the x-axis) allows ${}^{0}R_t$ to be determined. According to equation 1, ${}^{0}R_t = R_0 - \exp(b)$. Using the mean value of b (Table 3) determined by the Al-Thamir et al method gives values of R_0 of 1.19 (all data) and 1.26 (all data excluding MeOH and EtOH data points).

The final mathematical approach applied to the homologue data sets involved a linearization method. Bidlingmeyer et al [24] recently described a method whereby candidate values of dead time are used to derive plots of log (V_R/V_0) vs n, where n is the number of carbons in a side chain of an alkyl benzene. A range of candidate values of V_0 were selected. A graph of R^2 (the square of the correlation coefficient) vs. candidate V_0 value was generated, and the 'true' value of V_0 is the one at the maximum R^2.

As a large number of repetitive calculations are required by this approach, it is ideally suited to personal computer. One of us recently described a similar linearization method for data analysis using a programmed Excel spreadsheet [26]. That spreadsheet was modified to fulfill the requirements of this application. By entering the retention data, and selecting the range and increment of candidate dead time, the spreadsheet generated the R^2 vs candidate R_0 plot, and gave the R_0 value corresponding to the maximum value of R^2.

Re-analysis of some of the data presented in the Bidlingmeyer et al [24] article, using the programmed spreadsheet method, indicated that the authors' results were based on the linear regression analysis of plots of log $(V_R - V_0)$ vs n rather than log (V_R/V_0) vs n as stated. Presumably, this is a consistent typographical error in that paper. On further analysis of the Bidlingmeyer et al data, it was found that plotting log k' (again using candidate values V_0 to determine log k') gave the same optimum V_0 value as did linearization of log $(V_R - V_0)$ vs n (equation 5)

$$\text{Log } k' = a' n + b' \qquad [6]$$

To assess the reliability of the method, this approach was examined both with the full sets of data from the alkanol and alkyl benzene series, and with reduced sets of data,

Table 5. Estimated R_0 values from the alkyl alcohol and alkylbenzene series using equation 2 and selected ranges in the homologous series

	Alkyl alcohols		Alkylbenzenes		
C_T to C_1					
r =	Ro	R^2	Ro	R^2	n
8	1.19	0.99975	1.37	0.99991	8
7	1.18	0.99970	1.49	0.99989	7
6	1.17	0.99956	1.74	0.99988	6
5	1.16	0.99930	1.80	0.99979	5
4	1.14	0.99866	2.30	0.99972	4
3	1.26	0.99999	3.52	1.00000	3
C_T to C_8					
r =					
1	1.19	0.99976	1.37	0.99991	8
2	1.19	0.99964	0.74	0.99997	7
3	1.26	0.99993	1.01	0.99997	6
4	1.22	0.99998	0.34	0.99996	5
5	1.24	0.99998	-0.94	0.99994	4
6	1.34	1.00000	5.38	1.00000	3

taking sets from C_n - C_8 (n = 2 to 6), and C_1 - C_n (n = 3 to 7). The results are tabulated in table 5. These results show the maximum value of R^2 for each data set, with the corresponding value of R_0, based on equation 1. The values of R^2 and R_0 were identical to those shown when these linearization calculations were based on equation 6 rather than equation 2.

As in the other mathematical treatments, described above, the alkanol series appear to provide the most reliable results. Taking the full data set, or reduced sets which included MeOH and EtOH, values of R_0 were consistently below 1.2 (1.14-1.19). With these two lower alkanols excluded, R_0 averaged 1.24. When the number of data points were <4, the values of R_0 deviated considerably from the others, indicating that this method is unreliable with so few data points. The values for R_0 derived from the alkyl benzene series varied widely, again indicating that this homologous series cannot be used for dead time determination of the PRP-1 HPLC system.

The programmed spreadsheet method was also modified to be applied to equation 1; that is, candidate R_0 values were applied to plots of (R_t - R_0) vs exp(n), and plots of R^2 vs R_0 were generated. The results are shown in table 6. For the alkanol series, the results are similar (but not identical) to those in table 5.

Table 6. Estimated R_0 values from the alkyl alcohol and alkylbenzene series using equation 1 and selected ranges in the homologous series

	Alkyl alkohols		Alkylbenzenes		
C_T to C_1 r =	Ro	R^2	Ro	R^2	n
8	1.21	0.99991	0.96	0.99997	8
7	1.19	0.99988	0.97	0.99993	7
6	1.19	0.99971	1.63	0.99996	6
5	1.16	0.99939	1.41	0.99991	5
4	1.10	0.99878	1.77	0.99977	4
3	1.26	1.00000	3.52	1.00000	3
C_T to C8 r =					
1	1.21	0.99991	0.96	0.99997	8
2	1.22	0.99992	0.72	0.99998	7
3	1.25	0.99997	0.75	0.99997	6
4	1.23	0.99998	0.42	0.99996	5
5	1.26	0.99998	0.28	0.99995	4
6	1.34	1.00000	5.36	1.00000	3

It can be seen that all of these mathematical approaches for the determination of column dead time give reasonably consistent values (1.24 - 1.27) for the system under study, using the alkanol series when data derived from MeOH and EtOH are excluded. By comparison, the values of R_0 derived from the alkyl benzene series vary widely. Others, using alkyl-silica reversed-phased columns have noted that the determination of dead time from alkyl benzene retention data can be "unreliable" [23,24]. The reasons for this observation remain unclear. For the styrene-divinylbenzene PRP-1 packing material used in this study, a dual retention mechanism (the proposed pi-pi retention mechanism [29] attributed to aromatic substrates PRP-1 column, as well as purely hydrophobic interaction) may be a contributing factor.

It is clearly undesirable to use a homologous series (particularly one without a chromophore) for the determination of column dead time when a single compound might be used instead. Several dead time markers were evaluated on the PRP-1 system, and the observed retention times of these markers are recorded in table 2. The values for water and sodium nitrate are in good agreement with the R_0 values calculated from the alkanol series data. Uracil, urea, and formamide show partial retention, while acetone is clearly unsuitable. From this list, nitrate appears to be the most suitable. Berendsen et al [25]

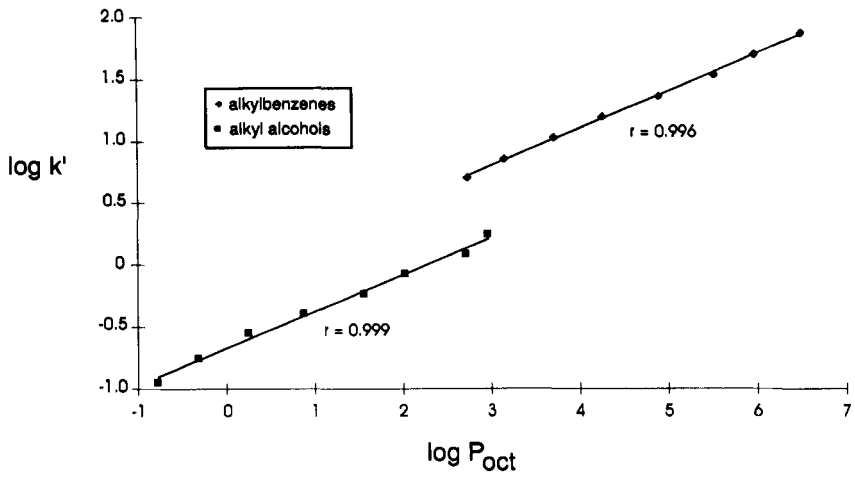

Figure 2. Graph of log k' vs log P_{oct}

have demonstrated a concentration effect when using salts as dead time markers, although the presense of a buffer in the aqueous phase appears to eliminate variability due to solute concentration [30]. Given that nitrate is readily detected at low concentration by UV (210 nm) and RI detectors, it should be the most appropriate of the systems tested in this study for routine use.

As the HPLC system reported in this study was established for the determination of lipophilicity [18], one advantage of the homologous series approach to dead time determination is that the same study can be used to calibrate the system. In figures 2 and 3 are shown graphs of log k' values of the alkanol and alkyl benzene series. As found previously [18], the calibration line for hydroxy compounds is different to that for non-hydrogen-bonding compounds, when log k' is plotted against log P_{oct}. However, plotting log k' vs log P_{hex} gives two calibration lines which are less separated than those in the log k'-log P_{oct} graph.

Analysis of covariance was used to determine whether log k' vs log P (octanol or hexadecane) data for the alkyl alcohols (group 1) were different from that for alkylbenzenes (group 2). The model used was:

log k' = constant + group + log P + (group x log P)

where log k' is the dependent variable, group is the dependent variable,
and log P is the covariate.

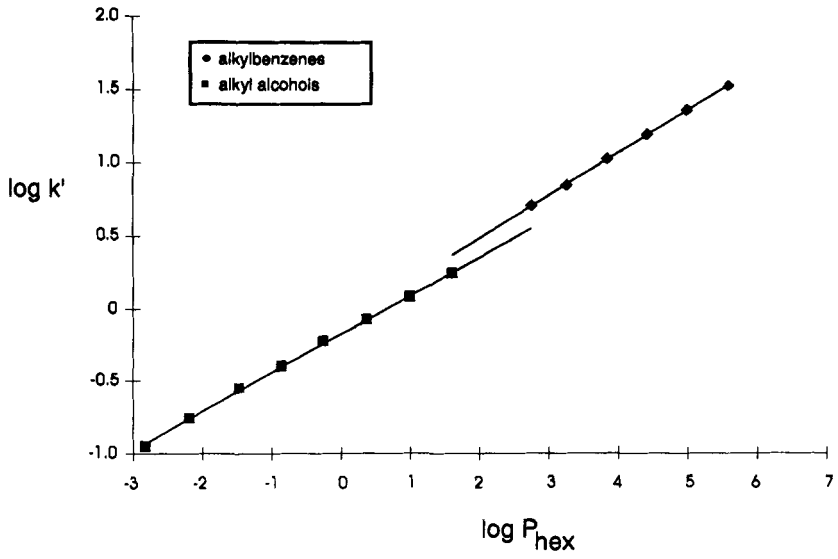

Figure 3. Graph of log k' vs log P_{hex}

In the analysis, the interaction term (group x log P) was checked first to see if the regression lines are parallel. The analysis of the data log k' vs log P_{oct} showed that the lines for the two groups are indeed parallel. However, the adjusted treatment mean of group 1 is significantly different from that of group 2 ($p < 0.001$). For the second set of data, log k' vs log P_{hex} the regression lines for the two groups are not parallel, thus invalidating the major assumption in the analysis of covariance. Moreover, the adjusted means of the two groups are significantly different ($p < 0.001$)

These data indicate that this acetonitrile/buffer PRP-1 HPLC system should provide a good estimate of log P_{oct} or log P_{hex} values using compounds drawn from a single set of congeners when the system is calibrated with compounds (of known log P) from the same class. Additionally, this system may provide a somewhat less accurate estimation of log P_{hex} of compounds drawn from several classes when the system is calibrated by compounds drawn either from a single set, or multiple sets, of congeners.

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