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SIMPLIFIED DESCRIPTION OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION UNDER OVERLOAD CONDITIONS, BASED ON THE CRAIG DISTRIBUTION MODEL

V. GRADIENT ELUTION SEPARATION

J. E. EBLE* and R. L. GROB
Chemistry Department, Villanova University, Villanova, PA, 19085 (U.S.A.)
P. E. ANTLE
Medical Products Department, E. I. Du Pont de Nemours & Co., Concord Plaza, Wilmington, DE 19898 (U.S.A.)
and
L. R. SNYDER*
LC Resources Inc., 26 Silverwood Court, Orinda, CA 94563 (U.S.A.)
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SUMMARY

The present model of mass-overloaded high-performance liquid chromatographic separation has been extended to the case of gradient elution. It is shown that the separation of two compounds by gradient elution varies with mass overload in the same way as in isocratic elution. If gradient conditions are selected to be equivalent to those in isocratic elution [for small samples, value of $\bar{k}$ (gradient) equal k' (isocratic)], resolution is the same in either gradient or isocratic runs when the sample size is the same. Therefore gradient separations in a mass-overload mode can be predicted by means of model simulations for corresponding isocratic systems (where $k_0 = \bar{k}$). Additional relationships are derived for use in optimizing gradient separations in a mass-overload mode.

INTRODUCTION

Preceding papers[1–4] have developed a general model for isocratic high-performance liquid chromatographic (HPLC) separation under mass-overload conditions. However an increasing number of preparative HPLC separations are being carried out in a gradient elution mode, especially for the case of biological samples[5]. Separations by gradient elution involve additional experimental parameters, and are more difficult to describe than are corresponding isocratic separations. We have also seen

* Present address: Agricultural Chemicals Department, E. I. Du Pont de Nemours & Co., Experimental Station, Wilmington, DE 19898, U.S.A.

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that mass-overloaded isocratic separations are more complicated than are corresponding small sample separations. It might therefore be assumed that the use of gradient elution in a mass-overload mode would be hopelessly complicated, so far as our ability to predict the outcome of separation. Fortunately this is not the case.

Other work[6–8] has shown that small sample separations by gradient elution are essentially equivalent to those by isocratic elution, when gradient and isocratic conditions are matched to give comparable "average" retention of the sample during separation. Gradient elution is then comprehensible in terms of the same concepts used to describe isocratic elution. In this paper we will proceed from this starting point to a general description of gradient elution carried out under mass-overload conditions.

THEORY

Gradient elution without mass overload

A good understanding now exists of HPLC separation carried out with gradient elution[6,7,9,10], when the injected sample is small. The assumption of linear-solvent-strength (LSS) conditions further simplifies the interpretation and prediction of gradient separations[6–8]. The LSS model of gradient elution assumes that sample retention (k') as a function of the time after the start of separation can be approximated by

$$\log k_i = \log k_{0g} - b(t/t_0) \tag{1}$$

Here k_i is the value of k' (at the column inlet) for the solute at any time t during separation, k_{0g} is the value of k' at the beginning of separation (referred to elsewhere as k_0[6–8]), b is a parameter related to gradient steepness, and t_0 is the column dead-time. Eqn. 1 need not be obeyed exactly for the LSS model to be useful[8,11]. The conclusions we will draw in this paper are based on the LSS model, but our findings will apply qualitatively even for systems that are quite different from LSS gradients (*e.g.*, ion exchange with linear gradients).

In reversed-phase HPLC, sample retention can usually be described by the empirical relationship[8]

$$\log k' = \log k_w - S\varphi \tag{2}$$

Here k_w refers to the k' value of the solute for water as mobile phase, φ is the volume fraction of organic in the organic–water mobile phase, and S is a constant that depends on both the solute and the organic solvent used. If linear gradients are employed, and eqn. 2 is valid, then the resulting gradient separation will be of the LSS type. It has been shown that minor modification of the LSS model allows its use for systems that deviate widely from eqn. 2; *e.g.*, ion-exchange chromatography[7,8]. The conclusions presented here for mass-overloaded gradient elution will therefore be applicable to any HPLC separation (reversed-phase, ion-exchange, normal-phase, etc.).

Retention time t_g in LSS gradient elution is given as[6–8]*

* The following approximate equations have been simplified for the case of small-molecule samples; see discussion of ref. 7.

$$t_g = (t_0/b) \log(2.3\, k_{0g} b + 1) + t_0 + t_D \tag{3}$$

with

$$b = V_m \Delta\varphi S / t_G F \tag{4}$$

t_D is the system dwell-time, V_m is the column dead-volume (equal to $[t_0 F]$), $\Delta\varphi$ is the change in φ during the gradient, t_G is the gradient time and F is flow-rate. The value of k' when the band has migrated halfway along the column ("average" or "effective" k') is of special importance for preparative separation:

$$\bar{k} = 1/1.15b \tag{5}$$

The mobile phase composition at this point in the separation (where $k' = \bar{k}$) is defined as $\bar{\varphi}$. Eqn. 5 assumes a small sample.

For small samples, resolution R_s in gradient elution is given as

$$R_s = (1/4)(\alpha - 1) N^{\frac{1}{2}} \bar{k}/(1 + \bar{k}) \tag{6}$$

Here α is the separation factor and N is the plate number. If α and N are functions of φ, then values of N and α for $\varphi = \bar{\varphi}$ apply in eqn. 6 (see discussion of ref. 7). Eqn. 6 is seen to be of the same form as for isocratic separation[12],

$$R_s = (1/4)(\alpha - 1) N^{\frac{1}{2}} k'/(1 + k') \tag{7}$$

except that k' (isocratic) replaces $\bar{k}$ (gradient). Eqn. 6 therefore predicts that resolution in isocratic and gradient elution will be the same (for a small sample), when conditions have been selected so that φ (isocratic) equals $\bar{\varphi}$ (gradient) and k' (isocratic) equals $\bar{k}$ (gradient).

Gradient elution with mass overload

Gradient vs. isocratic separation. We will first examine the relationship between isocratic and gradient separation under overload conditions. We will show that when small-sample separations for isocratic and gradient runs are made equivalent ($k_0 = \bar{k}$), resolution will be essentially the same for both separations under mass-overload conditions (assuming the same sample size in both isocratic and gradient separations). This means that we can determine the effect of mass overload on a gradient separation, using model simulations for the corresponding isocratic case. Our discussion here neglects the effects of "blockage" as discussed in refs. 3, 4, but this is only for purposes of simplification. The conclusions are the same, with or without blockage effects, as will be demonstrated by experimental isocratic and gradient runs under overload conditions.

First, assume corresponding gradient and isocratic separations (*i.e.*, $k_0 = \bar{k}$) of a sample containing compounds X and Y, with no mass overload. This situation is illustrated in Fig. 1a. The solid lines represent the dependence of log k' on φ, and the vertical dashed line indicates the value of φ for the isocratic separation. The solid circles mark the k' values for this isocratic (small sample) separation. Separation by

LSS gradient elution is represented by the horizontal dashed line; the corresponding open circles mark the $\bar{\varphi}$ values for gradient elution of X and Y. For this example, it is seen that isocratic elution occurs with constant values of φ and different values of k' for the two solutes. The situation for gradient elution* (open circles) is just the opposite: values of k' (or $\bar{k}$) are constant, while values of φ (or $\bar{\varphi}$) differ. Despite this difference in isocratic *vs.* gradient elution, eqns. 6 and 7 show that resolution will be the same as long as average values (for the two solutes**) of N and k' (or $\bar{k}$) are the same for the two separations ("corresponding" conditions).

Now consider the effect of mass overload on the isocratic separation of Fig. 1a, as illustrated in Fig. 1b). The sloping solid lines correspond to the dependence of log k' on φ for a mass-overloaded sample (specified values of w_x and w_y). The circles show the original k' values (as in Fig. 1a) for a non-overloaded separation, while the squares mark retention under conditions of mass-overload. The dashed, slanting curves are for a small sample (from Fig. 1a). We have drawn the dashed and solid curves parallel, since it can be shown (Appendix I) that mass overload has only a minor effect on the value of S in plots as in Fig. 1b. We have also shown the same fractional decrease in k' for each solute, which will be the case when the masses of X and Y are similar and w_s is the same for each solute (*e.g.*, Table II of ref. 4).

Finally, consider the effect of mass overload on the gradient separation of Fig. 1a, as shown in Fig. 1c. The slanted lines for the dependence of $\bar{k}$ on $\bar{\varphi}$ are the same as in Fig. 1b, corresponding to similar sample sizes for the isocratic and gradient separations. The circles again mark the retention of each solute in the original (small sample) run of Fig. 1a, while the squares mark retention for the mass-overloaded separation. The values of $\bar{k}$ for each solute are maintained constant as sample size increases (eqns. 4 and 5), since (Appendix I) values of S remain approximately constant with overload.

The average value of N during gradient elution is expected to change with sample size in the same way as for isocratic elution. The average value of α will similarly be affected in the same way by mass overload, for either isocratic or gradient elution (see Fig. 1b and 1c). However, k' and $\bar{k}$ (average values for X and Y) are no longer equal for the overloaded separations of Fig. 1b and c. It is seen that $\bar{k} > k'$, from which it can be concluded that resolution in the overloaded gradient separation should exceed that in the corresponding (overloaded) isocratic separation. However, this effect will generally be quite small, and will almost always be subordinated to corresponding changes in N and α. This is illustrated in Table I, using data for the separation of two xanthines (HET and HPT) from the preceding paper[4]. Here it is seen that as sample size increases, the factor $k'/(1 + k')$ decreases only slightly, when compared to corresponding changes in $\alpha - 1$ and N. That is, the difference in values of k' and $\bar{k}$ in Fig. 1b *vs.* c will have a negligible affect on resolution (eqn. 6 or 7).

Since the only difference between gradient and isocratic separation under mass-overload conditions is the larger (average) k' value in gradient runs, and since this affects resolution only slightly (compared to corresponding change in α and N), it can be concluded that resolution in mass-overloaded gradient elution will be similar

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* Fig. 1 assumes (a) the S values of the two solutes are the same (parallel plots in Fig. 1) and (b) a linear gradient is used.

** Eqns. 6 and 7 each assume average values of k' (or $\bar{k}$) and N.

to that in corresponding isocratic separations having the same sample size. This means that mass-overloaded gradient separations can be approximated by model simulations of corresponding isocratic separations. However, there is a minor exception to this conclusion that will be discussed next.

Case of non-equal S values. The preceding analysis assumes that values of S for each solute are similar. This will often be the case for related compounds, as might occur together in a given sample. However there are frequent exceptions to this generalization[13]. The effect of differing S values on isocratic separation is shown in Fig. 2 (*cf.* Fig. 1). Consider first the case of a small sample (circles and sloping, dashed lines). The vertical solid line indicates sample retention for a particular value

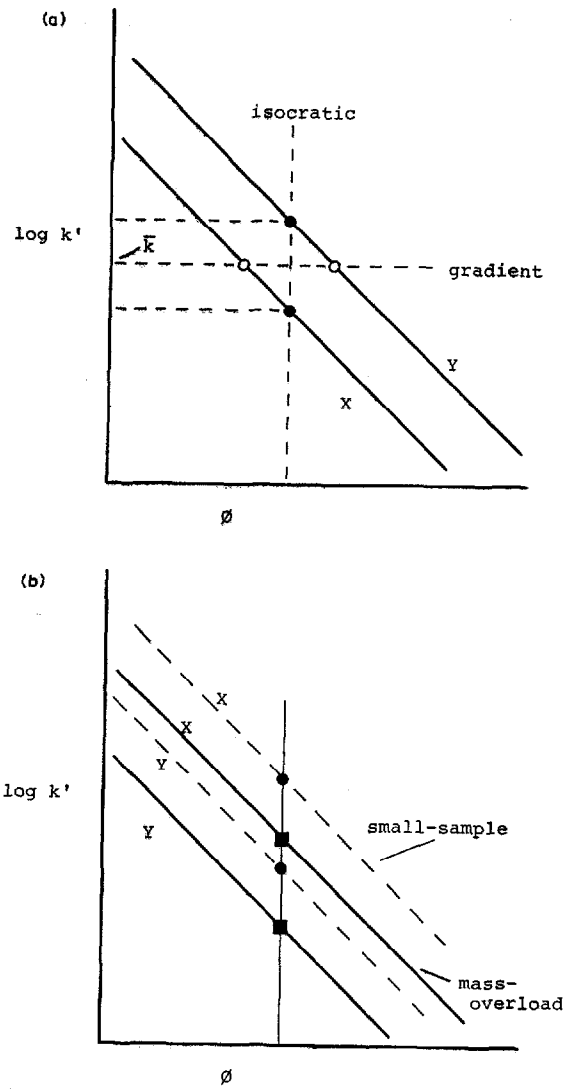

Fig. 1.

(*Continued on p. 56*)

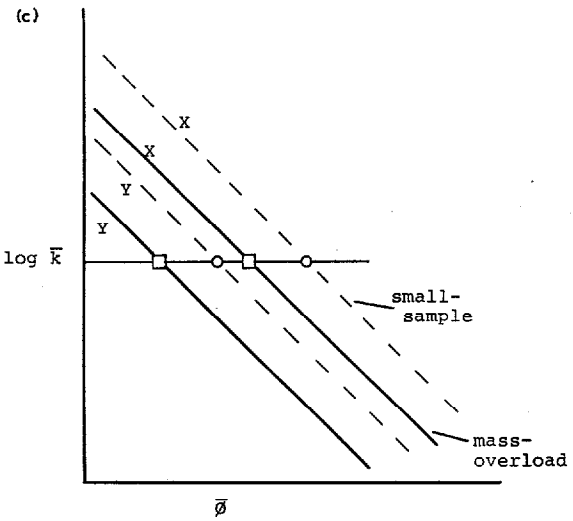

Fig. 1. Representation of corresponding separations of solutes X and Y by isocratic and gradient elution (see text). (a) For a small sample; (———) plots of log k' (or $\bar{k}$) *vs.* φ (or $\bar{\varphi}$); (b) isocratic separation with mass-overload; (– – –) small sample, (———) mass overload; (c) gradient separation with mass-overload; (– – –) small sample, (———) with mass overload.

of φ. The use of a weaker mobile phase in this case (smaller φ, indicated by the arrow) is seen to result in a decrease in α for the separation, with a resulting loss in resolution (other factors equal). This is due to the convergence of the log k' *vs.* φ plots at smaller values of φ. That is, a change in φ can result in changes in relative band spacing, when solute S values are not equal (see further discussion of ref. 13).

A similar situation exists for the mass-overloaded separation of Fig. 2 (squares and sloping, solid lines), where α and resolution will also be smaller for smaller values of φ. In the case of a corresponding gradient separation (same conditions as for

TABLE I

CONTRIBUTIONS TO RESOLUTION IN MASS-OVERLOADED SEPARATION

Xanthine sample (HET, HPT), data from runs of Figs. 1–5 of ref. 4.

Sample mass (mg)		$\alpha - 1$*	$N^{\frac{1}{2}}$**	$k'/(k'+1)$***
HET	*HPT*			
0.0	0.0	(1.00)	(1.00)	(1.00)
0.3	0.3	0.99	0.78	0.99
1.0	1.0	1.01	0.55	0.97
2.5	2.5	1.01	0.40	0.94
2.5	10	0.62	0.28	0.90
2.5	25	0.25	0.22	0.85

* Based on experimental (cumulative) k' values.
** Based on N/N_0 values from model simulation.
*** Based on experimental (cumulative) k' values.

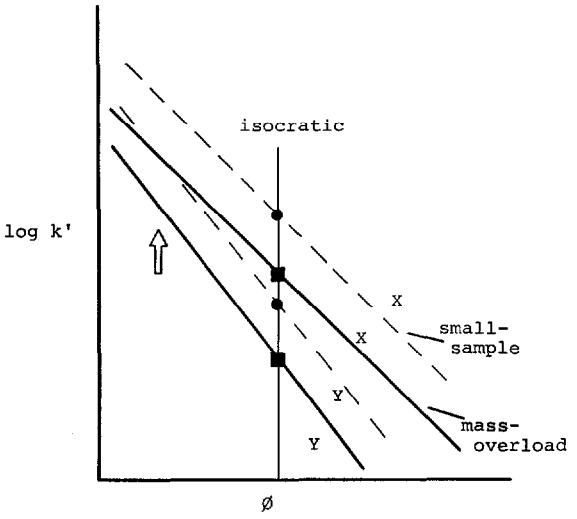

Fig. 2. Representation of corresponding separations of solutes X and Y by isocratic and gradient elution, for case where solute S values are unequal (see text). (– – –) log k' *vs.* φ for small sample, (———) log k' *vs.* φ for mass overload.

isocratic runs of Fig. 2), mass overload again results in elution of the sample at a lower average value of $\bar{\varphi}$ (as in Fig. 1c). However now (Fig. 2) this change in $\bar{\varphi}$ affects α in addition to $\bar{k}$. Thus for a separation where S is greater for the first-eluting solute (X), as in Figs. 1 and 2, the gradient separation should give poorer resolution than the isocratic separation under conditions of severe mass overloaded (and *vice versa*, for the case of a smaller S value for X). However a detailed analysis of this situation, with the assumption of (a) reasonable values of S for each solute and (b) moderate mass overloading as represented by k'/k_0, shows that this effect is generally small (see Appendix II). Thus only in exceptional cases, involving large differences in solute S values plus severe column overloading, will we see related differences in separation between corresponding isocratic and gradient runs in a mass-overload mode.

EXPERIMENTAL

Materials, equipment and procedures were described in the preceding paper[4]. The present studies required (in some cases) the use of rather large volumes of sample, because of the need to dissolve relatively large weights of sample in the mobile phase used at the beginning of the gradient. Retention data from these runs were used subsequently in various calculations. It was necessary in these cases to correct these retention times for the effect of sample-solvent volume. Let the sample volume be V_s. For isocratic runs, experimental values of t_R were reduced by the quantity $1/2\ V_s/F$. For gradient runs, experimental values of t_g were reduced by the quantity V_s/F. This correction for the case of gradient elution assumes that a large volume of a weak sample-solvent simply delays the arrival of the gradient at the column inlet, during which time the sample does not migrate along the column (k_{0g} large).

RESULTS AND DISCUSSION

The conclusions reached in the Theory section suggest that in most cases mass-overloaded gradient separations can be adequately approximated by corresponding isocratic separations. This means that we can predict how mass overload will affect gradient runs, using model simulations for the corresponding isocratic runs (as in ref. 4). The general discussion of mass-overload separation for isocratic elution (preceding paper[4]) will also apply equally for gradient separations.

We will first consider how a "corresponding" isocratic separation can be defined for model simulations of mass-overloaded gradient runs. Then we will examine some relevant experimental data.

Measurement of isocratic parameters N, k_0, and w_s from gradient runs

If we are to use isocratic model simulation to predict mass-overloaded gradient separations, we require values of N_0, k_0 and w_s for each solute X and Y. When gradient elution is preferred for separation in an overload mode, it will generally be more convenient to obtain values of N_0, k_0 and w_s directly from gradient runs, as opposed to carrying out corresponding isocratic separations. This is especially true for many large-molecule separations, where values of S can be large (*cf.* discussion of ref. 6), and values of k' then change rapidly with small changes in φ.

Measurement of k_0. Two small-sample gradient runs with only gradient time varying can be used to obtain values of k_{0g} and S for each solute, and values of $\bar{k}$ (equal to k' in a corresponding isocratic run) for each solute in each run[8]. We will assume that one of the two runs (*i.e.*, involving a particular value of t_G and/or resulting value of b) will then be repeated under mass-overload conditions. The values of $\bar{k}$ from the small-sample run for solutes X and Y can then be used as inputs (values of k_0) for model simulation.

Measurement of N. A value of R_s for the small-sample gradient separation can be obtained in various ways (*e.g.*, ref. 12). This value can be substituted into eqn. 6, along with values of k_0 and α obtained from the preceding section (*Measurement of k_0*). An average value of N (for X and Y) can then be obtained as

$$N_0 = [4\,R_s\,\alpha(1 + \bar{k})/(\alpha - 1)\bar{k}]^2 \tag{8}$$

Eqn. 8 recognizes that α may be large, unlike eqns. 6 and 7, which are derived for the case of small values of $\alpha - 1$.

Measurement of w_s. We require retention data from (a) a small sample and (b) a mass-overloaded run (gradient separations, all other conditions the same). For each solute, the retention times t_g are measured for each run: t_g (small sample) and t_{gm} (mass overload). It can then be shown (Appendix III) that

$$t_{gm} - t_g = (t_0/b) \log (k'/k_0) \tag{9}$$

Given a value of k'/k_0 for the mass-overloaded run (via eqn. 9), w_s can be determined as described in ref. 4.

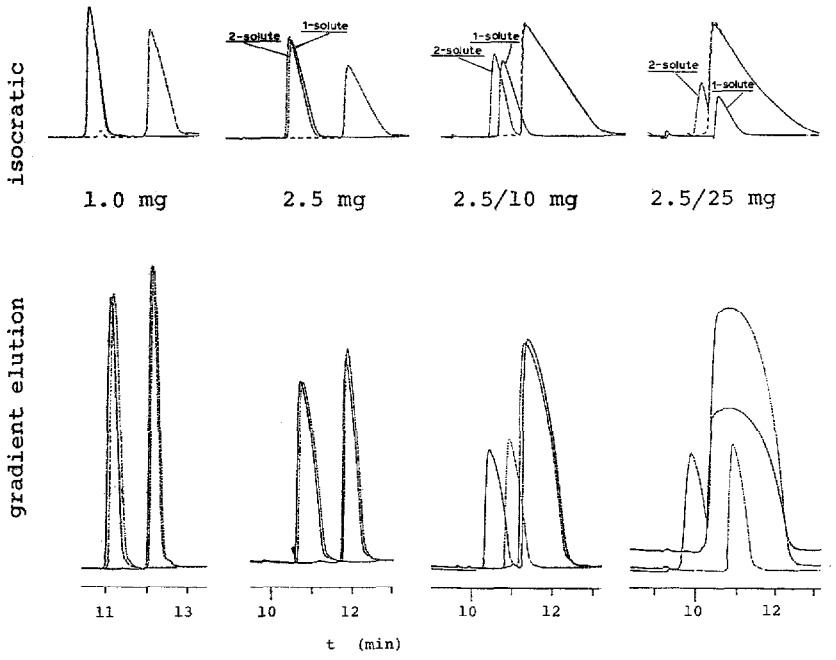

Fig. 3. Reversed-phase separation of two xanthines (HET and HPT) by isocratic (top) and gradient (bottom) elution. Gradient conditions (small sample) selected to correspond to isocratic separations of Figs. 2–5 of ref. 4 ($\bar{k} = k'$). Gradient is 5–100%v organic in 12 min, other conditions as in Table I. Sample sizes indicated in figure: 1 mg each solute; 2.5 mg each; 2.5 mg HET, 10 mg HPT; 2.5 mg HET, 25 mg HPT.

Separation of xanthine sample

Separations by gradient elution in a mass-overload mode should yield results that are equivalent to corresponding isocratic separations; *i.e.*, where all conditions are the same (including sample mass), and the gradient has been adjusted to give an

TABLE II

COMPARISON OF RESOLUTION BETWEEN CORRESPONDING ISOCRATIC AND GRADIENT SEPARATIONS OF TWO XANTHINES (HET AND HPT)

Comparison of separations of Figs. 2–6 of ref. 4 *vs.* Fig. 3 of present paper.

Sample size (mg)		*Resolution (R_s)**	
HET	*HPT*	*Isocratic*	*Gradient*
1.0	1.0	1.9	2.1
2.5	2.5	1.5	1.5
2.5	10	0.6	0.7
2.5	25	42%**	38%**

* R_s calculated as difference in retention times of two bands divided by sum of bandwidths at 50% of maximum peak height.

** Resolution measured in this case by height of valley (as %) between two bands, relative to smaller band; bandwidth at half-height was difficult to measure for HET.

effective retention ($\bar{k}$, small sample) that is the same as in the isocratic separation (k_0). In the present case (isocratic separations of HET and HPT as in Figs. 1–5 of ref. 4), the average value of S for HET and HPT was measured as 3.9, V_m equals 1.4 ml, a 5–100% gradient was selected ($\Delta\varphi = 0.95$), $F = 1$ ml/min, and the average k' value (isocratic) for HET and HPT is 2.5. From these data for the isocratic runs, we can calculate (eqns. 4 and 5) that the gradient time t_G should equal 14.8 min, if the gradient separations are to correspond to the isocratic runs of figs. 1–5 of ref. 4.

Fig. 3 (lower) shows four gradient separations of the HET–HPT mixture, using the same sample sizes as for corresponding isocratic runs in the preceding paper[4]. The corresponding isocratic separations (from Figs. 2–5 of ref. 4) are shown at the top of Fig. 3 for comparison. As predicted, we find essentially equivalent resolution in corresponding isocratic and gradient runs. A quantitative measure of separation in each case is afforded by values of resolution R_s calculated from the bandwidths at half-height. These R_s values are compared in Table II for corresponding gradient and isocratic runs of Fig. 3. For isocratic and gradient runs with the same sample mass, it is seen that the resolution is the same (within experimental error).

The symmetry of solute bands in the gradient runs of Fig. 3 is generally better than that observed in the isocratic runs. On average the asymmetry factors As for the gradient runs in Fig. 3 are about one-third smaller than As values for corresponding isocratic runs. However this difference in the gradient runs is not reflected in any improvement in actual resolution. That is, the beauty of the gradient runs is solely in the eye of the beholder.

There is also a noticeable change in shape of the more heavily overloaded bands of Fig. 3; the "right-triangle" appearance of the isocratic bands is replaced in the gradient runs by a more rounded ("shark-fin") shape as sample size increases. This change in shape reflects the fact that the band tail (in gradient elution) always travels in a stronger mobile phase. These band-shape changes will affect the exact form of the elution curve predicted by model simulation, but relative band overlap as a function of cutpoint will not be affected. This allows the use of model simulations for choosing a sample size that is optimum from the standpoint of preparative separation. However, the best cutpoint between two adjacent bands must then be determined empirically; *e.g.*, by collecting and analyzing fractions around the cutpoint.

Quantitative interpretation of xanthine data. The values of $\bar{k}$ for these two xanthines are rather small ($k_{0g} = 13$ and 22 for HET and HPT), so that the quantitative relationships derived for determining N_0 (eqn. 8) and w_s (eqn. 9) are imprecise. In cases like this, it is more convenient to determine values of k_0, N_0 and w_s from corresponding isocratic runs rather than from gradient runs. The following example, which involves the alkyl parabens as samples, provides a quantitative test of eqns. 8 and 9 (for larger k_{0g} values).

Separation of alkyl parabens sample

Methyl and ethyl parabens were selected as solutes, and their retention was determined as a function of %v methanol with a C_8 column (other conditions as in Table VII of ref. 4). Values of S could be derived from these measurements: S = 2.9 for methyl paraben, S = 3.7 for ethyl paraben. Separations of a 1:1 mixture of these two parabens were then carried out, as summarized in Fig. 4. The separations of Fig. 4a and b are isocratic separations, while the runs of Fig. 4c and d use gradient elution

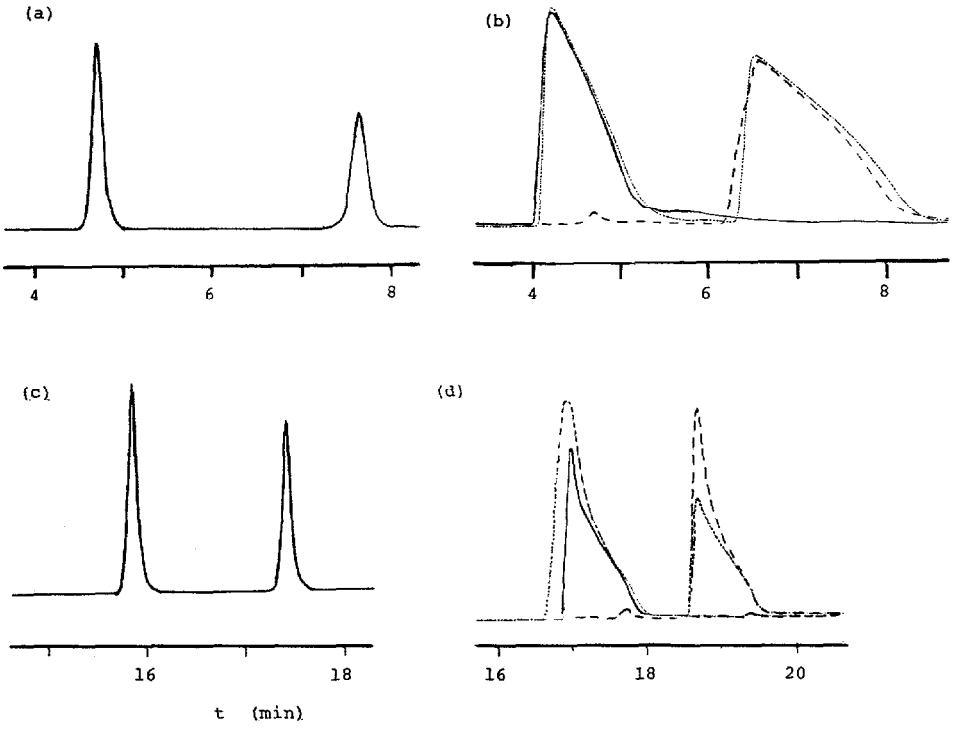

Fig. 4. Reversed-phase separation of methyl and ethyl parabens by isocratic (a, b) or gradient (c, d) elution. Zorbax C8 column (15 × 0.46 cm); 35°C; 1 ml/min; methanol–water (50:50) (a, b) or 10–100% methanol in water gradient (c, d); 1 μg each solute (a, c), 2 mg each solute (b, d); t_G = 17 min.

under "corresponding" conditions ($k' = \bar{k}$). The samples of runs a and c contain 1 μg each of the two parabens; runs b and d have 2 mg each of the two parabens. The mass-overloaded separations of Fig. 4 (b and d) show plots of both individual solutes (2 mg) and the mixture (2 mg each solute), as in Figs. 1–5 of ref. 4.

Table III summarizes a number of measurements and derived quantities from the separations of Fig. 4. The average value of k_0 for the two solutes in methanol–water (50:50) was determined first: k_0 = 3.3. This was used (eqn. 5) to calculate the value of b for a "corresponding" gradient separation. Other experimental conditions (V_m, F, $\Delta\varphi$, and an average value of S = 3.3) were then used with this value of b (eqn. 4) to calculate the required gradient time: t_G = 16 min. Resulting values of $\bar{k}$ for each solute (methyl, 4.0; ethyl, 3.1) are not the same as isocratic values (2.29 and 4.34), but this is not required (see Fig. 1a). Rather the average value of $\bar{k}$ must equal the average value of k_0, and this is approximately the case (values of 3.3, isocratic; 3.5, gradient). The small discrepancy here is unimportant (see footnote ** of Table III).

A comparison of corresponding runs a and c or b and d in Fig. 4 for the parabens shows similarities as seen in Fig. 3. The band shapes for the overloaded gradient run (d) differ from those observed earlier (Fig. 3, 10 and 25 mg of HPT), but this may be due to sample-injection artifacts caused by the low solubility of the

TABLE III

DATA FOR DEVELOPMENT OF GRADIENT SEPARATIONS THAT CORRESPOND TO THE ISOCRATIC SEPARATIONS OF PARABENS IN FIG. 4a AND b

Gradient separations use similar conditions, except 10–100% methanol (linear gradient).

Parameter	*Methyl paraben*	*Ethyl paraben*	*Average value*
k_0 (1 μg)	2.29	4.34	3.3
k' (2 mg)*	1.91	3.57	
S	2.9	3.7	3.3
b (eqn. 5, $k_0 = \bar{k}$)			0.26
$\bar{k}$ (1 μg)	4.0	3.1	3.5
t_G (eqn. 4) (min)			17**
Isocratic R_s***			
1 μg each			10.3
2 mg each			1.4
Gradient R_s***			
1 μg each			9.2
2 mg each			1.5
k'/k_0	0.83	0.82	
t_g (min)			
1 μg	15.83	17.39	
2 mg	14.99§	16.69§	
$\bar{k}/\bar{k}_0$§§	0.74	0.73	
N§§§			
Isocratic			267
Gradient			306
w_s†			
Isocratic	192	210	201
Gradient	130	147	139

* "Band" values (used for measuring w_s).

** Actual value calculated from above data is t_G = 16 min; discrepancy due to use of preliminary data to select t_G before final isocratic data (50% methanol) were obtained.

*** Baseline bandwidths calculated by drawing tangents to sides of each band.

§ Corrected for sample volume, as discussed in Experimental section.

§§ Calculated from Eqn. 9.

§§§ Eqn. 8.

† Calculated from values of k' or $\bar{k}$ (for small and large samples), N and w_x as described in ref. 4.

sample in the injection solvent. Resolution was measured for each separation in Fig. 3; as expected, values of R_s for corresponding (same sample size) runs are similar: R_s = 10.5 and 9.2 for the 1-μg sample; R_s = 1.4 and 1.5 for the 2-mg sample. That is, similar separation occurs in corresponding isocratic and gradient runs, when the sample size is the same.

Eqn. 9 was used to estimate k'/k_0 for the gradient run with the 2-mg sample. Similarly, eqn. 8 was used to estimate N for each run. These data permit estimates of w_s for each solute in both the isocratic and gradient runs. We expect to find the same value of w_s, for either isocratic or gradient conditions. The average values for isocratic (w_s = 201 mg) and gradient (139 mg) differ by 30%, which is a larger discrepancy than observed earlier for similar solutes and the same isocratic system. Each of these values is also smaller than w_s (283 mg) for the same column with

methanol–water (60:40) as mobile phase. However the latter discrepancy may reflect an increase in w_s for mobile phases with higher organic solvent concentrations, as discussed in ref. 4. We conclude that there is a rough equivalence of w_s values obtained from either isocratic or gradient runs with the same column and corresponding conditions.

CONCLUSIONS

A simple theory of mass-overloaded separations using gradient elution has been developed. For small samples, the separation of any two bands will be comparable in isocratic and gradient elution, when gradient conditions have been selected to match those in the isocratic run, and when gradient time t_G has been selected so that k' (isocratic) equals $\bar{k}$ (gradient). For such "corresponding" isocratic and gradient systems, larger samples of the same size give similar separations (same values of R_s) in either isocratic or gradient elution.

The prediction of separation in mass-overloaded gradient elution can be carried out in an analogous manner as described for isocratic elution in preceding papers[1–4]. Gradient elution data can be used to derive analogous isocratic parameters that can be used in model simulations as described previously. Examples of the essential similarity of isocratic and gradient elution under mass-overload conditions are shown for mixtures of both xanthines and parabens.

APPENDIX I

Effect of mass overload on values of S

We can assume some value of w_{xk} for a given mass-overloaded separation as in Fig. 1b, for $k_0 = 1$. The value of w_{xk} is then 20/11 larger for $k_0 = 10$ (other conditions the same; eqn. 2 of ref. 4). For various values of k'/k_0 at $k_0 = 1$, we can then calculate values of w_{xk} and k'/k_0 for $k_0 = 10$. This allows us to determine values of S as a function of k'/k_0 or the degree of mass overload (eqn. 2). In this way it can be shown that values of S under mass overload are related to values of S (S_0) for small samples as follows:

k'/k_0 ($k_0 = 1$)	w_{xk}	S/S_0
1.00	–	1.00
0.90	0.16	0.97
0.80	0.45	0.95
0.60	2.3	0.92
0.40	12	0.88

Note first that values of $k'/k_0 < 0.8$ are associated with severe mass overload. For smaller samples, the maximum change in S with sample size is about 5%. In the case of severe mass overload ($k'/k_0 < 0.8$), the maximum change in S will rarely exceed 10%. This was confirmed experimentally for HET and HPT.

APPENDIX II

Effect of non-equal solute S values on mass-overloaded gradient elution separation

We will approximate this case by analogous isocratic separations (Fig. 2), using two different values of φ: φ_a and φ_b. For simplicity we have drawn Fig. A1 for the special case of equal S values for both solutes, although it is understood that the actual situation may be as in Fig. 2. We will define φ_a as the value of φ chosen for a small-sample separation of solutes X and Y. A second value of φ (φ_b) will correspond to a mass-overloaded isocratic separation such that values of k' for X and Y (solid triangles) remain the same as for the original small-sample run. This approximately matches the case of mass-overloaded gradient elution, as illustrated in Fig. 2 by the open squares for the corresponding gradient separation. We will assume as in Fig. 2 that k'/k_0 for the mass-overloaded run has the same value for both X and Y, although this is not important to the following argument.

Eqn. 2 can be used to describe the retention of X and Y for values of φ equal to φ_a and φ_b. These relationships then yield the dependence of values of α on φ:

$$\log \alpha_a = \log(k_y/k_x)_a$$

$$= \log k_{wy}/k_{wx} + (S_x - S_y)\varphi_a \quad \text{(A1)}$$

and

$$\log \alpha_b = \log k_{wy}/k_{wx} + (S_x - S_y)\varphi_b \quad \text{(A2)}$$

Here k_x and k_y are k' values for X and Y, $(k_y/k_x)_a$ is the value of (k_y/k_x) for $\varphi = \varphi_a$, α_a and α_b refer to values of α for φ equal φ_a and φ_b, and S_x and S_y are S values for

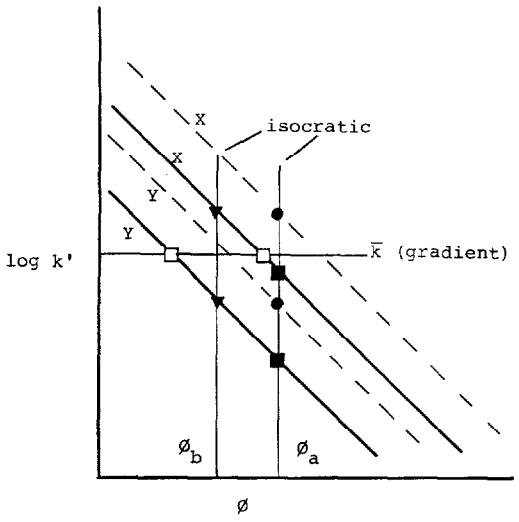

Fig. A1. Representation of "corresponding" separations of solutes X and Y by isocratic and gradient elution.

X and Y. We need to know how α changes with mass overload and φ. Eqns. A1 and A2 yield this information:

$$\log \alpha_b - \log \alpha_a = (S_x - S_y)(\varphi_b - \varphi_a) \tag{A3}$$

Now we want to relate this change in α to a corresponding change in k'/k_0 as a result of mass overload. Eqn. 2 for solute X can be written as

$$\log k_{xb}/k_{xa} = S_x(\varphi_a - \varphi_b) \tag{A4}$$

where k_{xa} and k_{xb} refer to values of k' for $\varphi = \varphi_a$ and φ_b, respectively. We initially chose φ_b to yield the same values of k' in the mass-overload separation as for a small sample and $\varphi = \varphi_a$. Therefore, we can equate k_{xa}/k_{xb} with the quantity k'/k_0 for mass overload at $\varphi = \varphi_a$ (see Fig. 2). This relationship with eqns. A3 and A4 then yields

$$\log \alpha_b - \log \alpha_a = (S_x - S_y)/S_x \log (k'/k_0) \tag{A5}$$

For small values of α and k'/k_0,

$$\alpha - 1 = 2.3 \log \alpha \tag{A6}$$

and

$$(k'/k_0) - 1 = 2.3 \log (k'/k_0) \tag{A7}$$

which allows eqn. A5 to be written as

$$\alpha_b - \alpha_a = [(S_y - S_x)/S_x][1 - (k'/k_0)] \tag{A8}$$

Now consider reasonable values of S_x, S_y and k'/k_0 — and their effect on α. A typical case might involve an initial α value of 1.5, a value of S_y that is 20% larger than S_x, and a value of k'/k_0 equal to 0.8 (moderate mass overload). Inserting these values into eqn. A7 gives a new value of α (for gradient elution with mass overload) equal to 1.46. This represents less than a 10% change in $\alpha - 1$ and R_s. While the combination of larger values of k'/k_0 and smaller values of α would have a greater effect on R_s, this same combination would also result in severe overlap of the two bands, with resulting failure of the present model for other reasons (see ref. 4). Values of S_x and S_y that are more than 20% different are possible but less likely[14], and would also lessen the similarity of mass-overloaded separation in isocratic *vs.* gradient modes. Our conclusion, therefore, is that differences in S values will in most cases not lead to differences in corresponding gradient *vs.* isocratic separation (mass-overload conditions).

APPENDIX III

Determination of a w_s value from two gradient runs

Gradient elution normally involves strong retention of the sample at the be-

ginning of the gradient (large k_{0g} values). Under these conditions, eqn. 3 can be approximated by

$$t_g = (t_0/b) \log (2.3\ k_{0g}b) + t_0 + t_D \quad \text{(A9)}$$

Mass overload results in a shifting of the log k' *vs.* φ curve as illustrated in Fig. 2a. This shift is equivalent to a change in k_{0g} for the gradient run (new value k_{0gm}), by the same ratio k'/k_0, or

$$k_{0gm}/k_{0g} = k'/k_0 \quad \text{(A10)}$$

Now we can write

$$\text{(small sample)} \quad t_g = (t_0/b) \log (2.3\ k_{0g}b) + t_0 + t_D \quad \text{(A11)}$$

and

$$\text{(mass-overload)} \quad t_{gm} = (t_0/b) \log (2.3\ k_{0gm}b) + t_0 + t_D \quad \text{(A12)}$$

Eqns. A11 and A12 combine to give

$$t_{gm} - t_g = (t_0/b) \log (k_{0gm}/k_{0g}) \quad \text{(A13)}$$

Eqns. A10 and A13 then yield eqn. 9.

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