*Journal of Chromatography*, 174 (1979) 35–50


CHROM. 11,746

# GRADIENT ELUTION IN LIQUID CHROMATOGRAPHY

## X. RETENTION CHARACTERISTICS IN REVERSED-PHASE GRADIENT ELUTION CHROMATOGRAPHY

PAVEL JANDERA, JAROSLAV CHURÁČEK and LADISLAV SVOBODA
*Department of Analytical Chemistry, University of Chemical Technology, Pardubice (Czechoslovakia)*
(First received October 20th, 1978; revised manuscript received January 15th, 1979)

SUMMARY

The reversed-phase chromatographic behaviour of selected barbiturates, alkaloids and substituted alkyluracils on a $C_{18}$ bonded phase column was investigated in both isocratic and gradient elution experiments using a binary mobile phase composed of water and methanol. Experimental capacity ratios of sample compounds under isocratic conditions fitted well with the linear relationships of log $k'$ *versus* concentration of methanol in the mobile phase. The approach for the calculation of retention volumes and peak widths in gradient elution chromatography presented earlier was extended to reversed-phase chromatography and verified experimentally. The agreement between the experimental and theoretically predicted values was satisfactory.

## INTRODUCTION

The number of reversed-phase liquid chromatography separations has increased considerably during the past few years. At present, the reversed-phase technique is by far the most important and versatile liquid chromatographic method which permits separations of various organic compounds of a wide polarity range (hydrocarbons to sulphonic acids)[1,2], including various separations of natural products, biochemicals and organic pollutants in water[9,16–19]. Natural and environmental samples usually contain compounds with large structural differences and the elution of all sample compounds in one run usually requires the application of the gradient elution technique, in which good reproducibility of the gradient profile and flow-rate is essential for reliable quantitation and identification of sample compounds[3].

The possibility of calculating retention data in reversed-phase gradient elution chromatography would be useful for the identification of peaks. Schoenmakers *et al.*[4] described a method for the calculation of retention times based on a quadratic function between log $k'$ and the content of the organic solvent in the mobile phase, but gave no experimental verification of their complex calculations. In most practical examples, a linear function can be used instead of the quadratic function in the concentration range of practical importance for chromatographic separations. In the

present work, our theoretical approach for the prediction of retention data in gradient elution operation, published previously for liquid–liquid chromatography[5], has been extended to reversed-phase chromatography, yielding a simplified model that has been verified experimentally.

## RETENTION DATA IN REVERSED-PHASE CHROMATOGRAPHY

The calculation of retention volumes and peak widths in gradient elution chromatography from isocratic data is possible only if two basic assumptions are fulfilled:

(1) the mathematical form of the function describing the influence of the composition of the mobile phase on the capacity ratios of chromatographed compounds under isocratic conditions is known;

(2) the mathematical form of the gradient profile [the relationship between the composition of the mobile phase at the inlet of column and time (or volume of the eluate)] is known and can be expressed in an adequately simple manner.

The calculations are considerably simplified if the following assumptions can be accepted. Firstly, it is assumed that no gradient profile distortion occurs during the gradient elution. This requirement is reasonably fulfilled in practice if the liquid chromatograph is designed in such a manner that any off-line void spaces and excessive volumes of the connecting tubing between the outlet of the gradient pump and the inlet of the chromatographic column are avoided. Further, a preferential retention of one component of the mobile phase on the column in comparison with the other components can be neglected, which is a realistic assumption in reversed-phase chromatography.

As most practical separation problems can be solved using binary mobile phases, one component being a much stronger eluent than the other, the calculations of retention characteristics are limited to binary solvent mixtures. In practical reversed-phase chromatography, eluents composed of water and methanol or water and acetonitrile are used almost exclusively.

The mechanism of retention on the hydrophobic stationary phase from aqueous–organic solutions is complex and still open to question. Horváth *et al.*[6] gave a detailed discussion of solvophobic effects, which play a role in the reversed-phase chromatographic process. According to these workers, the change in capacity ratio with change in the composition of a binary mobile phase such as methanol–water or acetonitrile–water is due primarily to the changes in the surface tension and in the ratio of the energy required to create a cavity for a molecule of the solute in the solvent to the energy required to expand the planar surface of the solvent by the same area. For non-ionized solutes, both the electrostatic and the Van der Waals forces and the entropy of mixing of the components of the mobile phase remain constant or tend to counterbalance each other with changing composition of the mobile phase.

Other workers[4] have attempted to apply the Hildebrand theory of regular solutions[7] to reversed-phase chromatography.

As we showed earlier[8], the following relationship between the capacity ratio ($k'$) and the concentration ($c$) of the more efficient eluting component in the binary mobile phase follows from this theory after certain simplifying assumptions:

$$k' = k'_0 \cdot 10^{-\alpha c} \qquad (1)$$

where $k'_0$ and $n$ are experimental parameters characteristic of the stationary and mobile phase and the solute.

Eqn. 1 is formally identical with the relationship found empirically for the dependence of the capacity ratio on the concentration of the organic component of the eluent in reversed-phase chromatography. The linear character of experimental log $k'$ *versus* $c$ curves in reversed-phase chromatography has been demonstrated experimentally by a number of workers[2,3,6,9–11], at least over a limited composition range of the mobile phase. $k'_0$ in eqn. 1 represents the capacity ratio of the solute in the pure weaker eluent (water). It is interesting that the influence of various structural and system factors on $k'_0$ and $n$ in eqn. 1 can be interpreted in qualitative agreement with the expressions expected for these parameters from the simplified regular solution theory[7]. Thus, from this theory it follows that the retention in water should increase with increasing size (molal volume) of the solute, increasing difference in polarity between the solute and the eluent (water) and decreasing difference in polarity between the solute and the stationary phase, which is fully confirmed by the experimental chromatographic behaviour of different organic compounds in reversed-phase systems. Further, a linear increase of log $k'$ in water with increase in the term $1/T$ would be expected and has been measured experimentally for aromatic phenolic compounds[6] and other aromatics[9].

The parameter $n$ represents the slope of the log $k'$ *versus* $c$ relationship. According to the above-mentioned theory[7], $n$ is expected to increase with increasing size (molal volume) and/or decreasing polarity of the solute. This was confirmed experimentally for *n*-hexanol and *n*-octanol in a mobile phase composed of water and methanol[10] and for phenol, benzophenone, biphenyl and *o*-terphenyl in water–methanol and water–acetonitrile[11]. $n$ should also increase with decreasing polarity of the organic solvent used. Experimentally observed $n$ values for chloronaphthalene and anthraquinone in dioxane–water are higher than in methanol–water[9] and $n$ for phenol is higher in isopropanol than in methanol[11]. The influence of temperature on $n$ has not yet been studied. Further, $n$ should not depend on the properties (solubility parameter) of the stationary (reversed) phase. Karch *et al.*[2] found identical experimental values of $n$ for butanol and phenol on reversed-phase $C_{18}$ and $C_4$ in water–methanol mobile phases. The qualitative agreement between the interpretation of the parameters $k'_0$ and $n$ in eqn. 1 based on the regular solution theory[8] and the experimentally found dependence of these parameters on certain structural and system factors do not mean that the solubility parameters from the literature can be used to calculate these parameters, which should always be evaluated from the experimental data measured in a given system. In an attempt to correlate these data[4], it was found that the discrepancies obtained were too large to allow for the meaningful utilization of $k'_0$ and $n$ calculated from the solubility parameters.

In our opinion, there is enough experimental evidence to encourage the application of eqn. 1 as a sound basis for calculations of retention characteristics in isocratic and gradient elution chromatography. Of course, as the derivation of this relationship implies simplified assumptions, deviations from eqn. 1 can be expected, mainly for concentrations of the organic solvent in the mobile phase close to 0 or to 100%.

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As we showed previously[5], the gradient function [concentration ($c$) of the more efficient eluting component in the mobile phase *versus* volume of the eluate ($V$)] can be chosen with advantage to be in the form

$$c = (A^{\frac{1}{\varkappa}} + BV)^{\varkappa} \tag{2}$$

where $A$ represents the initial concentration at the beginning of gradient elution, $B$ is the slope of the gradient function and $\varkappa$ is used for the convenient characterization of the shape (curvature) of the gradient. However, the mathematical derivation of relationships for retention characteristics in gradient elution chromatography in the explicit form would not be possible using gradient function according to eqn. 2. For this reason, the derivation is limited to the linear gradient function with $\varkappa = 1$:

$$c = A + BV \tag{3}$$

According to the mathematical solution given earlier[5], the net retention volume, $V'_{R(g)}$, in reversed-phase gradient elution chromatography is then given by the relationship

$$V'_{R(g)} = \frac{1}{nB} \cdot \log (2.31nBV_m k'_0 + 10^{nA}) - \frac{A}{B} \tag{4}$$

or, if the gradient elution is started at zero concentration of the efficient eluting component in the mobile phase ($A = 0$):

$$V'_{R(g)} = \frac{1}{nB} \cdot \log (2.31nBV_m k'_0 + 1) \tag{5}$$

The width of the peak in gradient elution chromatography can be understood as a result of three effects: the spreading of the solute band with time as it moves along the column, the value of $k'$ at the moment of elution of the peak maximum and the compression resulting from the fact that the front of the band moves in the mobile phase with a lower eluting strength than the end of the band[12]. Neglecting the last effect, we derived the relationship for the band width, which can be used in reversed-phase gradient elution chromatography[5]:

$$w_{(g)} = \frac{4V_m}{\sqrt{N}} [1 + k'_0 \cdot 10^{-n(A + BV'_{R(g)})}] \tag{6}$$

Eqn. 6 can be written in another form as

$$w_{(g)} = \frac{4V_m}{\sqrt{N}} [1 + k'_0 (2.31nBV_m k'_0 + 10^{nA})^{-1}] \tag{7}$$

$N$ is the plate number from isocratic elution chromatography, which is supposed not to depend significantly on the composition of the mobile phase.

Convex concentration gradients of different shapes can be generated using a logarithnic gradient function:

$$c = \log (A^{\frac{1}{\varkappa}} + BV)^{\varkappa} \tag{8}$$

where the concentration of the efficient eluting component in the mobile phase at the beginning of the gradient is given as log $A$. With this function, the following equations for the net retention volume, $V'_{R(g)}$, and peak width, $w_{(g)}$, can be derived:

$$V'_{R(g)} = \frac{1}{B}\left[(\varkappa n + 1)Bk'_0V_m + A^{\frac{\varkappa n+1}{\varkappa}}\right]^{\frac{1}{\varkappa n+1}} - \frac{A^{\frac{1}{\varkappa}}}{B} \quad (9)$$

and

$$w_{(g)} = \frac{4V_m}{\sqrt{N}}\{1 + k'_0[(\varkappa n + 1)Bk'_0V_m + A^{\frac{\varkappa n+1}{\varkappa}}]^{-\frac{\varkappa n}{\varkappa n+1}}\} \quad (10)$$

## EXPERIMENTAL

### *Gradient elution instrumentation*

The concentration gradient was generated in a low-pressure gradient two-plunger pump (PPM-68005, Workshops of the Czechoslovak Academy of Sciences, Prague) based on a photoelectric curve follower[13]. The mixed mobile phase was introduced into a Waters M6000 pump (with an adapted inlet port, in order to minimize the void volume) and delivered via a Waters U6K injector on to the column. The detector was a Waters 254-nm UV detector. The stainless-steel column (300 × 4.2 mm I.D.) was packed with an octadecylsilica reversed phase prepared from LiChrosorb Si 100 (10 $\mu$m) by reaction with *n*-octadecyltrichlorosilane[14]. The results of testing of this system for gradient elution will be published later; the equipment is able to reproduce very well any desired mathematical form of gradient function when operated in the "LOAD" position of the U6K injector; a correction for the time delay of the mixed mobile phase between the outlet of the PPM-68005 pump and the column had to be made by performing the sample injection a pre-calculated time after the start of the gradient run (198 sec at 0.97 ml/min).

### *Components of the mobile phase*

Deionized water was doubly distilled with addition of potassium permanganate and sodium hydrogen carbonate, and was stored for a maximum of 2 days in glass bottles. Methanol was of spectroscopic grade.

### *Samples*

Alkaloids and barbiturates were commercial reagent-grade materials. Substituted alkyluracils were synthesized at the Department of Organic Chemistry, University of Pardubice.

## RESULTS AND DISCUSSION

### *Isocratic elution*

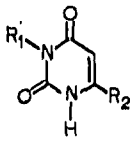
To verify the validity of the theoretical relationships for retention characteristics in reversed-phase gradient elution chromatography, relatively polar test compounds were chosen (barbiturates, xanthine alkaloids and substituted uracils). In the reversed-phase chromatography of such compounds, larger deviations from eqn. 1 can be expected than with less polar solutes.

Table I gives the values of $k'_0$ and $n$ evaluated by linear regression analysis from

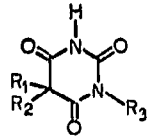
TABLE I

EXPERIMENTAL VALUES OF $k'_0$ AND $n$ (EQN. 1) FOR XANTHINE ALKALOIDS, SUBSTITUTED URACILS AND BARBITURATES IN REVERSED-PHASE CHROMATOGRAPHY ON A $C_{18}$ (OCTADECYLSILICA) COLUMN (4.2 × 300 mm) FROM ISOCRATIC EXPERIMENTS IN THE MOBILE PHASE METHANOL–WATER (5–80% METHANOL)

$k'$ values in the range 0.4–10 were used for regression analysis. $r$ are the correlation coefficients for regression of log $k'$ *versus* $c$.

| *No.* | *Compound* | *Substituents to basic structure** | *Number of carbon atoms in substituents** | $k'_0$ | $n$ | $r$ |
|---|---|---|---|---|---|---|
| | *Xanthine alkaloids* | | | | | |
| 1 | Hypoxanthine | | 0 | 2.18 | 4.84 | – |
| 2 | Xanthine | | 0 | 2.66 | 4.90 | – |
| 3 | Theobromine | | 2 | 13.14 | 4.65 | −0.9921 |
| 4 | Theophylline | | 2 | 12.87 | 3.45 | −0.9944 |
| 5 | Caffeine | | 3 | 32.28 | 3.78 | −0.9982 |
| | *Uracils* |  | | | | |
| 6 | 3,6-Dimethyluracil | $R_1 = CH_3$; $R_2 = CH_3$ | 2 | 9.51 | 4.40 | −0.9968 |
| 7 | 3-Ethyl-6-methyluracil | $R_1 = C_2H_5$; $R_2 = CH_3$ | 3 | 15.63 | 3.69 | −0.9947 |
| 8 | 3-*n*-Propyl-6-methyluracil | $R_1 = n\text{-}C_3H_7$; $R_2 = CH_3$ | 4 | 29.98 | 3.31 | −0.9939 |
| 9 | 3-Isopropyl-6-methyluracil | $R_1 = \text{iso-}C_3H_7$; $R_2 = CH_3$ | 4 | 31.39 | 3.32 | −0.9967 |
| 10 | 3-*sec.*-Butyl-6-methyluracil | $R_1 = sec\text{-}C_4H_9$; $R_2 = CH_3$ | 5 | 54.56 | 3.21 | −0.9990 |
| 11 | 3-Isobutyl-6-methyluracil | $R_1 = \text{iso-}C_4H_9$; $R_2 = CH_3$ | 5 | 61.16 | 3.25 | −0.9998 |
| 12 | 3-*n*-Butyl-6-methyluracil | $R_1 = n\text{-}C_4H_9$; $R_2 = CH_3$ | 5 | 67.20 | 3.25 | −0.9995 |
| 13 | 3-*tert.*-Butyl-6-methyluracil | $R_1 = tert.\text{-}C_4H_9$; $R_2 = CH_3$ | 5 | 96.25 | 3.40 | −0.9994 |

*Barbiturates*



| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 14 | Barbital | $R_1 = C_2H_5$; | $R_2 = C_2H_5$; | $R_3 = H$ | 4 | 21.81 | 3.20 | −0.9998 |
| 15 | Heptobarbital | $R_1 = CH_3$; | $R_2$ = phenyl; | $R_3 = H$ | 4.5 | 58.44 | 3.71 | −0.9995 |
| 16 | Allobarbital | $R_1$ = allyl; | $R_2$ = allyl; | $R_3 = H$ | 5 | 69.44 | 3.55 | −0.9991 |
| 17 | Aprobarbital | $R_1 = C_2H_5$; | $R_2$ = iso-$C_3H_7$; | $R_3 = H$ | 5 | 106.96 | 3.66 | −0.9994 |
| 18 | Phenobarbital | $R_1 = C_2H_5$; | $R_2$ = phenyl; | $R_3 = H$ | 5.5 | 117.57 | 3.81 | −0.9995 |
| 19 | Cyclobarbital | $R_1 = C_2H_5$; | $R_2$ = cyclohexenyl; | $R_3 = H$ | 6 | 169.94 | 3.73 | −0.9992 |
| 20 | Butobarbital | $R_1 = C_2H_5$; | $R_2$ = *sec.*-$C_4H_9$; | $R_3 = H$ | 6 | 187.41 | 3.78 | −0.9996 |
| 21 | Hexobarbital | $R_1 = CH_3$; | $R_2$ = cyclohexenyl; | $R_3 = CH_3$ | 6 | 252.29 | 3.77 | −0.9992 |
| 22 | Pentobarbital | $R_1 = C_2H_5$; | $R_2$ = *sec.*-$C_5H_{11}$; | $R_3 = H$ | 7 | 470.65 | 4.07 | −0.9994 |
| 23 | Amobarbital | $R_1 = C_2H_5$; | $R_2$ = iso-$C_5H_{11}$; | $R_3 = H$ | 7 | 617.73 | 4.29 | −0.9996 |

* An allyl substituent is considered as equivalent to 2.5, a phenyl ring to 3.5 and a cyclohexenyl ring to 4 carbon atoms (methylene groups) in aliphatic substituents.

the experimental values of the capacity ratios measured in the isocratic elution experiments with mobile phases containing different concentrations of methanol in water. The plots of log $k'$ against concentration of methanol in the mobile phase are shown in Figs. 1–3. The deviations from linearity are negligible with xanthine, hypoxanthine and all of the barbiturates tested and can be tolerated with the isomeric butyl-6-methyluracils. The plots for lower substituted uracils and especially for caffeine, theophylline and theobromine deviate significantly from linearity.

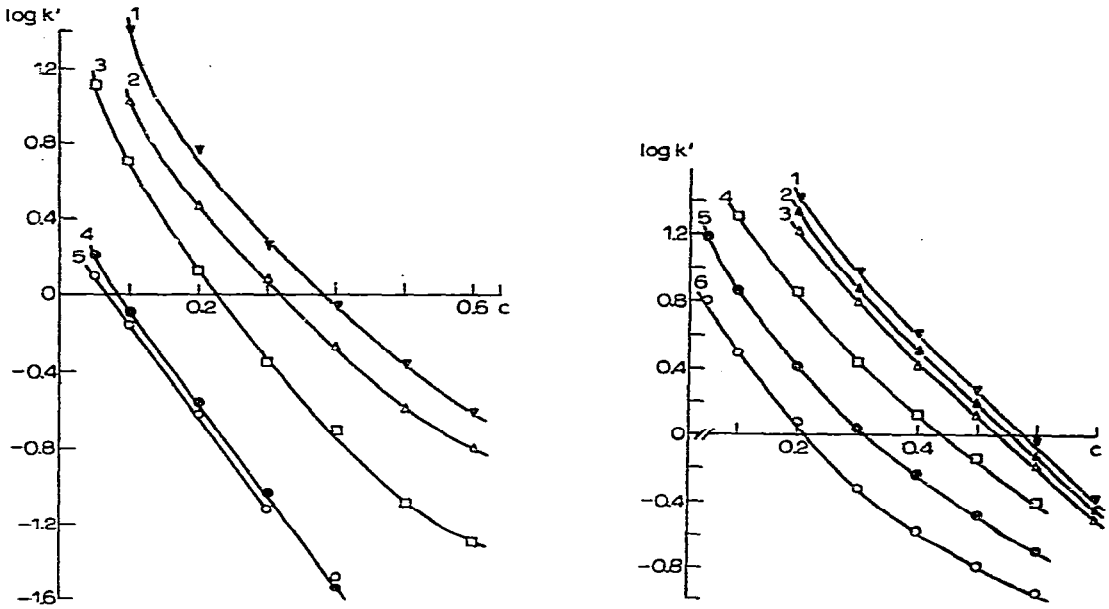
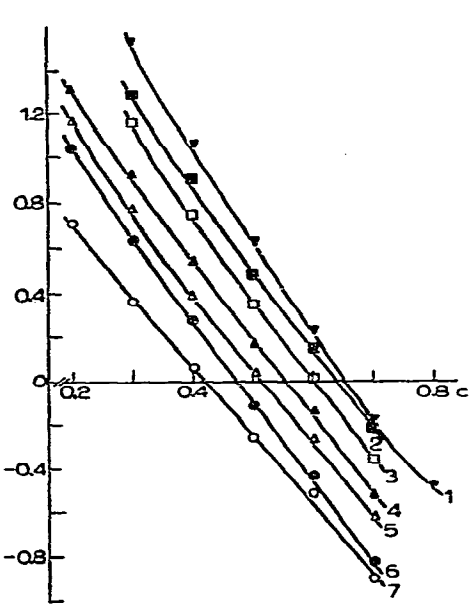



Fig. 1. Semilogarithmic plots of $k'$ of xanthine alkaloids *versus* the concentration of methanol in the water–methanol mobile phase ($c$ = volume % × $10^{-2}$) on $C_{18}$ reversed phase. Isocratic elution. Column: octadecylsilica chemically bonded on LiChrosorb Si 100 (10 $\mu$m); 300 × 4.2 mm; $V_m$ = 3.2 ml. Compounds: 1 = caffeine; 2 = theophylline; 3 = theobromine; 4 = xanthine; 5 = hypoxanthine.

Fig. 2. Semilogarithmic plots of $k'$ of substituted uracils *versus* the concentration of methanol in the water–methanol mobile phase ($c$ = volume % × $10^{-2}$) on $C_{18}$ reversed phase. Column as in Fig. 1. Compounds: 1 = 3-*tert.*-butyl-6-methyluracil; 2 = 3-*n*-butyl-6-methyluracil; 3 = 3-*sec.*-butyl-6-methyluracil; 4 = 3-*n*-propyl-6-methyluracil; 5 = 3-ethyl-6-methyluracil; 6 = 3,6-dimethyluracil.

In gradient elution chromatography, however, it is reasonable to expect chromatographed compounds to move along the column with actual instantaneous $k'$ values, which are within an intermediate range of $k'$ values (isocratic). Thus, it seems acceptable to suppose that the capacity ratios during gradient elution are not lower than 0.4 and that the mobility of solutes along the column can be neglected if $k' > 10$. In the corresponding range of log $k'$ values (—0.4 to 1.0), the lines can be fitted well to the experimental plots for all of the compounds. The values of $k'_0$ and $n$ were thus evaluated using only the experimental $k'$ values from the above region (Figs. 1–3). It should be mentioned further that the accuracy of the determination of $k'$ values lower than 0.4 is subject to a relatively large experimental error and thus $k'$ values of this magnitude should preferably be omitted in regression analysis in order not



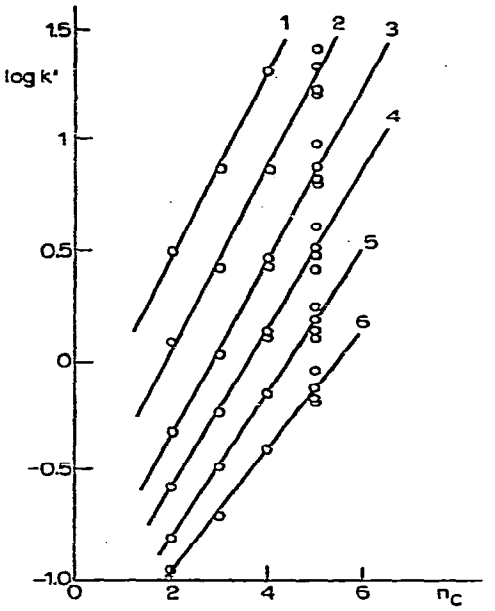




Fig. 3. Semilogarithmic plots of $k'$ of barbiturates *versus* the concentration of methanol in the water–methanol mobile phase ($c$ = volume % $\times 10^{-2}$) on $C_{18}$ reversed phase. Column as in Fig. 1. Compounds: 1 = amobarbital; 2 = hexobarbital; 3 = butobarbital; 4 = aprobarbital; 5 = allobarbital; 6 = heptobarbital; 7 = barbital.

Fig. 4. Semilogarithmic plots of $k'$ of substituted uracils *versus* the number of carbon atoms in aliphatic substituents ($n_c$) for different concentrations of methanol in the mobile phase: (1) 10%; (2) 20%; (3) 30%; (4) 40%; (5) 50%; (6) 60%. Column as in Fig. 1. The points on the lines are for $k'$ of *n*-substituted uracils.

to influence the results by a systematic error. The fit of the regression lines to the experimental data in the range of $k'$ values between 0.4 and 10 is demonstrated by the correlation coefficients in Table I.

As expected, the retention of all of the compounds studied (and $k'_0$ values) increases with increasing number of carbon atoms in the aliphatic chains. Unsaturated bonds and aromatic rings cause a decrease in polarity; thus, in the series of barbiturates, an allyl group has the same effect as about 2.5 carbon atoms, a phenyl ring about 3.5 carbon atoms and a cyclohexenyl ring about 4 carbon atoms. Branching of the aliphatic chain has only a minor effect on retention.

From the experimental data it follows that in the system studied, a good validity of eqn. 1 over a wide range of methanol concentrations in the mobile phase can be expected only if the substituents on the basic structure of xanthine, uracil or barbituric acid represent the equivalent of at least four carbon atoms.

Figs. 4 and 5 show linear relationships between $\log k'$ and the number of carbon atoms in the substituents for alkyluracils and barbiturates (phenyl and cyclohexenyl rings and allyl groups were substituted by 3.5, 4 and 2.5 aliphatic carbon atoms, respectively). The contribution of one carbon atom (one methylene group) to $\log k'$, $\Delta \log k'$, depends on the content of methanol in the mobile phase (Fig. 6), which is in agreement with experimental data for *n*-alkanols[10,15]. A decrease in $\Delta \log k'$ with increasing amount of organic solvent in the mobile phase can be predicted theoretically using

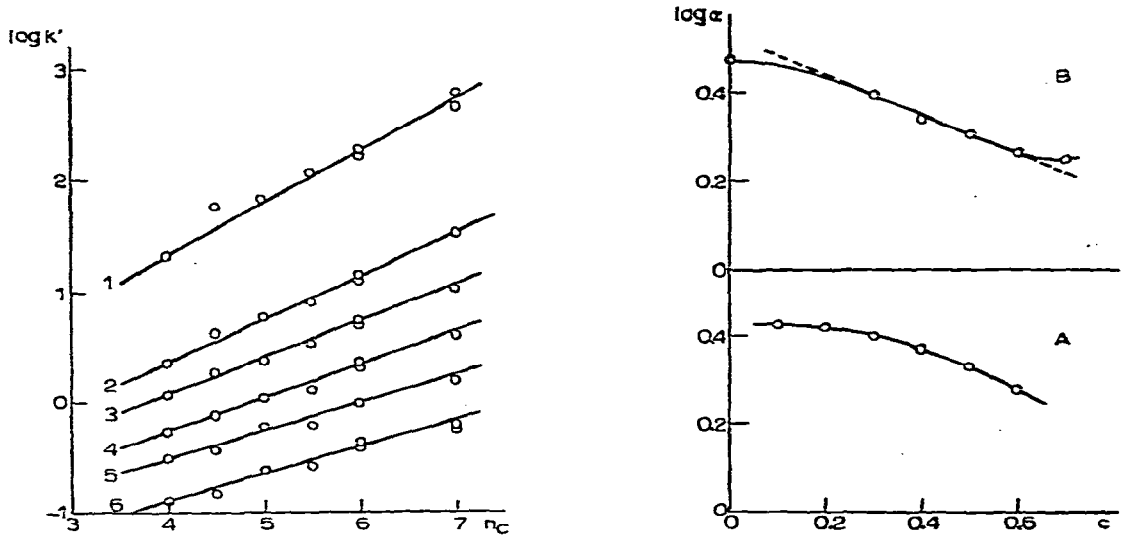



Fig. 5. Semilogarithmic plots of $k'$ of barbiturates *versus* the number of carbon atoms in hydrocarboneous substituents ($n_c$) for different concentrations of methanol in the mobile phase: (1) 0% (extrapolated from the $k'_0$ values); (2) 30%; (3) 40%; (4) 50%; (5) 60%; (6) 70%. Column as in Fig. 1. The lines were fitted through the points corresponding to $k'$ of barbital, butobarbital, pentobarbital and amobarbital. An equivalent of 2.5 $n_c$ was considered for an allyl group, $3.5n_c$ for a phenyl ring and $4n_c$ for a cyclohexenyl ring. The points are not given for aprobarbital ($n_c = 5$) and hexobarbital ($n_c = 6$).

Fig. 6. Plots of $\log\alpha = \Delta\log k'$ for substituted uracils (A) and barbiturates (B) *versus* the concentration ($c$ = volume % $\times 10^{-2}$) of methanol in the mobile phase. The values of $\log\alpha$ were taken from Figs. 4 and 5 as the slopes of $\log k'$ *versus* $n_c$ lines. The point at 0% methanol is extrapolated from $k'_0$ values.

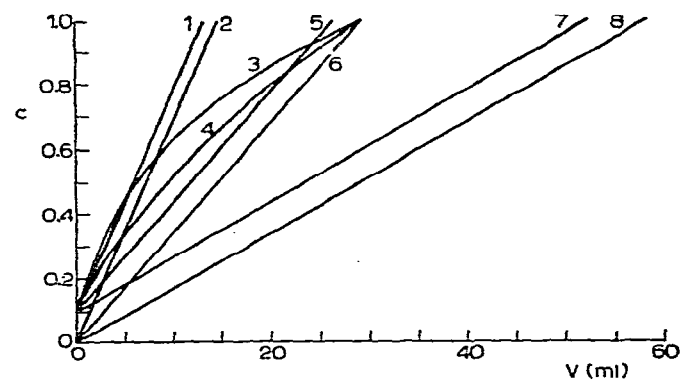



Fig. 7. Gradient functions used for verification of theoretical relationships for retention characteristics in reversed-phase gradient elution chromatography using water–methanol as the mobile phase. Linear gradient functions: $c = A + B \cdot V$. $A = 0$ for functions 2, 6 and 8; $A = 0.1$ for functions 1, 5 and 7. $B = 0.06872$ for functions 1 and 2; $B = 0.03436$ for functions 5 and 6 and $B = 0.01718$ for functions 7 and 8 ($B$ values were calculated for a flow-rate of the mobile phase of 0.97 ml/min). Logarithmic gradient functions: $c = \log(A^{\frac{1}{x}} + BV)^x$: $c = \log(1.2589 + 0.3004V)$ (function 3); $c \overset{c}{\Rightarrow} \log(1.1220 + 0.0701V)^2$ (function 4). $c$ = volume concentration of methanol in the mobile phase at the inlet of the chromatographic column (volume % $\times 10^{-2}$); $V$ (ml) = volume of the eluate.

eqn. 1. For two neighbouring members of a homologous series, $n$ and $(n+1)$, we can define $k'$ as $k'_n$ and $k'_{(n+1)}$. Then, $\Delta \log k' = \log \alpha$, where $\alpha$ is the separation factor for compounds $n$ and $(n+1)$, *i.e.*, $\alpha = k'_{(n+1)}/k'_n$. A contribution of a methylene group to the molal volume is approximately constant in a homologous series and, at a constant temperature, the corresponding contribution to $n$ should also be constant. Using eqn. 1 for $k'_n$ and $k'_{(n+1)}$, we can write

$$\log \alpha = \log k'_{(n+1)} - \log k'_n = \log k'_{0(n+1)} - \log k'_{0n} - c[n_{(n+1)} - n_n]$$
$$= \log \alpha_0 - c\Delta n \qquad (11)$$

A linear decrease in $\log \alpha$ is expected with increasing content of the organic solvent in the mobile phase ($c$). Similar deviations of experimental data from the theoretical line at low and high organic component concentrations in the mobile phase to those published by Karger *et al.*[10] for aliphatic alcohols were observed here; the numerical values of $\log \alpha$ are also close to their values for alcohols.

Thus, the selectivity contribution of a methylene group in reversed-phase chromatography seems to be approximately constant for different types of compounds, but it depends to a certain extent on the composition of the eluent and increases with decreasing content of the organic solvent.

The experimental $n$ values for barbiturates increase in a similar sequence to the $k'_0$ values, *i.e.*, with increasing number of carbon atoms in the substituent. The only significant difference is observed for the phenyl ring, which causes a much greater increase in $n$ than in $k'_0$. Owing to the experimental deviations from eqn. 1 for substituted uracils and alkaloids, the $n$ values for these compounds cannot be interpreted.

*Gradient elution*

To verify the validity of eqns. 4–7, 9 and 10 for retention volumes and peak widths in reversed-phase gradient elution chromatography, selected alkaloids, barbiturates and alkyluracils were chromatographed in experiments in which the concentration of methanol in the mobile phase was changed continuously according to six different linear and two logarithmic functions as shown in Fig. 7. The influence of the slope, the initial concentration of methanol and the curvature of the gradient function on the compounds chromatographed was examined. The experimental retention characteristics are compared in Tables II–V with the values calculated from eqns. 4–7, 9 and 10, using $k'_0$ and $n$ values from Table I. The differences between the experimental and calculated net retention volumes are less than 0.4–0.5 ml, which corresponds to *ca.* 5–10% relative, while the differences between the experimental and calculated peak widths are *ca.* 0.1–0.15 ml (*ca.* 20–25% relative). The experimental peak widths are narrower than the calculated values, probably owing to the compression effect being neglected in the calculations. Further, the calculations of peak widths were based on the average values of $N$ from isocratic elution experiments and the influence of the composition of the mobile phase on the chromatographic efficiency was also ignored (viscosity effects).

The errors in the calculated peak widths can be reduced if the values calculated from eqn. 6 or 7 are multiplied by an empirical "compression factor". This factor depends on the parameters of the gradient function and is difficult to calculate

TABLE II

EXPERIMENTAL AND CALCULATED VALUES OF NET RETENTION VOLUMES [$V'_{R(g)}$] AND PEAK WIDTHS [$w_{(g)}$] OF XANTHINE ALKALOIDS, SUBSTITUTED URACILS AND BARBITURATES IN REVERSED-PHASE GRADIENT ELUTION CHROMATOGRAPHY

Column: octadecylsilica on LiChrosorb Si 100 (10 $\mu$m); 300 × 4.2 mm; $V_m$ = 3.2 ml. Mobile phase: water–methanol, linear gradient (eqn. 3), 0.97 ml/min. Detection: UV, 254 nm, 0.16 a.u.f.s. The calculations were performed according to eqns. 4 and 7, using $k'_0$ and $n$ values from Table I and the average values of $N$ from isocratic experiments: $N$ = 1600 for alkaloids and 2000 for uracils and barbiturates. Numbers of compounds as in Table I.

| *Parameters of gradient function (eqn. 3)* | | *Compound No.* | $V'_{R(g)}$ *(ml)* | | $w_{(g)}$ *(ml)* | |
|---|---|---|---|---|---|---|
| *A* | *B* | | *Calc.* | *Exptl.* | *Calc.* | *Exptl.* |
| 0.0 | 0.06872 | 3 | 4.71 | 5.14 | 0.45 | 0.40 |
| | | 4 | 5.78 | 6.32 | 0.49 | 0.47 |
| | | 5 | 6.92 | 7.26 | 0.48 | 0.44 |
| | | 6 | 4.45 | 4.79 | 0.41 | 0.33 |
| | | 7 | 5.84 | 6.21 | 0.43 | 0.40 |
| | | 8 | 7.52 | 7.93 | 0.45 | 0.39 |
| | | 10 | 8.85 | 9.20 | 0.46 | 0.33 |
| | | 12 | 9.18 | 9.58 | 0.46 | 0.40 |
| | | 13 | 9.52 | 9.85 | 0.45 | 0.48 |
| | | 14 | 7.09 | 7.25 | 0.46 | 0.36 |
| | | 15 | 8.02 | 8.14 | 0.44 | 0.36 |
| | | 16 | 8.61 | 8.72 | 0.44 | 0.37 |
| | | 17 | 9.14 | 9.28 | 0.44 | 0.37 |
| | | 20 | 9.85 | 10.02 | 0.44 | 0.37 |
| | | 21 | 10.37 | 10.58 | 0.44 | 0.40 |
| | | 22 | 10.62 | — | 0.42 | — |
| 0.1 | 0.06872 | 3 | 3.33 | 3.54 | 0.44 | 0.42 |
| | | 4 | 4.42 | 4.74 | 0.49 | 0.42 |
| | | 5 | 5.50 | 5.64 | 0.48 | 0.41 |
| | | 6 | 3.11 | 3.38 | 0.40 | 0.34 |
| | | 7 | 4.46 | 4.75 | 0.43 | 0.39 |
| | | 8 | 6.11 | 6.40 | 0.45 | 0.40 |
| | | 10 | 7.42 | 7.68 | 0.46 | 0.44 |
| | | 12 | 7.74 | 8.02 | 0.46 | 0.47 |
| | | 13 | 8.08 | 8.33 | 0.45 | 0.47 |
| | | 14 | 5.69 | 5.84 | 0.45 | 0.38 |
| | | 15 | 6.58 | 6.68 | 0.44 | 0.36 |
| | | 16 | 7.17 | 7.31 | 0.44 | 0.31 |
| | | 17 | 7.70 | 7.90 | 0.44 | 0.31 |
| | | 20 | 8.40 | 8.63 | 0.43 | 0.32 |
| | | 21 | 8.92 | 9.13 | 0.44 | 0.33 |
| | | 22 | 9.16 | 9.41 | 0.42 | — |

exactly, but a value between 0.8 and 0.9 seems a reasonable estimate for many practical applications.

From Tables II–V, the influence of the parameters A and B (and $\varkappa$) of the gradient function (eqns. 3 and 8) on the chromatographic behaviour of the sample compounds is obvious. The retention volumes of the sample compounds increase with decreasing initial concentration of methanol in the mobile phase ($A$ and log $A$) and decreasing slope of the gradient function ($B$). If the initial and final concentrations of

TABLE III

EXPERIMENTAL AND CALCULATED VALUES OF NET RETENTION VOLUMES [$V'_{R(g)}$] AND PEAK WIDTHS [$w_{(g)}$] OF XANTHINE ALKALOIDS, SUBSTITUTED URACILS AND BARBITURATES IN REVERSED-PHASE GRADIENT ELUTION CHROMATOGRAPHY

Numbers of compounds as in Table I; experimental conditions as in Table II, except gradient function (linear, eqn. 3).

| *Parameters of gradient function (eqn. 3)* | | *Compound No.* | $V'_{R(g)}$ *(ml)* | | $w_{(g)}$ *(ml)* | |
|---|---|---|---|---|---|---|
| *A* | *B* | | *Calc.* | *Exptl.* | *Calc.* | *Exptl.* |
| 0.0 | 0.03436 | 3 | 7.61 | 7.78 | 0.58 | 0.46 |
| | | 4 | 9.17 | 9.60 | 0.66 | 0.54 |
| | | 5 | 11.57 | 11.39 | 0.64 | 0.66 |
| | | 6 | 7.04 | 6.99 | 0.52 | 0.44 |
| | | 7 | 9.41 | 9.44 | 0.57 | 0.52 |
| | | 8 | 12.47 | 12.60 | 0.61 | 0.53 |
| | | 10 | 15.02 | 15.23 | 0.63 | 0.54 |
| | | 12 | 15.69 | 15.97 | 0.63 | 0.58 |
| | | 13 | 16.48 | 16.56 | 0.62 | 0.58 |
| | | 14 | 11.55 | 11.56 | 0.62 | 0.51 |
| | | 15 | 13.71 | 13.55 | 0.59 | 0.45 |
| | | 16 | 14.78 | 14.68 | 0.60 | 0.47 |
| | | 17 | 15.91 | 15.88 | 0.59 | 0.47 |
| | | 20 | 17.39 | 17.39 | 0.58 | 0.47 |
| | | 21 | 18.42 | 18.35 | 0.59 | 0.50 |
| | | 22 | 19.19 | 19.23 | 0.55 | — |
| 0.1 | 0.03436 | 3 | 5.00 | 5.07 | 0.55 | 0.44 |
| | | 4 | 6.61 | 6.96 | 0.63 | 0.46 |
| | | 5 | 8.80 | 8.72 | 0.63 | 0.49 |
| | | 6 | 4.53 | 4.54 | 0.49 | 0.41 |
| | | 7 | 6.78 | 6.85 | 0.55 | 0.51 |
| | | 8 | 9.72 | 9.91 | 0.60 | 0.47 |
| | | 10 | 12.21 | 12.49 | 0.62 | 0.47 |
| | | 12 | 12.86 | 13.23 | 0.62 | 0.47 |
| | | 13 | 13.62 | 13.81 | 0.61 | 0.50 |
| | | 14 | 8.87 | 8.90 | 0.60 | 0.47 |
| | | 15 | 10.88 | 10.81 | 0.58 | 0.45 |
| | | 16 | 11.94 | 11.91 | 0.59 | 0.48 |
| | | 17 | 13.04 | 13.03 | 0.59 | 0.46 |
| | | 20 | 14.50 | 14.57 | 0.58 | 0.44 |
| | | 21 | 15.53 | 15.54 | 0.58 | 0.44 |
| | | 22 | 16.29 | 16.39 | 0.55 | 0.47 |

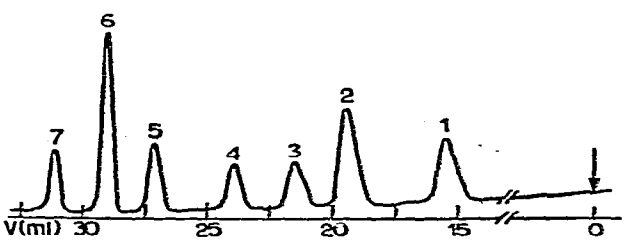



Fig. 8. Gradient elution separation of barbiturates in reversed-phase chromatography. Column: $C_{18}$ (10 $\mu$m), 300 × 4.2 mm. Mobile phase, methanol–water; concentration gradient of methanol according to the function $c = 0.1 + 0.01718V$; flow-rate, 0.97 ml/min; chart speed, 10 mm/min; detection, UV (254 nm), 0.16 a.u.f.s. Sample compounds: 1 = barbital; 2 = heptobarbital; 3 = allobarbital; 4 = aprobarbital; 5 = butobarbital; 6 = hexobarbital; 7 = pentobarbital.

TABLE IV

EXPERIMENTAL AND CALCULATED VALUES OF NET RETENTION VOLUMES [$V'_{R(g)}$] AND PEAK WIDTHS [$w_{(g)}$] OF XANTHINE ALKALOIDS, SUBSTITUTED URACILS AND BARBITURATES IN REVERSED-PHASE GRADIENT ELUTION CHROMATOGRAPHY

Numbers of compounds as in Table I; experimental conditions as in Table II, except gradient function (linear, eqn. 3), and plate numbers of uracils ($N$ = 3350) and barbiturates ($N$ = 2330) from isocratic experiments (repacked column).

| *Parameters of gradient function (eqn. 3)* | | *Compound No.* | $V'_{R(g)}$ *(ml)* | | $w_{(g)}$ *(ml)* | |
|---|---|---|---|---|---|---|
| *A* | *B* | | *Calc.* | *Exptl.* | *Calc.* | *Exptl.* |
| 0.0 | 0.01718 | 3 | 11.78 | 12.17 | 0.80 | 0.42 |
| | | 4 | 13.84 | 15.56 | 0.94 | 0.50 |
| | | 5 | 18.71 | 19.21 | 0.95 | 0.55 |
| | | 6 | 10.57 | 10.14 | 0.56 | 0.43 |
| | | 7 | 14.50 | 14.35 | 0.64 | 0.51 |
| | | 8 | 19.92 | 20.10 | 0.71 | 0.57 |
| | | 10 | 24.76 | 25.23 | 0.74 | 0.58 |
| | | 12 | 26.12 | 26.63 | 0.74 | 0.57 |
| | | 13 | 27.89 | 27.89 | 0.72 | 0.56 |
| | | 14 | 18.05 | 17.92 | 0.85 | 0.65 |
| | | 15 | 22.81 | 22.27 | 0.81 | 0.55 |
| | | 16 | 24.74 | 24.34 | 0.83 | 0.59 |
| | | 17 | 27.10 | 26.75 | 0.82 | 0.55 |
| | | 20 | 30.17 | 29.98 | 0.81 | 0.57 |
| | | 21 | 32.21 | 31.90 | 0.81 | 0.60 |
| | | 22 | 34.30 | 33.97 | 0.75 | 0.55 |
| 0.1 | 0.01718 | 3 | 7.04 | 6.93 | 0.71 | 0.53 |
| | | 4 | 9.26 | 9.85 | 0.85 | 0.49 |
| | | 5 | 13.43 | 13.11 | 0.90 | 0.47 |
| | | 6 | 6.16 | 5.51 | 0.48 | 0.41 |
| | | 7 | 9.70 | 9.06 | 0.58 | 0.49 |
| | | 8 | 14.72 | 14.37 | 0.67 | 0.55 |
| | | 10 | 19.30 | 19.25 | 0.72 | 0.53 |
| | | 12 | 20.60 | 20.65 | 0.72 | 0.56 |
| | | 13 | 22.27 | 21.85 | 0.71 | 0.53 |
| | | 14 | 13.07 | 12.36 | 0.79 | — |
| | | 15 | 17.30 | 16.23 | 0.78 | — |
| | | 16 | 19.19 | 18.37 | 0.81 | — |
| | | 17 | 21.46 | 20.79 | 0.81 | — |
| | | 20 | 24.45 | 23.94 | 0.80 | 0.87 |
| | | 21 | 26.46 | 25.86 | 0.81 | 0.76 |
| | | 22 | 28.51 | 27.91 | 0.75 | 0.73 |

methanol are constant, the retention volumes decrease with increasing convexity of the gradient function (logarithmic functions 3 and 4 in Fig. 7). The peak width increases slightly with decreasing slope of the gradient function; the effects of the initial concentration of methanol in the mobile phase and of the curvature of gradient, however, are almost negligible. All of the compounds eluted at one gradient function have approximately identical peak widths.

The influence of the gradient function on the chromatographic separation of a mixture of barbiturates is shown in Figs. 8 and 9. The speed of analysis increases significantly with increasing steepness of the gradient.

TABLE V

EXPERIMENTAL AND CALCULATED VALUES OF NET RETENTION VOLUMES [$V'_{R(g)}$] AND PEAK WIDTHS [$w_{(g)}$] OF XANTHINE ALKALOIDS, SUBSTITUTED URACILS AND BARBITURATES IN REVERSED-PHASE GRADIENT ELUTION CHROMATOGRAPHY

Numbers of compounds as in Table I; experimental conditions as in Table II, except gradient function (logarithmic, eqn. 8) and plate numbers for uracils ($N = 3350$) and barbiturates ($N = 2330$) from isocratic experiments (repacked column).

| *Parameters of gradient function (eqn. 8)* | | | *Compound No.* | $V'_{R(g)}$ *(ml)* | | $w_{(g)}$ *(ml)* | |
|---|---|---|---|---|---|---|---|
| $\varkappa$ | $A$ | $B$ | | *Calc.* | *Exptl.* | *Calc.* | *Exptl.* |
| 1 | 1.2589 | 0.3004 | 3 | 2.96 | 3.32 | 0.44 | 0.41 |
| | ($c_0 = 0.1$) | | 4 | 4.09 | 4.51 | 0.50 | 0.46 |
| | | | 5 | 5.32 | 5.59 | 0.51 | 0.41 |
| | | | 6 | 2.75 | 3.17 | 0.30 | 0.44 |
| | | | 7 | 4.13 | 4.53 | 0.34 | 0.48 |
| | | | 8 | 6.06 | 6.46 | 0.38 | 0.46 |
| | | | 10 | 7.84 | 8.29 | 0.42 | 0.47 |
| | | | 12 | 8.33 | 8.77 | 0.42 | 0.50 |
| | | | 13 | 8.89 | 9.26 | 0.43 | — |
| | | | 14 | 5.54 | 5.73 | 0.45 | 0.50 |
| | | | 15 | 6.71 | 6.79 | 0.45 | 0.46 |
| | | | 16 | 7.53 | 7.70 | 0.47 | 0.41 |
| | | | 17 | 8.34 | 8.62 | 0.48 | 0.43 |
| | | | 20 | 9.52 | 9.83 | 0.50 | 0.45 |
| | | | 21 | 10.44 | 10.70 | 0.52 | 0.48 |
| | | | 22 | 11.08 | 11.42 | 0.50 | 0.48 |
| 2 | 1.2589 | 0.0701 | 3 | 4.06 | 4.26 | 0.50 | 0.40 |
| | ($c_0 = 0.1$) | | 4 | 5.46 | 5.80 | 0.56 | 0.46 |
| | | | 5 | 7.20 | 7.30 | 0.58 | 0.41 |
| | | | 6 | 3.72 | 3.88 | 0.35 | 0.39 |
| | | | 7 | 5.57 | 5.75 | 0.38 | 0.41 |
| | | | 8 | 8.06 | 8.26 | 0.43 | 0.38 |
| | | | 10 | 10.28 | 10.52 | 0.45 | 0.40 |
| | | | 12 | 10.88 | 11.24 | 0.46 | 0.47 |
| | | | 13 | 11.58 | 11.82 | 0.46 | 0.49 |
| | | | 14 | 7.35 | 7.48 | 0.51 | 0.37 |
| | | | 15 | 9.00 | 8.98 | 0.50 | 0.36 |
| | | | 16 | 9.99 | 10.06 | 0.52 | 0.38 |
| | | | 17 | 11.00 | 11.17 | 0.53 | 0.37 |
| | | | 20 | 12.41 | 12.62 | 0.54 | 0.37 |
| | | | 21 | 13.45 | 13.59 | 0.55 | 0.41 |
| | | | 22 | 14.23 | 14.46 | 0.52 | 0.39 |

## CONCLUSIONS

The results show that the parameters $n$ and $k'_0$ in eqn. 1 in reversed-phase chromatography are influenced by various structural and system factors in a manner which is in qualitative agreement with the effects expected on the basis of the theory of regular solutions for liquid–liquid chromatography.

In our opinion, the agreement between the experimental and calculated values of retention volumes and peak widths in reversed-phase gradient elution chromato-

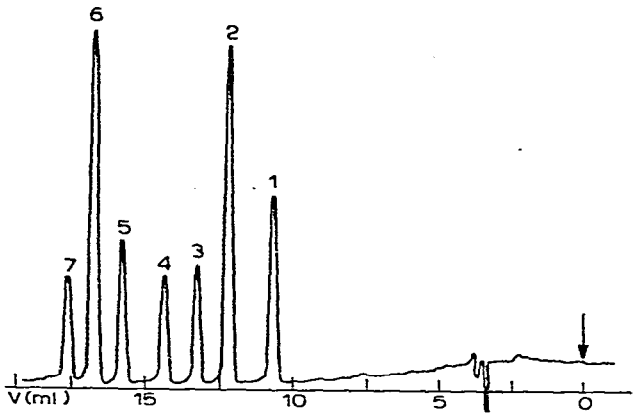



Fig. 9. Gradient elution separation of barbiturates in reversed-phase chromatography. Column: $C_{18}$ (10 $\mu$m), 300 × 4.2 mm. Concentration gradient of methanol in the mobile phase (methanol–water) according to the logarithmic function $c = \log (1.1220 + 0.0701)V^2$. Separation conditions and numbers of compounds as in Fig. 8.

graphy is acceptable and can be used as a good basis for calculations of retention characteristics and for the prediction of the optimal gradient profile in practical gradient elution experiments, even in systems in which significant deviations from eqn. 1 occur (as for alkaloids and substituted uracils in this work). The optimization approaches for gradient elution experiments will be discussed in the next part of this series.

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