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PRACTICAL APPROACH FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD DEVELOPMENT: ASSAYING SYNTHETIC INTERMEDIATES OF A LEUKOTRIENE INHIBITOR

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SUMMARY

High-performance liquid chromatographic (HPLC) methods for analyzing new drugs and their synthetic intermediates are needed as the synthesis is optimized and scaled up from making milligram amounts for initial evaluation of biological activity to producing kilogram amounts of the drug for thorough testing purposes. The most efficient solution is a single HPLC method that can be used for each step of the synthesis. A practical approach for the development of a single HPLC method is the use of computer-assisted method development to maximize the resolution within a reasonable analysis time. The computer program DryLab I was used in the development of an HPLC assay for the synthetic intermediates of a leukotriene inhibitor. The use of DryLab I with binary mixtures of organic solvents in the organic portion of reversed-phase HPLC systems is reported. With the retention data from two initial analyses, resolution can be optimized as a function of solvent strength.

INTRODUCTION

Much has been written about method development in high-performance liquid chromatography (HPLC) over the last decade. Schoenmakers and Mulholland[1] recently gave an overview of contemporary chromatographic techniques. The time and effort put into method development is closely related to the application of the analysis. Our laboratory handles compounds that are synthetic intermediates during the synthesis of a potential drug. Often an aliquot is removed from an ongoing synthesis and needs to be analyzed for purity and to check on the extent of the chemical reaction. Therefore, a preliminary method is needed as quickly as possible. We have found that the easiest way of analyzing an ongoing multi-step synthesis is to have a single method that can separate all the intermediates that are isolated in the synthesis, so that each step of the synthesis can be effectively monitored with the same HPLC method.

In Schoenmakers and Mulholland's[1] "method development staircase", this corresponds to the "purity check/optimum selectivity" stair. Gazdag *et al.*[2] divided method development into two branches, solvent strength and solvent type. Quarry *et al.*[3] reported that varying solvent strength is less powerful than varying solvent type. However, solvent strength can be readily optimized with only a few analyses and can

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significantly affect changes in band spacing. The use of relative retention maps[4] can quickly allow optimization based on solvent strength. These maps, a refinement of window diagrams developed by Laub and Purnell[5], can be generated by commercial computer software (DryLab I) after only two reversed-phase analyses with the same solvent in different proportions with water in the mobile phase.

THEORY

It has been shown by Schoenmakers *et al.*[6] that $\log k'$ as a function of the volume fraction of the organic modifier, φ, can be expressed as

$$\log k' = A\varphi^2 + B\varphi + C \tag{1}$$

This was reduced by Snyder *et al.*[7] to

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

where $1 \leqslant k' \leqslant 10$ and k'_w is the extrapolated capacity factor[8] in water as the mobile phase. When $k' < 1$, Schoenmakers *et al.*[9] found that the k' in water is greater than the extrapolated k'_w value in eqn. 2.

Isocratic retention data from two analyses with different ratios of water to organic solvent can be interpolated. This is because the plot of the log k' *versus* percentage of organic component in the mobile phase is nearly linear. Therefore, retention data for any mobile phase can be determined from these plots. When the plots are non-linear, errors in predicting values of k' can result, although they are generally small when $1 \leqslant k' \leqslant 10$ (ref. 10).

The determination of log k' *vs.* φ plots leads to the generation of relative resolution maps, which can easily be produced by a computer program such as DryLab I. For reversed-phase predictions, DryLab I assumes that only a single organic solvent with water is used. However, two organic solvents can be mixed together and still behave like a single solvent in a quasi-binary system[8] with water. Two solvents typically used in reversed-phase HPLC are methanol and acetonitrile (ACN).

Schoenmakers *et al.*[11], developed a transfer rule relating methanol–water volume fractions, φ_{CH_3OH}, to isoeluotropic acetonitrile–water volume fractions, φ_{ACN}.

$$\varphi_{ACN} = 0.32\,\varphi^2_{CH_3OH} + 0.57\,\varphi_{CH_3OH} \tag{3}$$

Herman *et al.*[12] refined this equation to a cubic polynomial:

$$\varphi_{ACN} = -0.490\,\varphi^3_{CH_3OH} + 0.953\,\varphi^2_{CH_3OH} + 0.447\,\varphi_{CH_3OH} \tag{4}$$

However, from 50 to 95% methanol, this cubic equation can be approximated by the linear equation

$$y = 1.038x - 0.117 \tag{5}$$

with a correlation coefficient of 0.999 (Fig. 1).

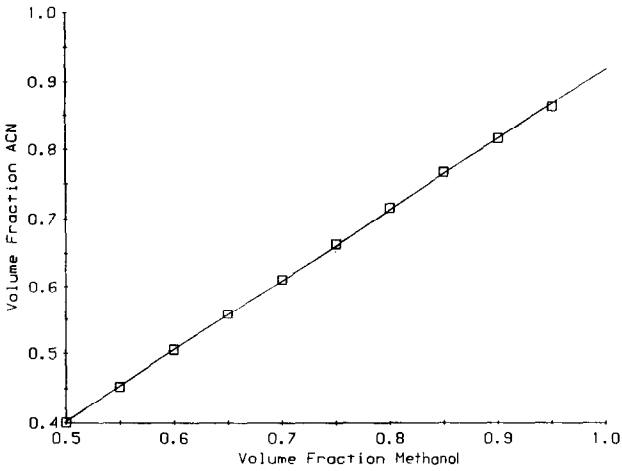

Fig. 1. Graph of transfer rule equation relating methanol–water volume fractions, φ_{CH_3OH}, to isoeluotropic ACN–water volume fractions, φ_{ACN}, from 50 to 95% methanol.

EQUIPMENT

Instrumentation

A liquid chromatograph consisting of a Model 590 pump (Water Assoc., Milford, MA, U.S.A.) with a flow-rate of 2 ml/min, an autosampler (Waters Assoc. 712 WISP), a Model 783 UV absorbance detector (Kratos, Ramsey, NJ, U.S.A.) set at 210 nm, a data system (DEC VAX 11/785 minicomputer with a G. D. Searle chromatography data system) and a Model 585 recorder (Linear, Irvine, CA, U.S.A.) were used. The separations were performed on a Zorbax C_{18} (DuPont, MacMod, Wilmington, DE, U.S.A.) column (250 × 4.6 mm I.D.). Computer simulations were made using DryLab I software (LC Resources, Lafayette, CA, U.S.A.) on an IBM AT personal computer (Boca Raton, FL, U.S.A.).

Reagents

HPLC-grade acetonitrile and methanol were obtained from Baxter Burdick and Jackson (Muskegon, MI, U.S.A.), water from J. T. Baker (Philipsburg, NJ, U.S.A.) and triethylamine from Aldrich (Milwaukee, WI, U.S.A.), which was added (0.1 vol.-%) to the water. Phosphoric acid (Mallinckrodt, Paris, KY, U.S.A.) was added to adjust the pH to 2.5 (*i.e.*, aqueous triethylammonium phosphate, TEAP). All solvents were filtered through 0.45-μm filters (Millipore, Milford, MA, U.S.A.) and degassed ultrasonically under vacuum.

Solvent systems

The following solvent systems were used: (1) ACN–TEAP (80:20, v/v); (2) methanol–TEAP (80:20, v/v); (3) ACN–methanol–TEAP (55:20:25, v/v/v); (4) ACN–methanol–TEAP (69:26:5, v/v/v); (5) ACN–methanol–TEAP (63:23:14, v/v/v); and (6) ACN–TEAP (77.7:22.3, v/v).

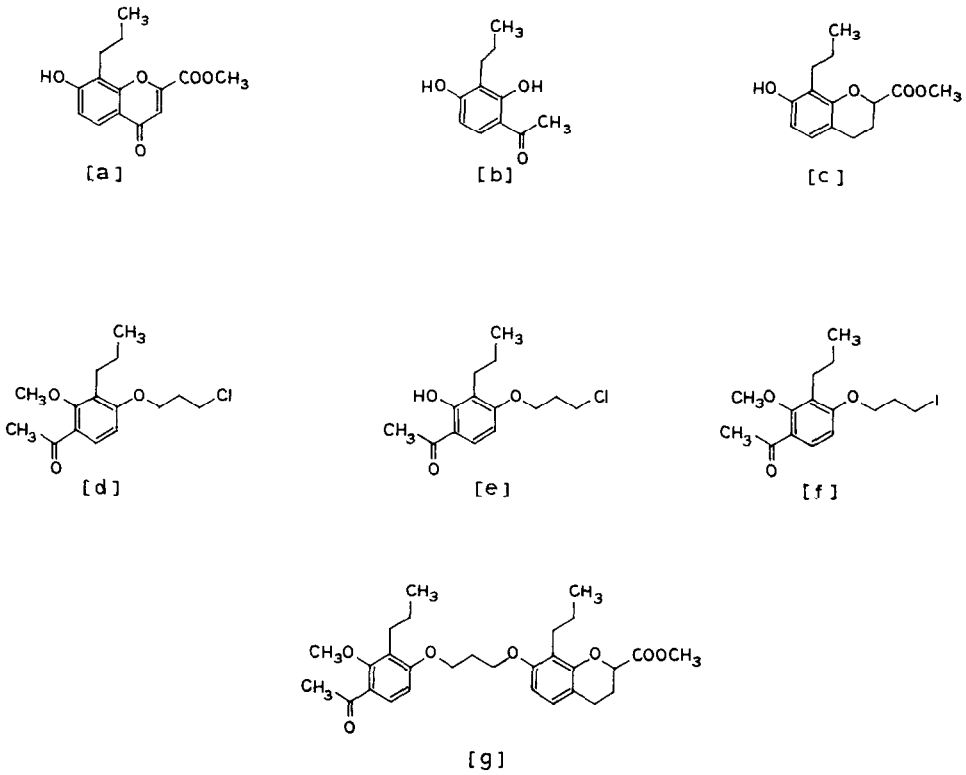

Fig. 2. Structures of the intermediates investigated.

Samples

All samples were synthesized in our laboratories and their structures are shown in Fig. 2.

RESULTS AND DISCUSSION

Typical binary solvent systems containing aqueous triethylammonium phosphate buffer (pH 2.5) with either acetonitrile or methanol (see Fig. 3) were initially investigated. The results indicated that system 1 eluted intermediate g much faster and thereby shortened the overall analysis time, but intermediates b and c co-eluted. System 2 merged intermediates a and b and eluted intermediate g much later. It can be seen from these two binary systems that the total percentage of organic component in the mobile phase has to be roughly 80%. As in any multi-component analysis, the later eluting peaks must elute rapidly enough so as not to be too broad, yet the solvent strength must be weak enough so as not to merge earlier eluting peaks. To achieve a complete separation of all seven intermediates with better resolution and short analysis time, binary solvent systems involving methanol–TEAP or ACN–TEAP would not succeed. It was decided to explore ternary solvent systems.

Two ternary solvent systems were tried (systems 3 and 4), each containing ACN

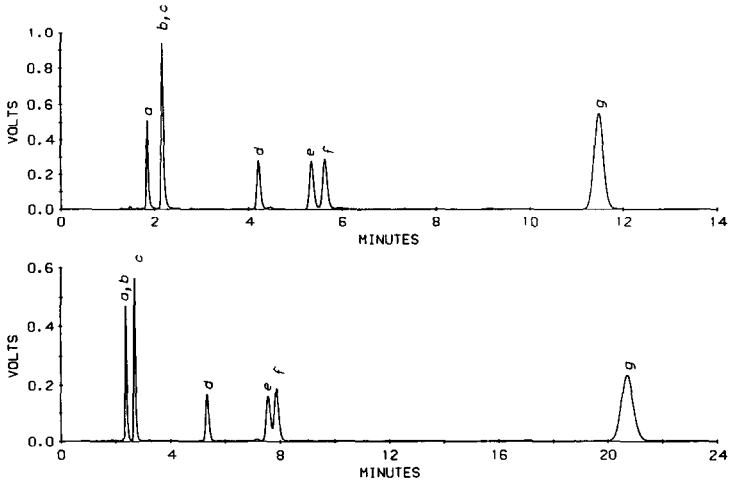

Fig. 3. Initial isocratic runs with 80% organic solvent in the mobile phase. Top: system 1 (ACN–TEAP, 80:20, v/v). Bottom: system 2 (methanol–TEAP, 80:20, v/v).

and methanol in a ratio of 2.7:1, thereby making these solvent systems quasi-binary mixtures with TEAP. This ratio of ACN to methanol was determined intuitively based on experience in HPLC. The major difference in the two ternary solvent systems was that the middle peak of the first three peaks, intermediate b, co-eluted with

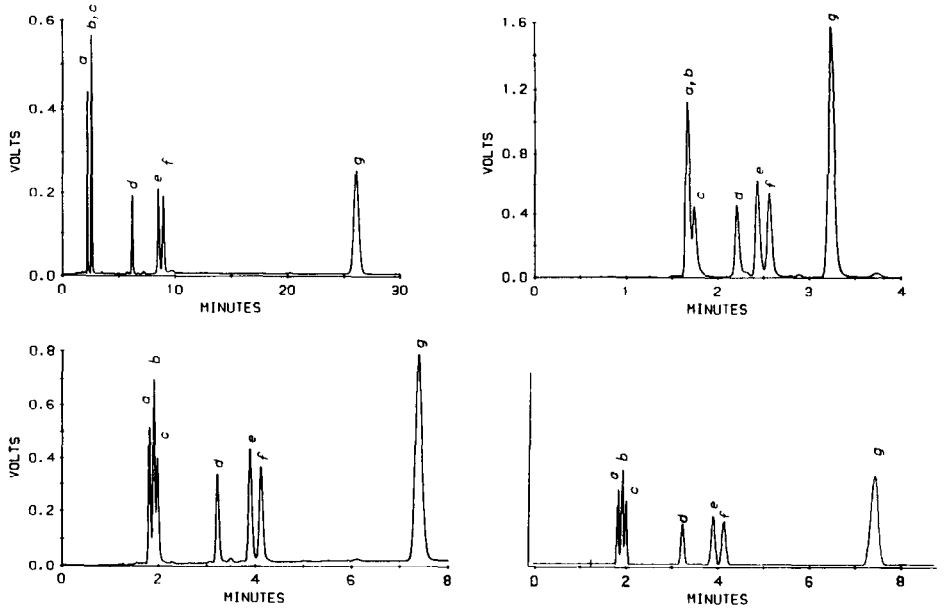

Fig. 4. Actual and simulated chromatograms from the two initial quasi-binary solvents systems and the predicted optimum solvent system. Top left: system 3, 75% organic component, actual run. Top right: system 4, 95% organic component, actual run. Bottom left: system 5, 86% organic component, actual run. Bottom right: 86% organic component, computer simulated.

intermediate a in the stronger solvent system and was partially merged with intermediate c in the weaker solvent system (Fig. 4). Intermediate g eluted much sooner in the stronger solvent system.

To help in the determination of the best ratio of the mixed binary organic solvents to water, DryLab I was used. The retention times of all the peaks from the two isocratic ternary runs were entered into the program. For the co-eluted peaks, the retention times were entered twice.

With the DryLab I software a relative resolution map could be created, showing the change in resolution between the two closest eluting peaks as a function of percentage of total organic solvent in the mobile phase. The latter value that gave the highest resolution becomes apparent from the map (Fig. 5).

The optimum percentage of total organic solvent in the mobile phase that maximizes the resolution and selectivity of the closest pair of peaks is found when the software tabulates the retention and resolution of the closest pair of peaks in 1% increments of total organic solvent. The maximum was found at 86% total organic solvent. An increase in the solvent strength would result in a loss of resolution between intermediates a and b and a decrease adversely affects the separation of intermediates b and c.

A simulated chromatogram with 86% total organic solvent is shown in Fig. 4. When an actual run (Fig. 4) was made at 86% total organic component (system 5), the results were very close to prediction. Then several different runs were made by varying the percentage of the mixed organic solvent from 75 to 95% and the log k' of each of the intermediates was plotted against organic volume fraction. Each intermediate gave a linear plot over the range with an average correlation coefficient of 0.998 (Fig. 6). It is apparent that there is only a small range of organic volume percentage where intermediate b is separated from intermediates a and c.

When the retention times of each peak were plotted against the peak number, the resulting graphs shows the accuracy of the computer model (Fig. 7). When an isoeluotropic binary solvent system (system 6) was determined by replacing the

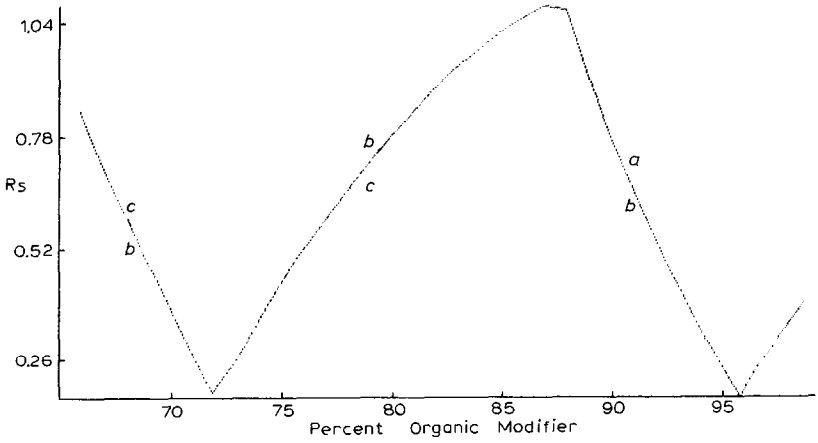

Fig. 5. Relative resolution map generated by the DryLab I software, showing a maximum resolution at 86% total organic component in the mobile phase.

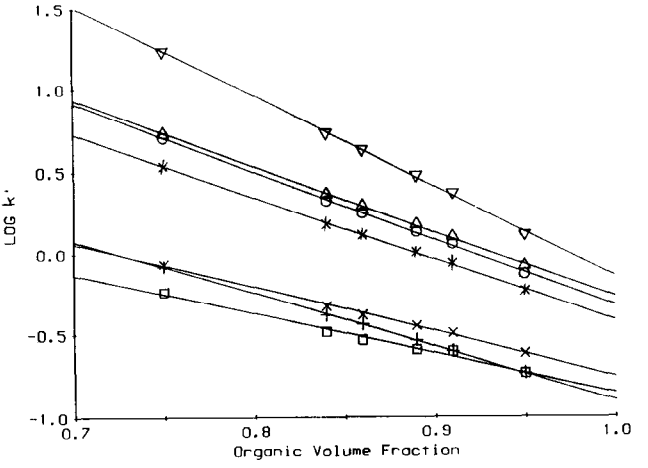

Fig. 6. Graph of the actual log k' of each of the intermediates *versus* percentage of organic component (eqn. 2). Intermediate: □, a; +, b; ×, c; ✱, d; ○, e; △, f; ▽, g.

methanol with an isoeluotropic amount of ACN determined from eqn. 4, the resulting chromatogram (Fig. 8) shows reasonable retention times but lacks selectivity between intermediates b and c. This solvent system is isoeluotropic with system 5, but its selectivity is similar to that of system 1.

Snyder and Quarry[10] found that the error in prediction using DryLab software should be about 3% for retention times and 2.5% for resolution. Our results gave average errors of 1.1% and 5.3%, respectively.

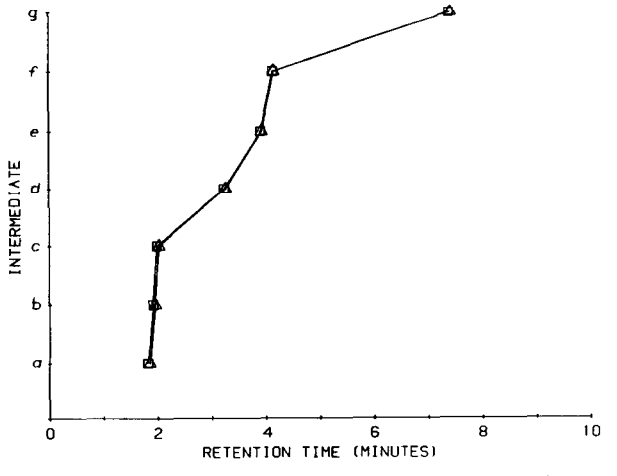

Fig. 7. Comparison of (□) actual and (△) computer-simulated retention times for the optimum solvent system with 86% total organic component.

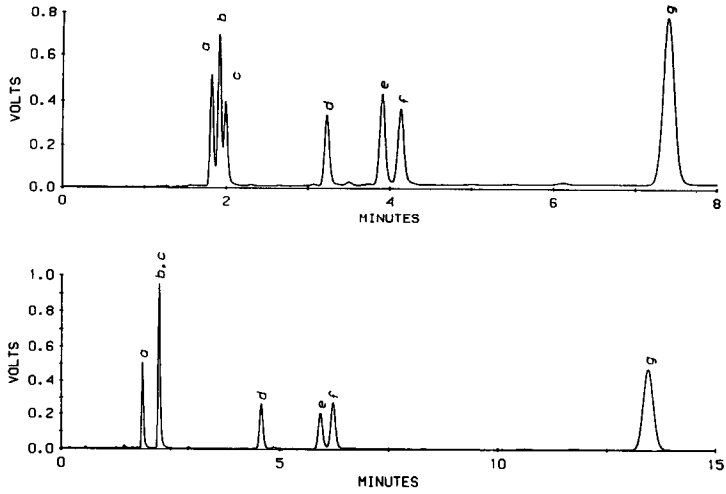

Fig. 8. Comparison of optimum quasi-binary solvent system containing 86% total organic component with isoeluotropic ACN–TEAP system. Top: system 5, DryLab optimized run. Bottom: system 6.

CONCLUSION

Rapid optimization of solvent strength is the key to rapid method development. Binary reversed-phase solvent systems can be quickly optimized for solvent strength and selectivity using the DryLab computer program. When selectivity is not sufficient, blending of organic solvents to generate quasi-binary solvent systems can quickly improve selectivity. Blended organic solvents have solvent strengths intermediate between those of the individual organic solvents and show selectivities different to those of the individual solvents. Blended organic solvents can then be treated as a single organic solvent and their ratio with respect to water can be optimized for solvent strength and improved selectivity using DryLab. This is because the relationship between $\log k'$ and percentage total organic modifier in the mobile phase is nearly linear for $1 \leqslant k' \leqslant 10$. Making just two runs with different ratios of the blended organic solvent to water gives retention data sufficient for DryLab to predict accurately the optimized ratio of the blended organic solvent to water.

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