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GRADIENT ELUTION IN LIQUID CHROMATOGRAPHY

XIII. INSTRUMENTAL ERRORS IN GRADIENT ELUTION CHROMATOGRAPHY

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SUMMARY

Instrumental sources of experimental errors in gradient elution liquid chromatography are considered. The performance of the equipment generating the concentration gradient in the low-pressure part has been investigated and it is shown that the equipment is able to reproduce the required ratio of the two components of the mobile phase and to maintain the flow-rate with an error of less than 1%. The deviations from the gradient profile due to the mixing in a reciprocating high-pressure pump are negligible under normal gradient elution conditions.

INTRODUCTION

The importance of the gradient elution technique in the liquid chromatography of mixtures of compounds with a wide range of capacity ratios is widely acknowledged. With increasing requirements on precise and reproducible quantitative results, it is important to evaluate and, as far as possible, to eliminate errors connected with the application of gradient elution. If the sample size and the chromatographic system are sufficiently stable and appropriately chosen, the errors in retention volumes and peak widths are due primarily to the limits of precision and reproducibility of three chromatographic variables: the composition and the flow-rate of the mobile phase and the temperature¹, their relative importance decreasing in that order. In order to achieve sufficiently reproducible results, it is important that the three variables can be reproduced in repeated experiments and their random fluctuations should be minimized. These three variables, however, should also be kept as close as possible to the expected values, otherwise the results are influenced by additional parameters that are properties not of the chromatographic system but of the instrumental design, are often very difficult to predict and hinder any comparison of results achieved with different instruments. In general, the reproducibility and accuracy of the experimental conditions can be far more easily controlled in isocratic elution chromatography than in gradient elution chromatography, but the latter technique can yield results as reproducible and accurate as those obtained under isocratic con-

ditions if appropriate attention is paid to the instrumental design and to the operation of the chromatographic system.

INSTRUMENTAL DESIGN AND ERRORS IN GRADIENT ELUTION CHROMATOGRAPHY

From the three parameters that influence the reproducibility in gradient elution chromatography, the temperature can be easily and satisfactorily controlled by simple thermostating of the column by means of a water-jacket connected to a circulating constant-temperature bath, if necessary².

The actual flow-rate and the change in the composition of the mobile phase with time (the profile of the gradient) may deviate from the required pre-set values for a number of reasons:

(a) The more efficient eluting component of the mobile phase is preferentially retained on the column, its content in the mobile phase decreases and consequently the gradient profile deviates from the pre-set value (solvent demixing effect). This effect is much more significant in adsorption than in reversed-phase chromatography and increases with the difference in polarities between the two components of the mobile phase³. The retention behaviour of the early eluted compounds may be subject to serious deviations due to the solvent demixing effect and these compounds may occasionally be eluted at some point of the gradient as badly resolved or unresolved narrow peaks. The elution of more strongly retained substances, however, is much less affected by solvent demixing, which can often be neglected or included in the delay of the gradient⁴. Further, this effect is much more significant if the gradient elution is started at zero concentration of the more efficient eluting component in the mobile phase than for the initial concentration of at least a few per cent of this component.

(b) Random or systematic deviations from the pre-set volume ratio of the two components and the flow-rate of the mobile phase (at atmospheric pressure), caused by the imperfect functioning of the mechanical parts of pumps (plungers, valves, seals) or electronic part of the system. In addition, the gradient profile can be influenced by the thermodynamic volume changes connected with the mixing of the two components of the mobile phase. As will be shown later, this effect is negligible with mixtures of organic solvents commonly used in adsorption chromatography, but can cause errors of up to a few per cent in the flow-rate of the mobile phase with solutions of polar organic solvents in water, which are frequently used in reversed-phase chromatography.

(c) The influence of the compressibility of the two components of the mobile phase, which may cause significant deviations from pre-set gradient profile and flow-rate at the operating pressure.

(d) Discrepancies between the actual and expected gradient profiles can be caused by additional mixing of the mobile phase in on- and off-line void spaces between the gradient mixing chamber and the column and subsequently, in the mixing chamber itself.

The instrumental design can influence significantly the errors included in groups (b)–(d). The most frequently used commercial instruments for gradient elution chromatography have recently been reviewed^{5–7} with respect to their design and

operational principles. The accuracy and reproducibility of the gradient profile and flow-rate have also been discussed in detail⁵⁻⁷.

Gradient devices are usually classified into two types: instruments in which the solvents are mixed in the low-pressure part and mixed solvent is pumped through the column, and those in which the solvents are mixed at high pressure before being delivered to the column^{6,7}. Instruments of the latter type may be subdivided according to the type of pumps used (the hydraulic part of the system) and according to the way in which the gradient is controlled by the electronic part of the instrument. As the errors in the formation of a gradient usually originate in the hydraulic part of the device, the classification according to the pumps used is more meaningful for our present purpose. At this point, it would be useful to recall briefly possible sources of instrumental errors connected with different types of devices forming gradients from two solvents. (Two-solvent gradients are by far the most frequently used in practice and very few separation problems, if any, can be expected to require gradients composed of more than two solvents⁷.) In systems in which pneumatic or hydraulic amplifier pumps deliver two solvents into a mixing chamber of small volume connected to the column, the flow-rate varies with time because of changing viscosity and compressibility of the mobile phase during the gradient run. These devices cannot accurately form the gradient required unless precise feedback control of the flow-rate is employed. The systems based on two large-volume syringe pumps are strongly influenced by compressibility effects. The differences in the compressibilities of the organic solvents or water, usually used as the two components of the mobile phase, can lead to profiles completely different from the theoretically expected gradients, which leads to irreproducibility of retention times^{2,6-9}. These effects can be suppressed by operating the two pumps at an equal and constant pressure, higher than the column operation pressure. A constant back-pressure valve can be used for this purpose².

The instruments using two reciprocating pumps without a flow-feedback control cannot reproduce accurately the gradient profile in the initial and final part owing to the limited speed range of the driving stepping motors⁶⁻⁸.

A major part of the drawbacks of the above systems is overcome in a new generation of gradient elution systems based on the use of one reciprocating flow-feedback pump, usually controlled by a microprocessor. The solvents (two or three) are introduced directly into the pump via time-proportioning electrovalves and mixed in the pump plunger chamber and in the lines between the pump and the column, *i.e.*, essentially in the high-pressure part of the instrument. Devices of this type can, in principle, provide accurately and reproducibly the gradient required⁷.

The instruments forming the gradient in the low-pressure part mix the solvents before they reach the inlet port of a high-pressure reciprocating pump. The low-pressure gradient mixing units of simple design, utilizing gravity as the driving force, which were used in classical column chromatography have been reviewed by Snyder¹⁰. Because of poor accuracy and reliability of performance, devices of this type can be hardly used for precise gradient formation as the low-pressure part of a gradient system. Rather, the solvents fed to the high-pressure pump should be controlled by time-proportioning electrovalves or via auxiliary low-pressure precision pump(s).

These gradient systems are little influenced by compressibility effects and can

completely eliminate errors connected with thermodynamic volume changes due to mixing of the solvents. For several years we have been using such a low-pressure system, forming the gradient in a low-pressure reciprocating pump.

In this work, the performance of this system has been tested with respect to the accuracy and precision of the gradient profile and flow-rate.

DESCRIPTION OF THE SYSTEM

The equipment for gradient elution is shown schematically in Fig. 1. Components A and B of the mobile phase are stored in glass reservoirs (erlenmeyer flasks, 500 or 1000 ml) (2), which can be heated and agitated (1). They are pumped by a gradient-generating low-pressure device (3) (PPM-68005, Workshops of the Czechoslovak Academy of Sciences, Prague), which is the reciprocating pump with two plunger blocks operating out-of-phase with a programmed stroke ratio and a constant total flow-rate. The total flow-rate is fixed by adjusting the plunger stroke frequency by means of a gear system. The ratio of the strokes in each plunger block delivering one solvent is continuously adjusted by a servo-motor, controlled by a photoelectric element, which follows the gradient drawn as a broad black trace on a sheet of paper fastened on a slowly rotating drum. Thus, an infinite number of gradients can be formed and reproduced well, as the sheets with drawn gradients can be kept. The pump has been slightly adapted in order to minimize the volume of solvents delivered in each stroke cycle (the total volume of the two solvents delivered in one stroke cycle is $19 \mu\text{l}$; this means, for example, a frequency of 62 strokes/min represents a

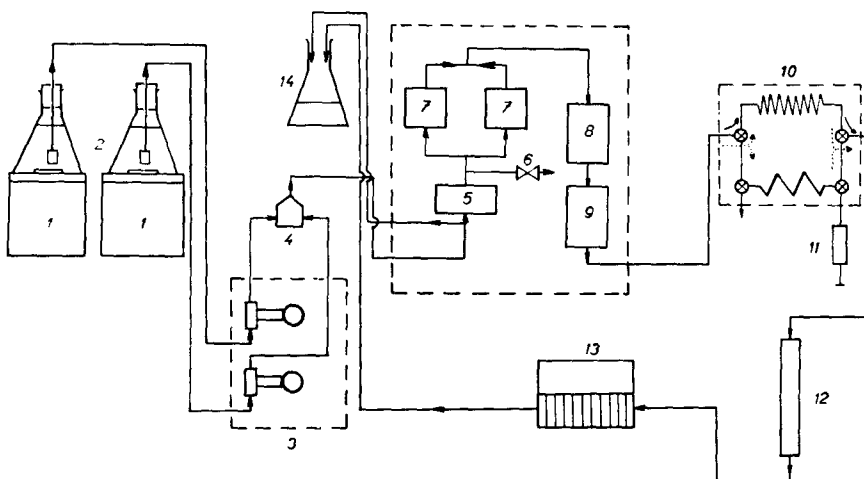


Fig. 1. Gradient-elution equipment with gradient generation in the low-pressure part, using the PPM 68005 gradient-generating device in series with the Waters Assoc. M6000 high-pressure pump. 1 = Electromagnetic stirrers and heaters; 2 = glass reservoirs (erlenmeyer flasks, 500 or 1000 ml); 3 = PPM 68005 two-plunger block gradient-generating device with programmed stroke ratio; 4 = gradient mixing chamber; 5-9 = M6000 high-pressure pump (Waters Assoc.); 5 = adapted inlet port; 6 = flushing vent; 7 = plunger heads; 8 = flow-through pulse damper; 9 = flow-through pressure sensor; 10 = U6K injector (Waters Assoc.); 11 = syringe septumless injection; 12 = column, reversed-phase C_{18} on LiChrosorb Si-100 ($10 \mu\text{m}$), $300 \times 4.2 \text{ mm}$; 13 = detector (UV, 254 nm, in gradient-elution operation; RI in performance tests; both Waters Assoc.); 14 = waste reservoir.

flow-rate of 1.18 ml/min). For further details on the performance of this pump, see ref. 11.

From the mixing chamber (4), the mixed liquid is delivered to a Waters Assoc. M6000 pump (reciprocating, stepping motor driven) with an adapted inlet port (5), enters the two plunger heads (7) of the pump, passes through a flow-through pulse damper (8) and pressure sensor (9) of the pump and is delivered through a sample injector (10) (Waters Assoc. U6K, but septum injectors can also be used) on to the column (12), flows through the detector (13) (UV, 254 nm, Waters) into the waste reservoir (14).

For accurate functioning of the equipment, it is essential to set the total flow-rate of the gradient-forming device (3) approximately 10–20% higher than the flow-rate of the high-pressure pump. The overflowing liquid is by-passed to the waste reservoir (14), which must be placed higher than the pump inlet (5).

The inlet port (5) of the high-pressure pump was adapted by inserting a small-bore Teflon cylinder with a disc frit, in order to minimize the void volume in this part.

TESTS OF THE PERFORMANCE OF GRADIENT ELUTION EQUIPMENT

The gradient-generating pump is capable of reproducing any gradient profile according to the selected mathematical function drawn on a sheet of paper fastened to the rotating drum of the photoelectric curve-follower. To investigate the performance of the system, the flow-rates of components A and B of the gradient delivered by the individual plunger blocks were compared with the expected values at different pre-set volume ratios of B to A, the agreement of the flow-rate at the outlet of the mixing chamber with the sum of the individual flow-rates of A and B was tested and the experimental and required gradient profiles were compared, especially in the initial and final parts of the gradient. The tests were performed for different instrumental arrangements, to determine the influence of the individual parts of the system on the experimental errors. These arrangements included: (a) PPM 68005 and M6000 pumps in series, with the refractive index (RI) detector connected to the outlet of the M6000 pump; (b) the M6000 pump was connected to the column and RI detector; (c) the sample injector (U6K, Waters Assoc.) in the "LOAD" position was connected between the M6000 pump and the column; and (d) the arrangement as (c), but with the injector in the "INJECT" position. A reversed-phase column (C_{18} , 10 μm), 300 \times 4.2 mm, was used in the tests of the system performance.

The results are shown in Tables I–III and Figs. 2–11. As shown in Table I, the flow-rates of the individual components of the organic (*n*-heptane–*n*-propanol) and aqueous (water–methanol) mobile phases delivered by plunger blocks of a PPM 68005 gradient-generating device correspond to the expected values with a standard deviation of *ca.* 0.5%, which is comparable to or better than in the commercial Waters Assoc. equipment (M6000 + M6000A pumps + M660 solvent programmer). The total flow-rate of the mobile phase at the outlet from the mixing chamber of the gradient-generating device (PPM 68005) is independent of the volume ratio of A and B, with a standard deviation of less than 1% for the *n*-heptane–*n*-propanol system, which is comparable to the gradient instrumentation from Waters Assoc. With the water–methanol system, however, the deviations of the total flow-rate from the expected values are systematic and reach maximal values of *ca.* 4–5%

TABLE I
COMPARISON OF THE FLOW-RATE OF THE LIQUID DELIVERED BY THE INDIVIDUAL PLUNGER BLOCKS (PUMPS) (F_A , F_B) AND THE TOTAL FLOW-RATE OF THE MIXED LIQUID (F_C) AT DIFFERENT SETTINGS OF THE VOLUME OF SOLVENT B FOR TWO TYPES OF GRADIENT ELUTION EQUIPMENT BASED ON TWO RECIPROCATING PLUNGER PUMPS

Type I, programmed stroke-ratio gradient-generation equipment, PPM 68005; II, programmed stepping motor speed equipment (M6000 + M6000A pumps controlled by M660 solvent programmer, Waters Assoc.). F_A , F_B and F_C are in ml/min; $\Delta = (F_A + F_B) - F_C$, as % of $F_A + F_B$; a and b are the parameters of the regression lines $F = a + bc$ ($c =$ pre-set % volume of B) and s is the corresponding standard deviation of F values, evaluated by regression analysis; F (mean) represents the arithmetic mean of the corresponding values F for different c .

In- stru- ment	B (%)	A = n-heptane, B = n-propanol					A = water, B = methanol					
		F_A	F_B	$F_A + F_B$	F_C	Δ (%)	F_A	F_B	$F_A + F_B$	F_C	Δ (%)	Δ (theoretical) (%)
I	0	1.220	0	1.220	1.220	0	1.200	0	1.200	1.200	0	0
	10	1.110	0.115	1.226	1.224	+0.16	1.070	0.142	1.211	1.199	+0.79	+0.70
	20	0.992	0.238	1.230	1.230	0	0.961	0.252	1.213	1.189	+1.85	+1.49
	30	0.873	0.358	1.231	1.224	+0.49	0.835	0.375	1.210	1.173	+3.08	+2.26
	40	0.750	0.478	1.228	1.223	+0.43	0.719	0.497	1.216	1.158	+4.21	+2.90
	50	0.620	0.600	1.219	1.221	-0.17	0.601	0.618	1.219	1.152	+4.71	+3.35
	60	0.513	0.723	1.236	1.220	+1.38	0.474	0.732	1.206	1.157	+4.18	+3.55
	70	0.378	0.842	1.220	1.212	+0.65	0.359	0.850	1.209	1.174	+2.98	+3.43
	80	0.250	0.960	1.210	1.210	+0.01	0.238	0.970	1.207	1.190	+1.77	+2.91
	90	0.132	1.085	1.217	1.210	+0.62	0.116	1.087	1.203	1.202	+0.74	+1.88
	100	0	1.210	1.210	1.210	0	0	1.209	1.209	1.209	0	0
	a	1.236	-0.006	1.230	1.227		1.198	0.002	1.210	1.179		
	b	-0.0123	0.0121	-0.00015	-0.00018		-0.0120	0.0120	-0.00002	0.000055		
	s	$7.3 \cdot 10^{-3}$	$2.0 \cdot 10^{-3}$	$8.7 \cdot 10^{-3}$	$8.3 \cdot 10^{-3}$		$4.5 \cdot 10^{-3}$	$6.7 \cdot 10^{-3}$	$8.4 \cdot 10^{-3}$	$2.13 \cdot 10^{-2}$		
	F (mean)	—	—	1.222	1.218		—	—	1.209	1.182		

II	0	0	1.041	1.041	1.041	0	0	1.064	1.064	1.064	0	0
	10	0.080	0.915	0.995	1.008	-1.33	0.070	0.950	1.020	1.003	+1.7	+0.70
	20	0.184	0.817	1.002	1.015	-1.34	0.165	0.837	1.002	0.997	+0.5	+1.49
	30	0.306	0.706	1.011	1.005	+0.61	0.263	0.725	0.988	0.982	+0.6	+2.26
	40	0.401	0.614	1.015	1.009	+0.60	0.374	0.630	1.004	0.968	+3.4	+2.90
	50	0.498	0.508	1.007	1.009	-0.26	0.476	0.521	0.997	0.952	+4.5	+3.35
	60	0.608	0.410	1.017	1.007	+0.97	0.566	0.420	0.986	0.951	+3.6	+3.55
	70	0.684	0.307	0.991	1.005	-1.39	0.669	0.314	0.983	0.950	+3.3	+3.43
	80	0.806	0.205	1.011	1.008	+0.27	0.765	0.223	0.988	0.964	+2.4	+2.91
	90	0.921	0.100	1.021	0.998	+2.28	0.862	0.112	0.974	0.963	+1.0	+1.88
	100	1.018	0	1.018	1.018	0	0.966	0	0.966	0.966	0	0
	<i>a</i>	-0.013	1.018	1.016	1.007		-0.020	1.052	1.031	1.016		
	<i>b</i>	0.0103	-0.0102	-0.00017	0.00002		0.00982	-0.0105	-0.00068	-0.00075		
	<i>s</i>	$7.0 \cdot 10^{-3}$	$4.4 \cdot 10^{-3}$	$10 \cdot 10^{-3}$	$5.4 \cdot 10^{-3}$		$9.4 \cdot 10^{-3}$	$7.6 \cdot 10^{-3}$	$1.50 \cdot 10^{-2}$	$2.35 \cdot 10^{-2}$		
	<i>F</i> (mean)	-	-	1.009	1.008		-	-	0.997	0.978		

TABLE II

ELIMINATION OF THE VOLUME CONTRACTIONS IN THE SYSTEM USING A PPM 68005 DEVICE FOR THE GENERATION OF CONCENTRATION GRADIENTS IN THE LOW-PRESSURE PART, PRIOR TO THE INLET OF THE M6000 HIGH-PRESSURE PUMP (FIG. 1)

F_C = flow-rate at the outlet from the column, ml/min; Δ = $100[F_C - F(\text{mean})/F(\text{mean})]$, % (rel.); I, component A = water, component B = methanol; II, component A = *n*-heptane, component B = *n*-propanol. Other symbols as in Table I.

<i>B</i> (%)	<i>System I</i>		<i>System II</i>	
	F_C	Δ	F_C	Δ
0	0.971	-0.4	0.988	+0.1
10	0.980	+0.5	0.986	-0.1
20	0.973	-0.1	0.989	+0.2
30	0.980	+0.6	0.986	-0.1
40	0.976	+0.1	0.984	-0.3
50	0.976	+0.1	0.987	0
60	0.973	-0.1	0.988	-0.1
70	0.973	-0.1	0.984	-0.3
80	0.978	+0.3	0.986	-0.1
90	0.971	-0.4	0.987	0
100	0.971	-0.4	0.992	+0.5
<i>a</i>	0.976		0.987	
<i>b</i>	-0.00004		-0.000004	
<i>s</i>	$3.4 \cdot 10^{-3}$		$2.2 \cdot 10^{-3}$	
$F(\text{mean})$	0.975		0.987	

TABLE III

DEVIATIONS OF THE EXPERIMENTAL GRADIENT PROFILES FROM THE PRE-SET VALUES FOR DIFFERENT INSTRUMENTAL ARRANGEMENTS USING AN M6000 PUMP WITH A PPM 68005 LOW-PRESSURE GRADIENT-GENERATING DEVICE

Arrangements: (a) PPM 68005-RI detector; (b) PPM 68005-M6000-RI detector; (c) PPM 68005-M6000-column-RI detector; (d) PPM 68005-M6000-U6K injector in "LOAD" position-column-RI detector; (e) PPM 68005-M6000-U6K injector in "INJECT" position-column-RI detector; column: reversed-phase C_{18} on LiChrosorb Si-100 (10 μm), 300×4.2 mm; void volume 3.2 ml. Gradient components: A = methanol; B = 0.3% nitromethane in methanol. B = slope of the gradient function $c = BV$ (c = volume ratio of B; V ml = volume of the eluate); V_z = volume between the mixing chamber of PPM 68005 and the detector; Δ_0, Δ_{100} = experimental deviations from the expected content of component B at the pre-set values 0% and 100% B, respectively (in %B). Flow-rates: (a) 1.18 ml/min; (b-e) 0.97 ml/min.

<i>Instrumental arrangement</i>	<i>B (theoretical)</i>	<i>B (experimental)</i>	V_z (ml)	Δ_0	Δ_{100}
a	0.0035	0.0035	0.18	--	--
	0.0565	0.0564		0	0
b	0.0043	0.0043	3.08	--	--
	0.0687	0.0687		+1.53	-1.47
c	0.0086	0.0086	6.31	--	--
	0.0687	0.0686		+1.19	-1.56
d	0.0086	0.0086	6.49	--	--
	0.0687	0.0681		+1.72	-1.33
e	0.0086	0.0087	9.09	--	--
	0.0687	0.0684		+3.41	-2.87

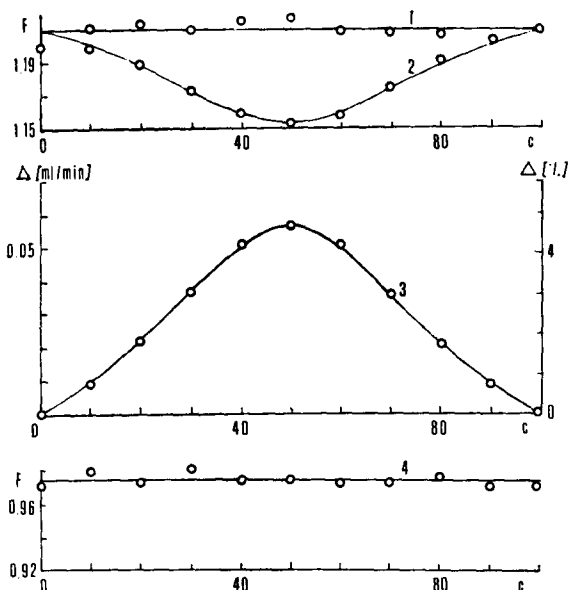


Fig. 2. Dependence of the flow-rate (F) on the pre-set composition of the mobile phase (c , in % of component B) in the system water (component A)–methanol (component B), using the PPM 68005 gradient-generating device (curves 1-3) and the device shown in Fig. 1 (curve 4). Curves: 1 = $F_A + F_B$ versus c ; 2 = F_c versus c ; 3 = $\Delta = F_A + F_B - F_c$, in ml and % (rel.) of $F_A + F_B$, versus c ; curve 4 = F_c versus c . F_A and F_B = flow-rates at the outlet of the individual blocks delivering components A and B, respectively; F_c = total flow-rate at the outlet from the mixing chamber of PPM 68005 (curve 2) and at the outlet from the column in the system shown in Fig. 1 (curve 4).

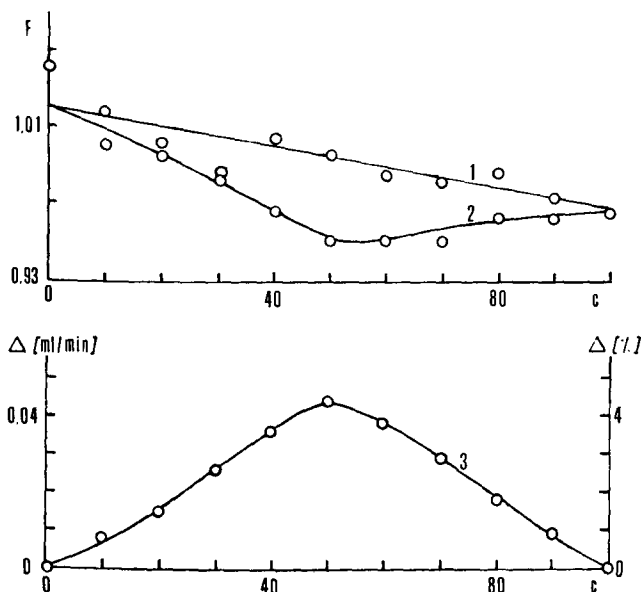


Fig. 3. Dependence of the flow-rate (F) on the pre-set composition of the mobile phase (c , in % of component B) in the system water (component A)–methanol (component B), using the Waters Assoc. gradient-elution system (pumps M6000 + M6000A + M600 solvent programmer). Curves: 1 = $F_A + F_B$ versus c ; 2 = F_c versus c ; 3 = Δ versus c . Symbols as in Fig. 2.

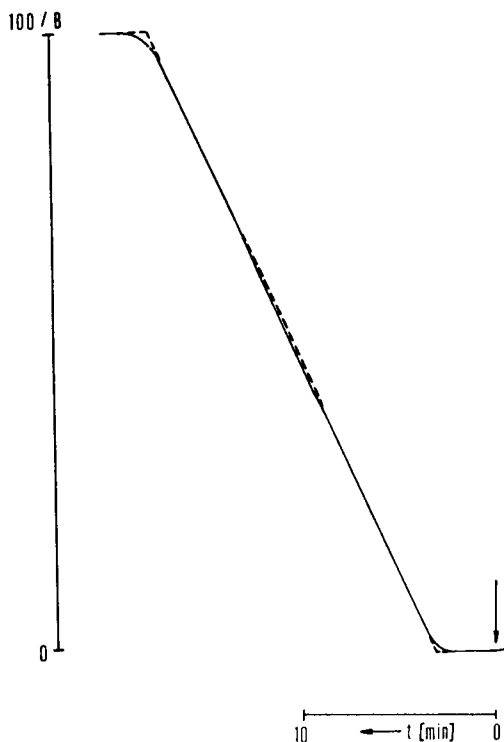


Fig. 4. Comparison of the experimental (full line) and expected (broken line) gradient profiles at the outlet of the M6000 pump connected in series to a PPM 68005 gradient-generating device. Component A = methanol; component B = 0.3% (v/v) solution of nitromethane in methanol. Gradient: $c = 0.06872V$, where c represents the volume ratio of B to A; V ml = volume of the mobile phase delivered from the mixing chamber. The experimental profile is measured as the signal of the R-401 RI detector (Waters Assoc., sensitivity 32).

in 40–60% methanol–water with both PPM 68005 and Waters Assoc. gradient equipment, as illustrated by Figs. 2 and 3. This is in approximate agreement with theoretically expected volume contractions due to the mixing of methanol with water (Table I).

This effect is eliminated in the gradient-generation system using a PPM 68005 gradient device in series with an M6000 high-pressure pump. As shown in Table II, the experimental values of the flow-rate at different settings of %B do not show any systematic decline as in the two above systems and the random errors are approximately the same in the water–methanol and *n*-heptane–*n*-propanol systems, with standard deviation of less than 0.5% of the total flow-rate in both systems. This precision is superior to that achieved with PPM 68005 or Waters Assoc. equipment (see Table I for a comparison). The elimination of the volume contractions in this system is illustrated in Figs. 2 and 3. It is further important that the flow-rates of the two plunger blocks (on pumps) be properly adjusted to achieve equal flow-rates at 0 and 100% B, as shown in Fig. 2, otherwise systematic shifts in flow-rate can be superimposed on to the other effects (Fig. 3, Waters Assoc. system incorrectly adjusted).

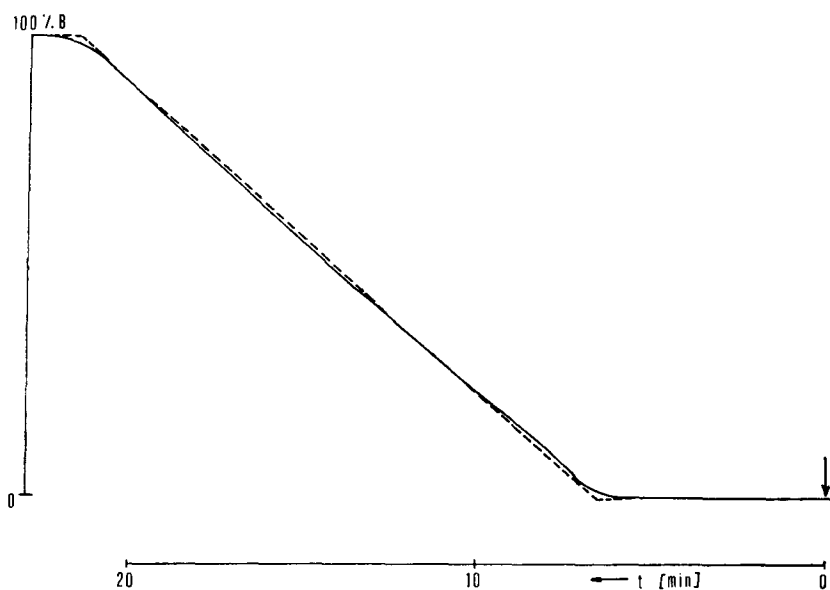


Fig. 5. Comparison of the experimental (full line) and expected (broken line) gradient profiles at the outlet of the column connected in the arrangement PPM 68005-M6000-column (reversed-phase C_{18} , $10 \mu\text{m}$, $300 \times 4.2 \text{ mm}$). Conditions and symbols as in Fig. 4.

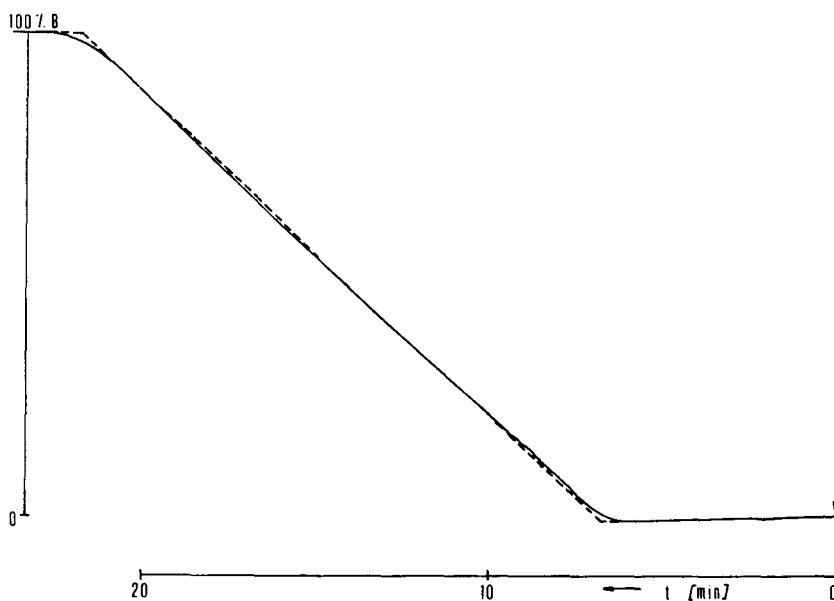


Fig. 6. Comparison of the experimental (full line) and expected (broken line) gradient profiles at the outlet of the column connected in the arrangement PPM 68005-M6000-U6K injector (in the "LOAD" position)-column. Conditions and symbols as in Fig. 4.

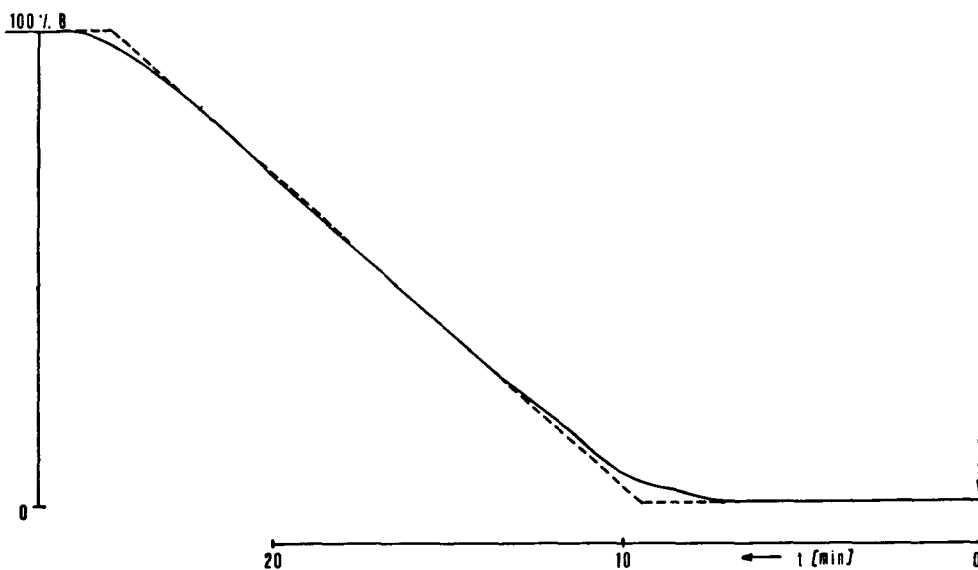


Fig. 7. Comparison of the experimental (full line) and expected (broken line) gradient profiles at the outlet of the column connected in the arrangement PPM 68005–M6000–U6K injector (in the “INJECT” position)–column. Conditions and symbols as in Fig. 4.

The equipments generating gradients in the low-pressure part inevitably contain void spaces between the mixing chamber at the inlet and the outlet of the high-pressure pump, which are given by the volumes of the plunger blocks, valve systems and the necessary connecting tubing. These void volumes, if incorrectly designed, can lead to deviations from the expected gradient profile. In any case, a corresponding volume delay must be considered before the gradient is operative in the column. Table III gives experimental values of this delay (V_z) for the PPM 68005 pump alone (0.18 ml), for the low-pressure and high-pressure pumps PPM 68005 and M6000 in series (3.08 ml) and the contribution of the U6K injector (0.18 ml in the “LOAD” position and 2.78 ml in the “INJECT” position). Thus, in the system shown in Fig. 1, the volume delay is 3.26 ml if the injector is switched to the “LOAD” position at the time corresponding to less than 3 ml of the mobile phase. This delay volume is not much larger than those in most commercial liquid chromatographs, where it is usually 1–2 ml.

Figs. 4–7 show experimental (full lines) and expected (broken lines) gradient profiles in gradient elution equipment with and without the column and U6K injector. As is further demonstrated in Table III, the gradient slope (B in Table III) is reproduced with a precision of better than 1% (relative). The expected and the experimental gradient profiles at the outlet from the PPM 68005 device cannot be distinguished and the discrepancies are less than 1% of B for other instrumental arrangements tested, with the exception of the initial and final part of the gradient, where it is *ca.* 1.5% for *ca.* 0.5 ml of the mobile phase (Figs. 8–11). These systematic errors in the gradient profile are relatively insignificant and approximately equal, regardless of whether the column and U6K injector (in the “LOAD” position) are connected to the M6000 pump or not. However, the deviations are significantly larger (1–3% of B)

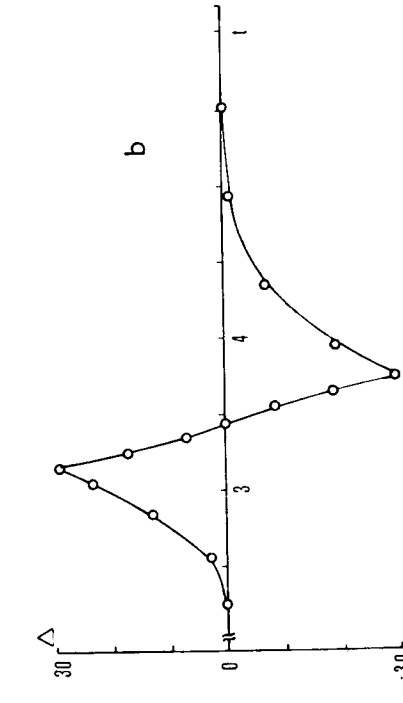
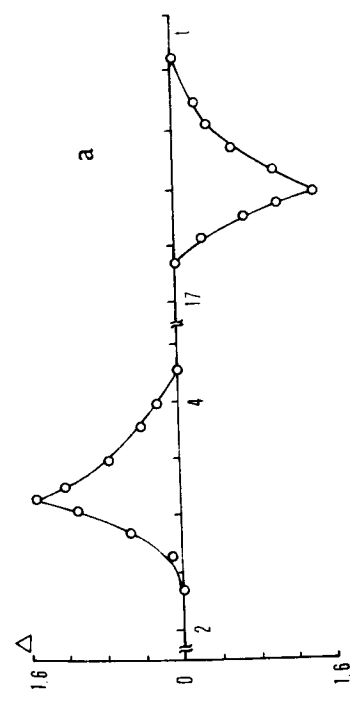
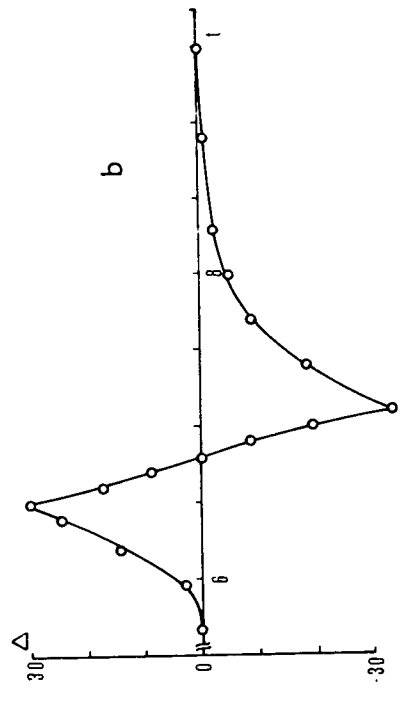
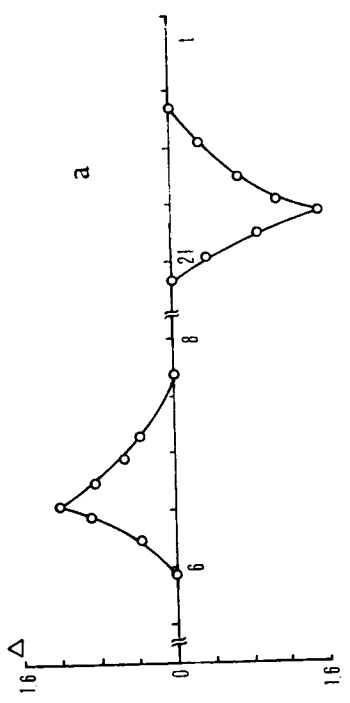


Fig. 8. Deviations of the experimental from the expected gradient profile (Δ) in the initial and final parts of the gradient in the arrangement PPM 68005-M6000 pump. $\Delta = \%$ of component B. (a) Gradient $c = 0.00687V$ (15 min); (b) gradient $c = 1.6628V$ (0.6 min). Symbols and conditions as in Fig. 4.

Fig. 9. Deviations of the experimental from the expected gradient profile (Δ) in the initial and final parts of the gradient in the arrangement PPM 68005-M6000-column. Conditions and symbols as in Figs. 4 and 8.

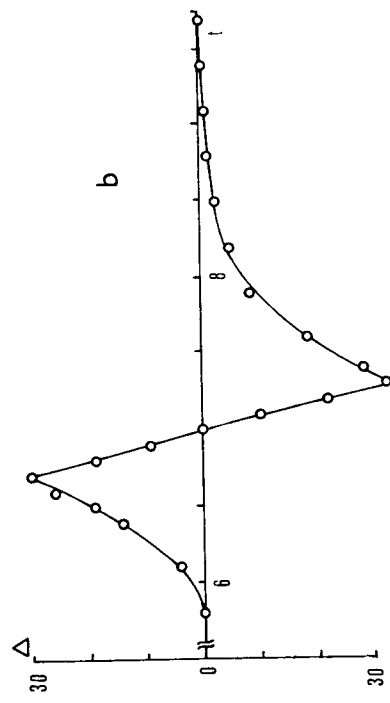
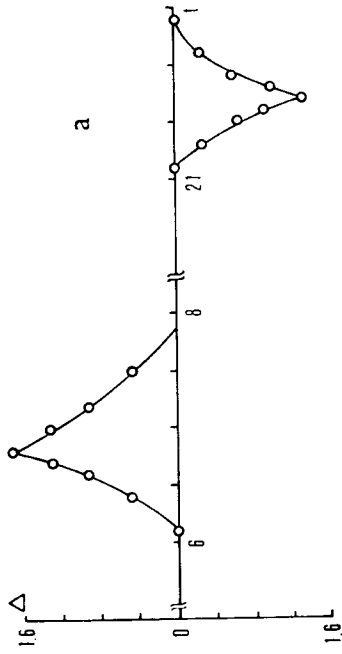
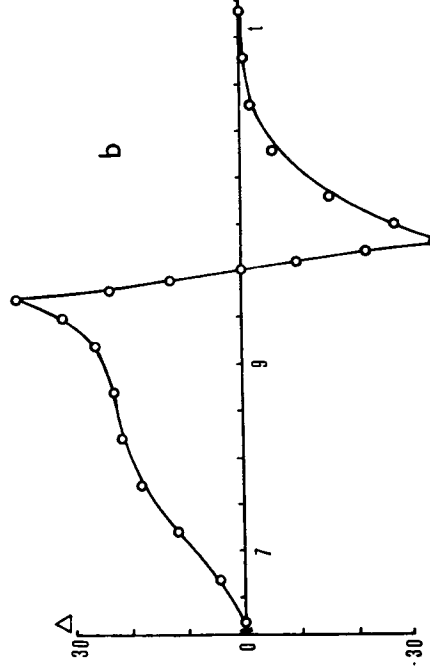
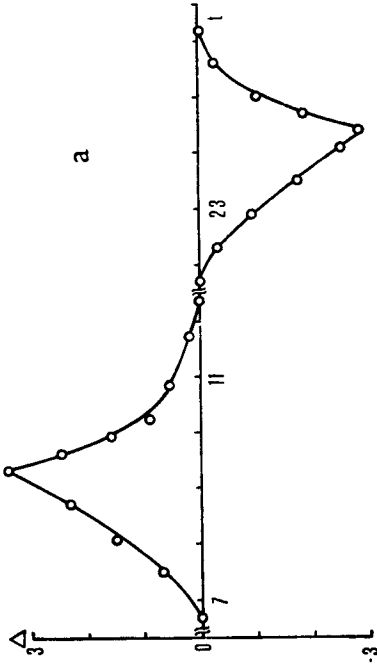


Fig. 10. Deviations of the experimental from the expected gradient profile (Δ) in the initial and final parts of the gradient in the arrangement PPM 68005-M6000-U6K injector (in the "LOAD" position)-column. Conditions and symbols as in Figs. 4 and 8.

Fig. 11. Deviations of the experimental from the expected gradient profile (Δ) in the initial and final parts of the gradient in the arrangement PPM 68005-M6000-U6K injector (in the "INJECT" position)-column. Conditions and symbols as in Figs. 4 and 8.

for a considerably longer part of the gradient (*ca.* 2 ml) if the injector is operated in the "INJECT" position, with a large-volume inner coil contributing to the instrumental void volume. Errors of this magnitude can be expected under the usual conditions of gradient elution operation (15-min gradient, 1 ml/min), but they increase with increasing slope of the gradient and can be considerably larger at extremely short gradients such as those shown in Figs. 8b–11b, which are, however, not used in practice.

CONCLUSIONS

The tests of the performance of the gradient elution device described here demonstrate the utility of this equipment for gradient elution operations, where both good reproducibility and good agreement between the experimental and pre-set gradient profiles and flow-rates of the mobile phase are required. The equipment, if operated with a U6K injector in the aLOAD" position, is able to reproduce accurately the required gradient profile with an error of less than 1% of solvent B during the gradient run, with the exception of a small part (*ca.* 0.5 ml) at the beginning and the end of the gradient, where the deviations are *ca.* 1.5%. The discrepancies between the pre-set and the experimental gradient profiles increase to some extent with increasing slope of the gradient, but this influence is relatively insignificant under the operating conditions usually used in gradient elution chromatography. A random error in the flow-rate of the mobile phase of *ca.* 0.5% (relative) can be expected and the total flow-rate does not depend on the ratio of solvents A and B being mixed. Eventual volume contractions due to the mixing of two liquids, such as a polar organic solvent with water, are eliminated with the system described. The performance of the gradient equipment was further demonstrated by the agreement between the experimental and calculated values of retention volumes in the gradient elution reversed-phase chromatography of various compounds in the water–methanol system, which was better than 5% (relative)¹².

The volume delay of the gradient in the equipment has to be considered and corrected for mathematically in calculations of retention volumes¹³, or it can be compensated for by delaying the sample injection with respect to the start of the gradient at the time calculated from the known volume delay and the flow-rate of the mobile phase.

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