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SEPARATION STRATEGIES FOR *o*-PHTHALALDEHYDE - MERCAPTOETHANOL DERIVATIVES OF AMINO ACIDS FOR REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATROGRAPHY

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SUMMARY

Twenty-three *o*-phthalaldehyde-mercaptoethanol derivatives of primary amino acids in serum were separated with Waters C₁₈ 5- μ m Radial-Pak Resolve columns, using aqueous phosphate-methanol mobile phase with acetate and tetrahydrofuran as modifiers. Resolution is critical in this system for glutamine/histidine, citrulline/glycine/threonine/3-methylhistidine, and tryptophan/methionine derivatives, and is affected by small changes in column properties or mobile phase. However, pre-run adjustments in gradient scheme and/or mobile phase composition can usually be used to obtain chromatograms in which all derivatives can be quantitated. For this purpose, and for general method development, we have written two very fast, interactive programs for the Zenith Z-100 Microsoft BASIC compiler: RTGRAPH, for retention, separation, and resolution plots; and LCSIM for simulations of multisegment binary gradient elution. These programs are shown here to be useful and accurate in most aspects required for developing strategies for this method.

INTRODUCTION

Over the past few years several reports have described the use of reversed-phase chromatography of *o*-phthalaldehyde-mercaptoethanol (OPA-ME) derivatives for amino acid analysis. In some cases excellent separation of most detectable OPA-ME derivatives of amino acids in serum has been demonstrated [1-7]. Because of putative advantages of radial compression technology for column integrity and stability over large numbers of injections, we sought to refine the

method for the Waters Radial-Pak column of Resolve chemistry (non-end-capped, recommended by Waters for OPA-ME amino acid derivatives). Use of the Waters WISP 710B autoinjector made possible automation of the mixing reaction to form the derivatives (Autotag) with concomitant improvement in quantitative accuracy [5-7]. However, with this system we found that the separation of all OPA-ME amino acid derivatives in serum was difficult to attain with consistency. First, the separation is critically dependent on concentrations of methanol and tetrahydrofuran (THF). Second, the columns vary individually and on aging, so that small adjustments in solvent modifiers and gradient schemes are frequently required to maintain separation of all derivatives.

We have written two programs in Microsoft BASIC for the compiler as an aid in planning separations, and making adjustments before runs; their outputs are used here to illustrate the effects of these modifiers. RTGRAPH provides isocratic retention, separation, and resolution plots, while LCSIM carries out simulation of multisegment gradient elution. Both programs use isocratic retention data for input. Test results are also provided in this report which substantiate the accuracy of LCSIM.

EXPERIMENTAL

Instrumentation

Two Waters 6000A pumps controlled by either a Waters Model 721 controller or a Waters Model 840 controller were used; both systems provided Multimethod-Autotag [7] capability for overnight runs with several gradient method programs and samples. The injection of reactants, mixing, and reaction to form fluorescent OPA-ME derivatives, and several gradient methods were pre-programmed and totally automated for the Waters WISP 710B autoinjector. Data were obtained and digitized with a Waters Model 730 integrator-recorder (used with the Model 721) or alternatively with the 840 controller-data system using 1-s detection intervals. A Kratos Model 970 fluorimeter was used for all experiments with excitation wavelength 330 nm and 418-nm emission filter. Waters Radial-Pak C₁₈ Resolve columns of 8 mm I.D. and 5- μ m packing were used in conjunction with an RCM-100 radial compression module. A Waters C₁₈ Guard-Pak precolumn and a precolumn particle filter were upstream to the analytical column. Between the injector high-pressure outlet and the particle filter, and close to the injector was placed the reaction chamber, a coil of 0.5 mm I.D. tubing ca. 0.8 m long, tightly wound.

Simulations, calculations, and graphics were carried out using a Zenith Z-100 desktop computer with the MS-DOS 2.21 operating system, and the Microsoft ZBASIC interpreter and compiler package [8]. Curve fitting was accomplished with Bevington's CURFIT Fortran program, revised for compiled BASIC [9].

Reagents

Research-grade Baker (Phillipsburg, NJ, U.S.A.) or Mallinckrodt (St. Louis, MO, U.S.A.) reagents were used for high-performance liquid chromatographic (HPLC) buffers. OPA and amino acids were from Sigma (St. Louis, MO, U.S.A.),

ME was from Kodak Labs. (Rochester, NY, U.S.A.), and methanol and THF were Burdick and Jackson HPLC grade (American Scientific Products, McGaw Park, IL, U.S.A.). Distilled water was further purified by a MILLI-Q Reagent water system (Millipore, Bedford, MA, U.S.A.). All mobile phase buffers were filtered using Millipore Durapore 0.23- μm filters. Samples and reagents were filtered using Gelman 0.45- μm ACRODISC filters (Gelman Sciences, Ann Arbor, MI, U.S.A.) before injection.

Autotag precolumn derivatization method

OPA-ME reagent (31 mM OPA, 44 mM ME in 5 parts of 0.4 M boric acid, pH 10.4-1 part methanol) was prepared according to ref. 7 by first dissolving 12.5 mg OPA in 0.5 ml methanol; then 2.5 ml boric acid, pH 10.4, and 10 μl ME were added. Sample vials contained 2-4 μM amino acid, dissolved in acidic buffer (60 ml methanol-50 ml water-0.1 ml concentrated trifluoroacetic acid with resultant pH 2.5). In our procedure 8 μl OPA-ME reagent were injected; after a latent period of 1.5 min this was followed by injection of an 8- μl sample. Flow-rate was 0.1 ml/min for 2 min on injection of the sample aliquot, followed by a 1-min ramp to a final flow-rate of 1.2 ml/min. Before each injection the column was equilibrated with the appropriate mobile phase mixture for several minutes.

Mobile phase preparation

Total organic content of all buffer A solvents was 4% (methanol+THF), by volume. In the text only the THF is listed, for brevity. For buffers without acetate, sodium dibasic phosphate and sodium monobasic phosphate were combined so that the total phosphate in solution was 0.05 M and pH 6.9 after adding organic modifiers. For buffers containing acetate, 0.05 M sodium dibasic phosphate, 0.05 M sodium acetate, and organic modifiers were added; then the pH was adjusted to 6.9 by addition of glacial acetic acid. For reference, three buffers are listed below:

- (1) no acetate, 1.0% THF, 3.0% methanol;
- (2) acetate, 1.8% THF, 2.2% methanol;
- (3) No acetate, 3.0% THF, 1.0% methanol.

All three buffers, as well as others discussed in the text, contained phosphate as described above.

Solvent B always consisted of methanol-water (65:35), and contained no buffers.

Acquisition of isocratic data

Since the amino acid derivatives elute in clusters, three to four sample vials were necessary to avoid overlap between derivatives. A single isolated derivative on the chromatogram, e.g., that of Arg or α -aminobutyrate (Amb), was used as a local reference. Binary gradient schemes were written for the Waters Model 721 or 840 controllers, which employed the Autotag method as well as re-equilibration to the next required mobile phase for the next run in the multimethods, done overnight. Data for several (four to six) values of ϕ (solvent B fraction) for each derivative were acquired, chosen to give retention times in the range generally

8–35 min. In the case of the test of the gradient simulation program, additional precautions were taken. The isocratic data for derivatives over the whole chromatogram, not just one cluster, were acquired during the same multimethod overnight run as were the three different gradient runs, using the same solvents A and B. Only a few derivatives from each cluster were included for this test, due to the large number of runs required. Temperature of the RCM-100 was maintained at 24°C by enclosure with a large insulating box equipped with a copper coil; a VWR 1140 refrigerated–heated circulator was used (VWR Scientific, San Francisco, CA, U.S.A.).

Method for fitting isocratic data

Retention data acquired with the Autotag method were adjusted by subtracting 2.25 min, as determined by a separate run in which the derivatives were performed, and injected under isocratic flow conditions. The void volume (3.3 ml) for the system and resulting t_0 (2.75 min at a flow-rate of 1.2 ml/min) were determined by observing the elution time of acetone injected in mobile phase B. Values for $t_0 k' = (t_R - t_0)$ were then calculated and used as input data for the Bevington CURFIT program. Two forms of $F(\phi)$ were used to fit the data. The first, called the standard fit, assumes a linear dependence of $\ln k'$ on ϕ ; however, a correction term A_3 is added to the function:

$$F(\phi) = t_0 \cdot k' = A_1 \cdot \exp(A_2 \cdot \phi) + A_3$$

where A_1 and A_3 have units of time, A_2 is always negative ($-11 < A_2 < -4$), and A_3 is always small ($-2 < A_3 < 1$ min, generally). In the second alternative form, called the quadratic fit, the correction A_3 is applied as a term of the exponential which is quadratic in ϕ :

$$F_2(\phi) = A_1 \cdot \exp(A_2 \cdot \phi + A_3 \cdot \phi^2)$$

or

$$\ln k' = A'_1 + A_2 \cdot \phi + A_3 \cdot \phi^2$$

where

$$A'_1 = \ln(A_1/t_0).$$

Isocratic retention graphics program (RTGRAPH)

This program, written in BASIC for the compiler, enables retention times and separation to be shown on the monitor, as well as graphic presentations of retention time functions $t_R(\phi)$ of four different derivatives. The parameters of t_R are stored in disk files prepared with a line text editor after the parameters have been determined using the Bevington CURFIT program. The graphic outputs take on three forms: (1) plots of retention times versus ϕ ; (2) plots of retention time differences ($t_{R_i,ref} = t_{R_i} - t_{R,ref}$) versus ϕ where the reference amino acid derivative is menu-selected; (3) plots of resolution

$$R = \frac{(t_{R_i} - t_{R_j}) \cdot \sqrt{N}}{(t_{R_i} + t_{R_j}) \cdot 2} = \frac{2t_{R_N} \cdot (t_{R_i} - t_{R_j})}{W_N \cdot (t_{R_i} + t_{R_j})}$$

where the plates N are determined from an isolated peak having t_{R_N} and width W_N , and in which six plots may be overlaid and identified on the monitor by color. While this plot shows windows of resolution [10,11], it also displays and identifies all pairs, whether critical or not. One can then inspect plots from the data obtained with different sets of solvent modifiers, and predict critical situations by viewing the displayed cross sections of three-dimensional plots and conceptually visualizing any three-dimensional plot.

Multisegment gradient simulation program (LCSIM)

Input files for the retention function $F(\phi)$ as well as a gradient program file are required. Menu-selected input values are column length, dead time from formation of a gradient event to column inlet, mobile phase column transit time (t_0), column plates (N), solvent switch times, maximum increments of times, and compounds to be analyzed. Outputs include monitor and hardcopy listings of retention times (t_R), peakwidths (W), separation, and resolution (R). For each compound on a run, retention times are calculated as

$$t_R = \sum_{i=1}^M \Delta t_i$$

where M (the number of increments) is limited by the condition that the sum of the incremental distances Δs_i , corresponding to each increment of time Δt_i , $\sum_{i=1}^M \Delta s_i$, cannot exceed L_{anal} .

The program calculates the individual Δs_i increments as follows:

$$\Delta s_i = L_{\text{cal}} \cdot \Delta t_i / [F(\phi) + t_0]$$

where L_{anal} is the length of the analytical column, L_{cal} the length of the column on which isocratic functions were determined, and $F(\phi) = t_R(\phi) - t_0$, the retention function. For accuracy, the number of increments is large; our tests show that 10 per min are sufficient for complicated gradient schemes involving steep slopes. The retention function $F(\phi)$ requires calculation of ϕ as a function of the time t_p , thereby correlating the delay for travel of the solvent front up to the analyte position p with the corresponding gradient event initiated at real time t . The time t_p is a function of the distance of travel $s(t)$ of solute along the column to point p at time t , i.e. $t_p = t + \frac{\Delta t}{2} - [t_0 \cdot s(t) / L_{\text{anal}}] - t_{\text{grd}}$. The first term is the real time t ; the second term is half the current time increment Δt ; the third term is the time required for mobile phase to flow from column inlet to the solute point $s(t)$, and the fourth term (t_{grd}) is the lag time for a gradient formation event in

the gradient table to reach the column inlet, which we have determined to be approximately 3 min in our system.

On elution, the peakwidth is calculated: $W = (W_{\text{isocr}}/t_{\text{Risocr}}) \cdot J_{\text{anal}} \cdot \Delta t_M / \Delta s_M$. The factor $(W_{\text{isocr}}/t_{\text{Risocr}})$, where W refers to the peakwidth at the base or $1.6 \cdot \text{area}/\text{height}$, is input by the user, or alternatively, calculated from the user input value for the plate count N by $W_{\text{isocr}}/t_{\text{Risocr}} = 4/(N)^{1/2}$. The conversion factor 1.6 was determined from graphic Waters 840 Scanner outputs of peaks for which area and height values had been already determined.

The resolution between the eluting derivative k and the preceding one, $k-1$ is calculated as: $R_{k,k-1} = 2 \cdot (t_{Rk} - t_{Rk-1}) / (W_k + W_{k-1})$.

The program is made more useful by two additional features. First, hardcopies may be created giving results of single runs, or of Multimethod runs in which one or several events on the gradient table are systematically varied. Second, a graphic monitor display for individual runs indicates when and where along the column changes in levels of ϕ reach the solute in its course of travel. In this display, the value of ϕ at the solute band is plotted against real time on the ordinate and abscissa, respectively. Simultaneously, in a long rectangular box, representing the column, the solute band moves. As gradient events contact the solute band, small marks under the box appear, indicating where along the column these events took place. This shows how many column plates were used at various levels of ϕ , which is useful information if the resolution plots are then examined. Appropriate changes then can be made to effect improvements in separation and resolution, parameters which are listed on the monitor after completion of the run (which takes a minute or two for several compounds). The gradient changes can be done from the menu and a hardcopy can be made when necessary. The latter method is totally interactive and has the advantage of eliminating unnecessary hardcopies.

RESULTS AND DISCUSSION

Fitting function $k'(\phi)$

The dependence of k' on ϕ is often expressed as $\ln k' = C_1 + C_2 \phi + C_3 \phi^2 + \dots$; at times only the first two terms are used [12-14]. The sum on the right hand side is proportional to the free energy difference for the solute between solid and mobile phases; use of only C_1 and C_2 implies a simple linear relationship of free energy change as the solvent composition is varied in a linear fashion. On the other hand, in practice accurate representation of $k'(\phi)$ is often quite complicated. This matter is under investigation currently in several laboratories, with systems of a greater range in diversity than the one discussed here [15,16]. Our data required a three-parameter fit (see Experimental), and we have attempted to stay as close to the ideal thermodynamic form as is feasible. The standard fit cannot be forced into this form due to the correction term $A_3 = t_0 \cdot C_3$: $k' = C_3 + \exp(C_1' + C_2 \phi)$ or $k' = C_3 + C_1 \exp(C_2 \phi)$, where $C_2 < 0$, and C_3 is the correction term. The quadratic fit can: $\ln k' = C' + C_2 \phi + C_3 \phi^2$ or $k' = C_1 \exp(C_2 \phi + C_3 \phi^2)$. The quality of fit and appearance of resolution plots was different for the two types of fit only when ϕ is well outside of the range of isocratic data actually collected. However, graphic plots of parameter values C_1 , C_2 , and C_3 from our current data suggest that the

expectation of reasonable behavior of each parameter with small changes in solvent, or column replacement is fulfilled better with the standard fit function, especially for C_1 and C_2 , than with the quadratic fit function. Furthermore, with the Bevington CURFIT routine the standard fit function converged to the data much faster than did the quadratic fit function.

We tentatively conclude that in spite of the unorthodox form of the standard fit function, its more predictable behavior may make it a better choice. In any event, the results discussed here depend only marginally on which function is used.

Test of multisegment gradient simulation program (LCSIM)

The multisegment gradient simulation program LCSIM was written to enable the designer of a multisegment gradient scheme to visualize how changes in the ϕ values with time would affect elution times and separation between closely eluting compounds. In practice, we found the program to be very useful; however, we were able to document the correspondence between real and calculated elution times by carrying out a single overnight set of runs as follows. Several isocratic runs and three different gradient runs for the test were carried out with a limited number of compounds, but over a large range of retentivity, using a 1-1 aliquot each of solvents A and B, and with temperature carefully controlled at 24°C. For the test no solvent switches were used; only buffer 2 was used as solvent A. Two of the three gradient schemes are of interest for the separations, and they are listed in Table I. Results with ten OPA-ME amino derivatives for real data and simulations are given here in Table II only for the Quick, gradient method with switchback. For all three methods, and for nearly all derivatives the retention times are matched within a minor fraction of a minute, and the very worst match is within 2.6% of the data. The larger deviations, however, do appear to be systematic: the later eluting compounds appear in simulation to elute slightly ahead of the eluents in the real gradient runs. The separation between Ala/Tyr and Trp/Met derivatives is not predicted well, but the effects of the three gradient schemes in changing these separation values are ordered correctly. Separation between early eluting compounds is predicted quite accurately. Peak widths W predicted by the simulation may be compared with data for area/height; graphs of these versus elution time for all peaks (not shown) indicate excellent agreement between the changes in these two parameters for the different solutes as they eluted over the time course of each gradient scheme. Sources of error may include slew of the gradient events, errors in input parameters such as t_0 , and time of equilibration of solute at the solid/liquid interface of the column.

Mobile phase modifiers

While RTGRAPH produces plots of retention time, separation, or effective resolution versus ϕ there are no comparable plots for the mobile phase modifiers, acetate and THF. Since these modifiers have a relatively small effect on retention parameters, linear interpolation between parameters from real data is used to generate new three-parameter sets for intermediate concentrations of modifiers, when necessary. The three buffer systems chosen for data collection form a basis

TABLE I
GRADIENT SCHEMES

The curves are as defined by Waters for control of gradients, such that a linear approach to an event on a line M is carried out between the line $M-1$ and line M if curve = 6. If curve = 1 the event occurs on line $M-1$; if curve = 11 the event occurs abruptly when the time $t = t(M)$. For intermediate curves the approach is curvilinear. The programs allow sufficient time to equilibrate to the next run (steps 11-15 for method A). In the other version of the Quick program, used for the top chromatogram in Fig. 3, isocratic $\phi = 40\%$ up to 14 min was used.

Line No.	Time (min)	Flow-rate (ml/min)	ϕ (%)	Curve	Solvent A buffer
<i>A. Quick method with switchback</i>					
1	Initial	0.0	43	-	3
2	2.0	0.1	43	1	3
3	3.0	1.2	43	5	3
4	5.0	1.2	43	6	2
5	9.0	1.2	34	6	2
6	14.0	1.2	37	6	2
7	19.0	1.2	74	7	2
8	25.0	1.2	77	7	2
9	30.0	1.2	83	7	2
10	35.0	1.2	100	6	2
11	42.0	1.2	100	6	3
12	47.0	1.2	44	6	3
13	52.0	1.2	44	6	3
14	53.8	0.0	44	8	3
15	53.9	0.0	44	11	3
<i>B. Long method</i>					
1	Initial	0	28	-	1★
2	2.0	0.1	28	1	1★
3	3.0	1.2	28	5	1★
4	34.0	1.2	28	6	2
5	36.0	1.2	46	6	2
6	41.0	1.2	46	6	2
7	46.0	1.2	78	7	2
8	52.0	1.2	75	6	2
9	58.0	1.2	96	6	2
10	60.0	1.2	100	6	2
<i>C. Tyrosine separation method</i>					
1	0	0	47	-	3
2	2.0	0.1	47	1	3
3	3.0	1.2	47	1	3
4	16.0	1.2	47	5	3
5	21.0	1.2	75	6	3
6	23.0	1.2	78	6	3
7	25.0	1.2	100	6	3

★With 0.6% THF.

set for calculations and discussion of the early eluting clusters (see Experimental). The original buffer system suggested by Waters for this system used high acetate and high (2%) THF; we have found that 1.8% THF is most useful and

TABLE II
TEST OF LCSIM

Real data versus simulation, Quick gradient scheme with switchback*.

Derivative	t_R (min)			Separation (min)		W^{**} (min)	
	Real*	Simulated	Difference	Real	Simulated	Real	Simulated
Gln	10.23	10.25	0.02			0.87	0.62
His	11.17	11.04	-0.13	0.94	0.79	1.04	0.72
Gly	18.10	18.16	0.06			(Peaks	0.99
Thr	18.85	18.87	0.02	0.75	0.71	fused)	0.91
Arg	21.31	21.54	0.23			0.44	0.42
Ala	22.44	22.63	0.19			0.29	0.28
Tyr	22.70	22.64	-0.06	0.26	0.01	0.34	0.23
Amb	25.39	24.93	-0.46			0.45	0.39
Trp	27.94	27.66	-0.28			0.48	0.43
Met	28.46	28.11	-0.35	0.52	0.45	0.57	0.47

*Autotag delay 2.25 min has been subtracted out.

** W for real data is presented here as $1.6 \cdot \text{area/height}$, in min. W for simulation uses the same plate count ($N=6250$) for all derivatives.

this is buffer 2. Buffers 1, 2 and 3 comprise a set in which the effects of lowering acetate or THF or both can be studied.

Separation methods

The elution order of OPA-ME derivatives depends on the buffer system and gradient method. Preliminary and careful inspection of RTGRAPH resolution plots for early-eluting derivatives Asp, Glu, Asn, Ser, (Gln/His), (Citr/Gly/Thr/3-Mehis), Arg showed that partial resolution of the second clustered quartet could be achieved with buffer system 2 or 3 (high THF) with the above elution order at $\phi=0.4$. This results in a 1-h cycle time for all the amino acids (the Quick method). With lower THF and no acetate (Buffer 1) and $\phi=0.28$, however, a different elution order is obtained for this quartet, (Gly/Thr/Citr/3-Mehis). The lower organic fraction, of course, lengthens the separation considerably and the cycle time becomes > 75 min (the Long method). The latter method is very nearly identical to that proposed earlier by Quereshi et al. [1]. Details of various aspects of these two distinct methods are discussed below. The elution order for the later eluting derivatives is Arg, Taur, (Ala/Tyr), Amb, (Trp/Met), Val, Phe, Fph, Ile, Leu, Orn, Lys.

Early quartet cluster

Difference plots and resolution plots with high acetate and high THF (buffer 2) are shown for Citr, Gly, Thr, and 3-Mehis (Fig. 1). The order of elution is as above for $\phi=0.4$; for different ϕ values it can be ascertained from the difference plot (top). Resolution is partial at $\phi=0.4$, and about the same with a narrower ϕ range at $\phi=0.31$ (elution order Gly/Citr/Thr/3-Mehis), which confers no advantage. At $\phi=0.2$ elution is too slow. Notice that at $\phi < 0.4$ Citr/Gly coelute, but at

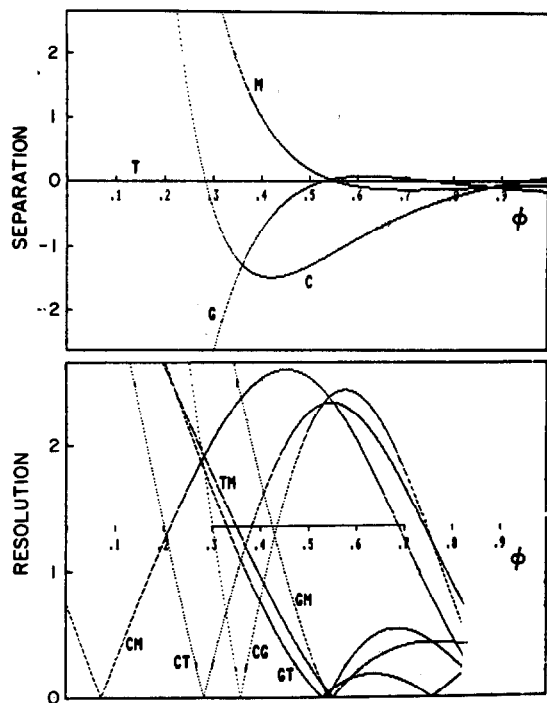


Fig. 1. RTGRAPH plots from isocratic data obtained with Radial-Pak RESOLVE columns. Mobile phase A is buffer 2 described in Experimental. Mobile Phase B fraction, ϕ , is abscissa, shown in plots. Horizontal solid bar on resolution plot is drawn through range of ϕ values for which isocratic data were collected. (Top) Separation plot; ordinate is retention time difference in min relative to that of Thr, located centrally. (Bottom) Resolution for all six pairs of amino acid derivatives. In all RTGRAPH plots the plate count $N=6400$ ($t_R/W=20$) has been arbitrarily assigned. C=Citr; G=Gly; T=Thr and M=3-Mehis.

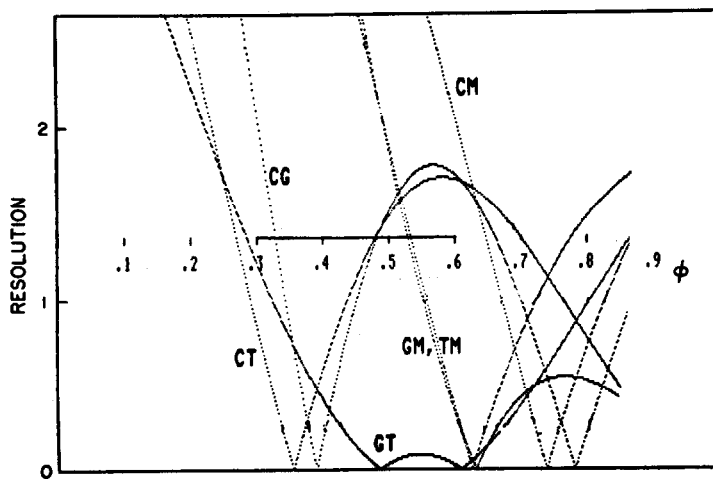


Fig. 2. RTGRAPH resolution plots for mobile phase with no acetate and low (0.6%) THF (extrapolation from parameters for 1 and 3% THF). C=Citr; G=Gly; T=Thr and M=3-Mehis.

$\phi > 0.4$ Gly/Thr and Thr/3-Mehis coelute. Thus, the plot serves as a guide to adjust the value of ϕ , which is critical for the separation. Inspection of these plots for buffer 3 (low acetate, high THF) reveal very similar features (not shown). However, if both acetate and THF are lowered as with buffer 1 (see resolution plot, Fig. 2) note that at $\phi = 0.28$ resolution is obtained for all compounds. While the elimination of acetate speeds the elution, the low ϕ value results in slow elution of these derivatives, hence, the Long method. Note in Fig. 2 that the resolution line for Thr/3-Mehis indicates excellent resolution of 3-Mehis for $\phi < 0.5$.

Successive linear interpolative mixing with data obtained with buffer 3 results in a series of parametric sets; if these are used to generate plots, it is seen that adding THF has the effect of speeding up the elution of 3-Mehis relative to the other derivatives, and to a much greater extent than separating Gly/Thr (for which THF is usually used). These effects can be visualized in a rough way by comparing directly with the resolution plot in Fig. 1. Therefore, it can be demonstrated quantitatively by interpolation, or by a series of runs at $\phi = 0.4$ in which THF is successively titrated in buffer A in increments of about 0.1%, that adjusting the THF concentration is very critical. The concentration of THF necessary for 3-Mehis to follow Thr too closely is somewhat higher (3%) when no acetate is present, than when high acetate is present (1.8%). This exact concentration of THF varies for individual columns. Although the titration must usually be done just before a run, these plots enable one to adjust methanol and THF to partially resolve all four derivatives. The resolution which can usually be obtained for the quartet with such isocratic conditions at $\phi = 0.4$ is typified by the chromatogram shown in the top of Fig. 3.

Further improvement in the separation is possible if isocratic constraints are removed. As previously noted (and from inspection of Fig. 1 once more) Citr/Gly resolution is better at values of ϕ larger than 0.4, while lower values of ϕ enhance Gly/Thr and Thr/3-Mehis resolution. Since Citr elutes first, it is advantageous to start the separation at $\phi = 0.44$ then decrease the ϕ value to 0.34 at a carefully chosen point in time. Because Citr elutes before Thr or 3-Mehis, this drop in ϕ has relatively little adverse effect on the resolution of Citr/Gly, but makes up for the loss in resolution of the latter two eluting pairs, otherwise expected as a result of starting out at a higher ϕ value. The resulting improvement on applying this switchback gradient scheme, listed in Table IA, is apparent (bottom, Fig. 3); this chromatogram was obtained immediately after the first one with the identical solvents. The calculated peak positions predicted by LCSIM are shown as lines. Note that although the exact peak positions are not predicted exactly (due to a different individual column having been used for input data), the effect of the gradient scheme on the separation is predicted closely. The prediction for resolution of Citr/Gly, Gly/Thr, and Thr/3-Mehis, respectively, are 0.9, 1.0 and 1.0 (isocratic); and 1.45, 1.0 and 1.1 (backwards) which are in agreement with resolution actually obtained and can be observed visually on the traces shown.

The LCSIM graphic outputs for both Citr and 3-Mehis have been combined in Fig. 4 for the Quick switchback gradient method; note that lengths of the column for low ϕ values are preferentially used for 3-Mehis (regions labeled 5 to 6) while Citr experiences higher ϕ values (left of the arrow).

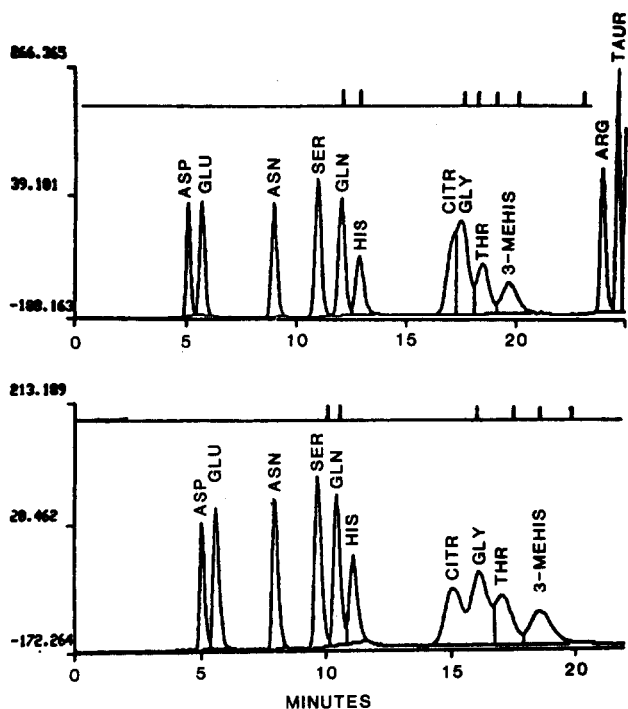


Fig. 3. Separation of early-eluting amino acids, using two versions of the Quick method with solvent switch A \rightarrow A' at 5 min, as discussed in text. (Top) Equilibration of column and isocratic separation through elution of 3-Mehis with $\phi=0.40$. (Bottom) Equilibration at $\phi=0.44$, and gradient scheme with switchback given in Table IA, to enhance resolution of Citr/Gly without materially affecting Gly/Thr or Thr/3-Mehis resolution. Retention times predicted by LCSIM are shown with lines above the chromatograms. Mobile phase A, no acetate, 2.4% THF (similar to buffer 3); mobile phase A', buffer 2.

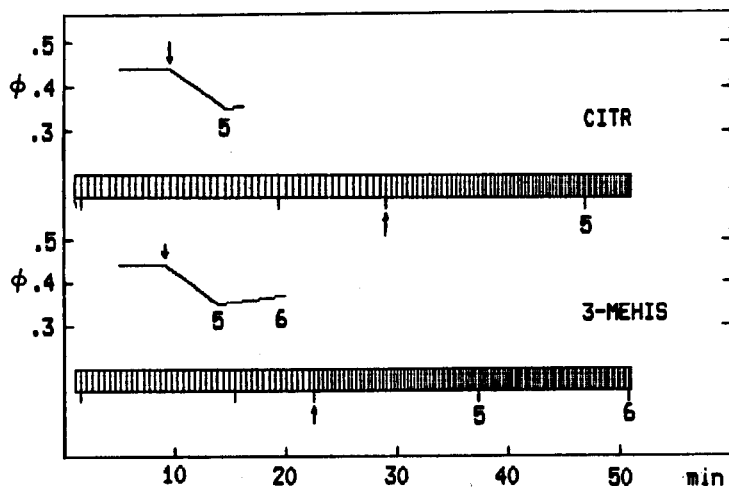


Fig. 4. Graphic LCSIM outputs for Citr and 3-Mehis, for Quick gradient method with switchback run shown in Fig. 3. Line numbers of the gradient segments appear under real time plot of ϕ value at the analyte point, while corresponding numbers under box (the column) indicate the analyte's position on column at that time. Arrows mark solvent switch. Note that the average ϕ value for Citr is greater than that for 3-Mehis, even though Citr elutes earlier.

Gln/His

Since in serum $[Gln] \gg [His]$, very good resolution of this pair is required. Actual gradient runs, as well as resolution plots from isocratic data, show that increased acetate concentration, increased ϕ , or increased THF concentration are detrimental to Gln/His resolution. They indicate further that use of 3% THF (necessary for Gly/Thr resolution as discussed above) with no acetate improves Gln/His resolution substantially over what can be attained with high acetate and 1.8% THF.

Solvent switch

In order to have good Gln/His resolution and still maintain the quartet's resolution the separation is initiated with lower acetate, high THF (2.6%) buffer A and switched to high acetate 1.8% THF buffer A' (see arrow, Fig. 4, LCSIM graphic output).

The use of acetate buffer after Gln/His have eluted slows down the separation, allowing the switchback gradient events to be used more effectively. The separations in Fig. 4 employ this strategy, as does the LCSIM program.

Late eluents

Resolution between Ala/Tyr and between Trp/Met is problematic, and depends on the individual column used, as well as its age. From isocratic data resolution plots have been made, which indicate for Ala/Tyr a sharp crossing at ϕ ca. 0.5 and for Trp/Met a sharp crossing at ϕ ca. 0.6. First, for $\phi < 0.5$ the Ala/Tyr elution order holds. Since Ala/Tyr follow closely behind Arg and Taur and Ala/Tyr are well-separated for $\phi < 0.45$, this would so far seem to cause no special problem. In fact, a quick analysis for tyrosine is easily devised (see the following section) which works well, even with old columns. The problem arises when resolution of Trp/Met is attempted. At $\phi < 0.6$ the elution order is Met/Trp, but since the time required for elution is very long (34 min), it is necessary to separate these derivatives at $\phi > 0.6$ where the elution order is Trp/Met. Therefore, since the order has already become inverted during the separation of the early eluents, it is necessary to proceed as quickly as possible with the gradient scheme to a high ϕ value, approximately 0.75, where separation of the Trp/Met pair takes place very efficiently. In fact, this must be done before Ala/Tyr elute to be effective; this can produce very narrow peakwidths and sometimes bad resolution of Ala/Tyr. The LCSIM graphic outputs in Fig. 5 show the distances along the column used at various ϕ levels (with consequences of relative motion on resolution inferred from the isocratic RTGRAPH plots). The output for Met with the Quick method is at the bottom and that for an implementation of the Long method is in the middle of the figure. Considerably more plates are consumed at ϕ values causing the detrimental order Met/Trp for the Long method. The shelf of the Long method a $\phi = 0.5$ has an adverse effect on the Trp/Met resolution since the order is Met/Trp at $\phi = 0.5$, but it is necessary to get Ala/Tyr off the column (Fig. 5, top); note that after the long separation of the early-eluting compounds at $\phi = 0.28$, label "4", only 1/3 of the column has been traversed. Therefore while total reliance on the LCSIM program to predict resolution is not feasible with the late-

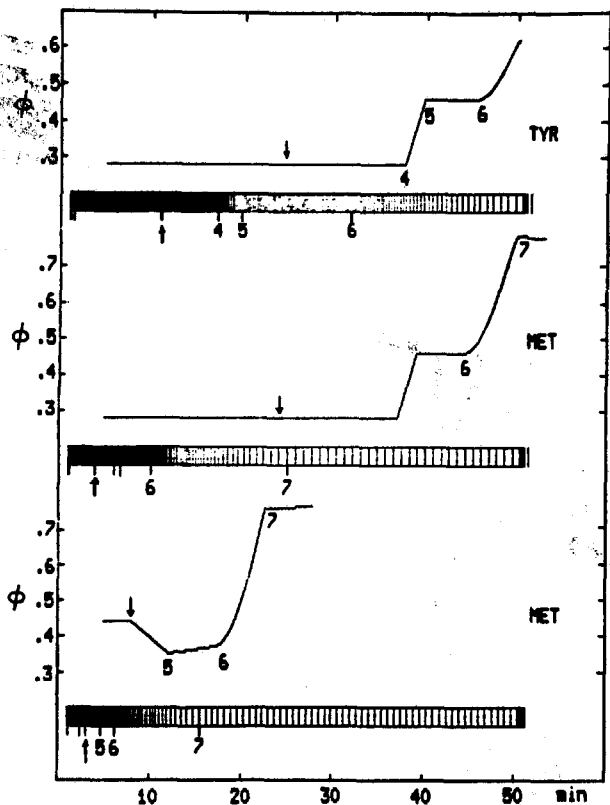


Fig. 5. Three graphic LCSIM outputs for Tyr and Met derivatives to show effects of gradient scheme on availability of column plates for separation of closely eluting solutes at specific ϕ values. (Upper two outputs) Long method; (lower output) Quick method.

eluting compounds, it seems likely that the Long gradient scheme will be detrimental to Trp/Met resolution. Several trial gradient runs in which we attempted to obtain good Trp/Met resolution gave results which were generally worse for the late eluents with the Long method than with the Quick method.

Consideration of the chemical structures of Ala, Tyr, Trp, and Met derivatives might lead one to expect that solvent modifier effects which would improve the resolution of one pair (Ala/Tyr) would be detrimental to resolution of the other pair (Trp/Met), and this has been found to be the case. Briefly, due to small shifts of the crossover points, THF improves, and acetate worsens Ala/Tyr resolution. Attempts to further improve Trp/Met resolution by solvent changes have not been successful.

An example of a typical chromatogram of a normal serum sample (Fig. 6, top) and of equimolar standards (bottom) obtained with a column several months old using the Quick method shows that all derivatives can be recognized and approximately quantitated. The method is a compromise which favors branched chain and aromatic amino acids. Usually better resolution for Trp/Met can be obtained, especially with a newer column. The steep gradient to $\phi=0.75$ has

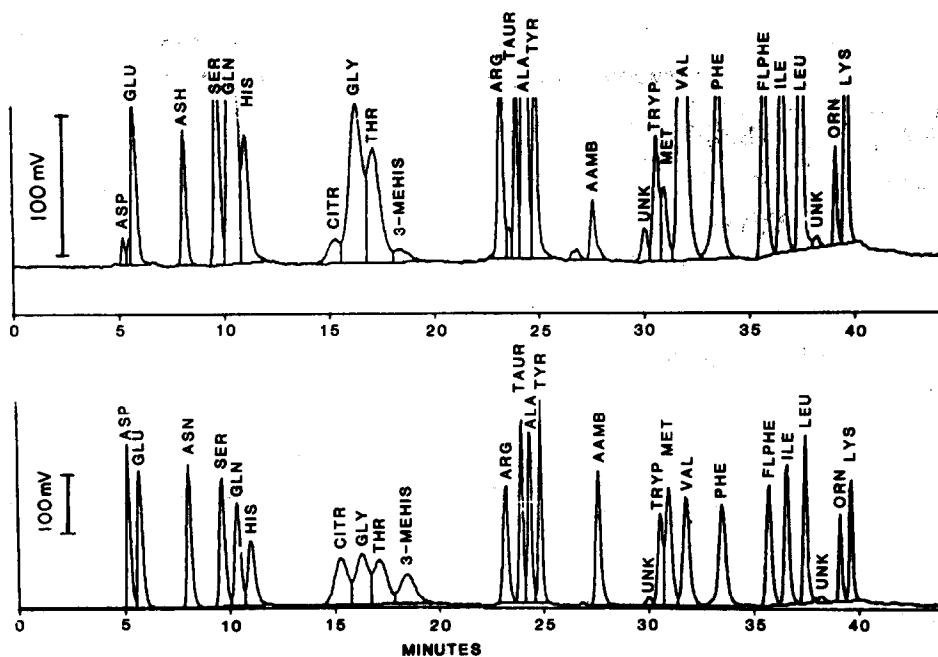


Fig. 6. Separation of 23 amino acids, with conditions and Quick method, gradient scheme with switch-back (Table IA) as used for bottom chromatogram, Fig. 3. (Top) human serum; (Bottom) amino acid standards (16 pmol each).

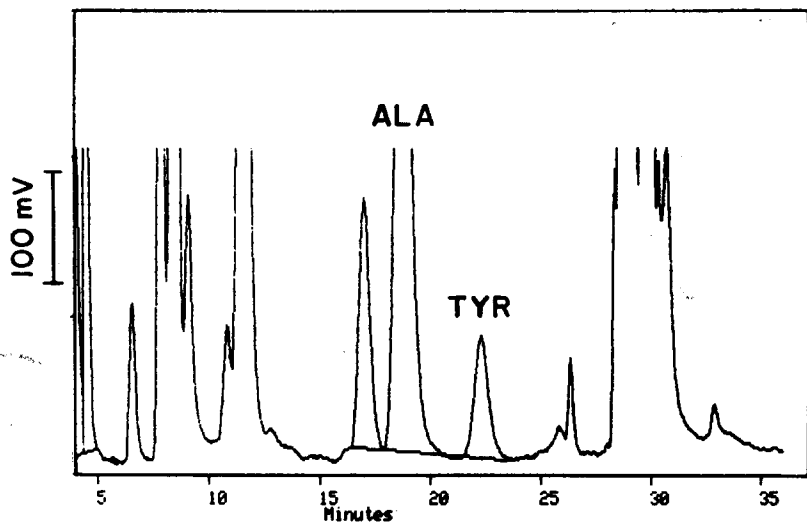


Fig. 7. Separation of Ala/Tyr and other components from culture media, for quantitation of tyrosine. Mobile phase: buffer 2.

resulted in narrow peak widths for Taur, Ala, and Tyr. Note that in the serum sample the large glutamine and alanine concentrations make Gln/His and Ala/Tyr resolution more critical than is the case with the equimolar standards.

Special applications

The computer-assisted methods discussed above make development of special methods for particular amino acids very simple. A quick gradient method listed in Table IC for tyrosine (of interest in certain metabolic studies with tissues) has been formulated by doing most of the separation at $\phi=0.48$ which takes 16 min, then eluting at $\phi=0.75$ (resulting in narrow line widths and sensitive detection). In accord with predictions based on RTGRAPH and LCSIM, excellent Ala/Tyr resolution is obtained when the mobile phase A contains a high THF concentration and no acetate (Fig. 7). Quantitation, as in all cases discussed in this report, is done with area, not peak height.

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