Optimization of Mobile Phase Conditions for TLC Methods Used in Pharmaceutical Analyses

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Abstract

A simple empirical procedure is proposed for optimizing thin-layer chromatography mobile phases. This approach is based on an adaptation of the window diagrams technique and requires only a limited amount of data because acceptably accurate intermediate capacity factors and spot width values can be estimated from three initial experiments.

Introduction

Thin-layer chromatography (TLC) is a mature chromatographic technique that is widely used within the pharmaceutical industry. It is used throughout the drug development process, mainly in purity tests for drug substances, reference standards, stability samples, and key intermediates. There are many reasons why TLC continues to be used today in spite of the availability of several high-efficiency forms of chromatography. Much of the appeal of TLC lies in its simplicity and low cost. It is even possible to use TLC with minimal training and experience. As an examination technique, it is complimentary to high-performance liquid chromatography (HPLC) because it often employs a different mode of chromatography and because, in TLC, all the sample components are potentially visible on the plate. Even substances that remain near the origin or are carried to the solvent front have some possibility of being detected because of the wide range of visualization techniques available. As part of an array of analytical tests, TLC offers a distinct value.

However, in spite of the attractiveness of TLC, poorly optimized systems can be subject to considerable error. TLC is relatively inefficient, and because of the complex interaction between the mobile phase, stationary phase, and vapor or gaseous phase, it may be subject to more problems than column chromatography. There are several factors that contribute to TLC's variability, including the effects of ambient humidity, how the sample is applied to the plate, the size and type of the developing chamber, plate conditioning in the presence of mobile phase vapors, and mobile phase composition. The importance of these factors will vary from system to system, but they all need to be evaluated and controlled if the final method is to be truly rugged (i.e., the method yields reproducible results under normal operation, including different laboratories and different analysts). In turn, each of these method parameters has its own degree of robustness, or ability to withstand deliberate stresses (1). If all the method parameters are sufficiently robust or tightly controlled, the final method will be rugged. The challenge facing the method developer is to provide sufficient control over these parameters or establish the limits of their robustness in day-to-day use. For TLC as well as other chromatographic methods, this goal is best accomplished through the optimization of the mobile phase (2–6). The present study presents a practical procedure for optimizing resolution by controlling mobile phase conditions that is consistent with the normal qualities of TLC.

Strategy for optimizing TLC resolution

Resolution

In order to be effective, chromatographic methods must provide adequate resolution on a day-to-day basis. As an aid to measuring resolution, chromatographers have devised a large number of parameters. Even though these parameters have been used successfully for column chromatography, most of them are only marginally useful for TLC. The reasons for this limitation will be considered in the following section. It is important that TLC resolution be properly defined if progress is to be made in developing an optimization strategy.

First, resolution needs to be expressed in terms applicable to TLC. In general, resolution (R_s) is characterized by solute capacity factors (k), relative retention factors (a), and plate efficiency (N).

$$R_S = \frac{N^{1/2}}{4} \times \frac{\alpha - 1}{\alpha} \times \frac{k_2}{1 + k_2} \qquad \text{Eq 1}$$

In the case of HPLC, it is assumed that the number of theoretical plates does not change throughout the column and that it also does not vary substantially with solvent composition. This means that resolution can be optimized using

methods that are based on estimation of k as a function of solvent composition. With TLC, however, the number of theoretical plates changes considerably, depending on the retention factor (R_f) of each solute. As the mobile phase moves up the plate, the flow rate and efficiency decrease. This situation is further complicated by changes in solvent viscosity and surface tension; demixing occurs as the more polar portions are retained by the stationary phase. The result is that TLC resolution cannot easily be related to capacity factors and mobile phase composition. In keeping with the simplicity of TLC, a more direct approach to optimization is warranted. By definition, resolution is obtained by dividing the distance between solute pairs ($X_2 - X_1$) by their average peak or spot widths ($W_1 + W_2$):

$$R_S = \frac{2(X_2 - X_1)}{(W_1 + W_2)} \qquad \text{Eq 2}$$

In terms of the retention factor:

$$R_S = \frac{2\Delta R_f \times DD}{(W_1 + W_2)} \qquad \text{Eq 3}$$

where DD is the development distance of the mobile phase as measured from the spotting origin to the solvent front. ΔR_f can be expressed in terms of capacity factors k_1 and k_2, and TLC resolution becomes:

$$R_S = \frac{2(k_1 - k_2) \times DD}{(W_1 + W_2)\ (1 + k_1)\ (1 + k_2)} \qquad \text{Eq 4}$$

Three-point window diagrams

Three-point diagrams provide a simple means of establishing TLC resolution. This approach is based on the estimation of intermediate capacity factors and spot widths from three chromatographic runs. This information is used to calculate resolution for sample components that are difficult to resolve. Window diagrams are constructed by plotting resolution versus mobile phase composition. The actual window diagram consists of overlapping plots of resolution versus mobile phase composition for all the compounds that are difficult to resolve. This approach only requires three TLC runs, and it provides considerable insight into the chromatographic system for a fixed set of mobile phase conditions. This allows the chromatographer to quickly scan several different mobile phases and ultimately arrive at optimum conditions. Application of this approach is demonstrated later in this work.

Estimation of capacity factors

Except for simple binary combinations (7,8), there is no published procedure for relating TLC mobile phase composition and analyte retention factors. For most TLC systems, an empirical approach is needed. This can be accomplished by employing the usual chromatographic approach of starting with simple mobile phases of approximately equal solvent strength and combining them to form more complex systems. For example, after an initial mobile phase is found (A), other iso-eluotropic mobile phases (B, C, etc.) can be determined from solvent strength relationships (5), depending on the mode of chromatography employed. Data for the three-point window diagrams are obtained from two initial systems and a 50:50 mixture of each. This provides three points of data, which is sufficient to estimate intermediate values by simple regressions. Alternatively, if a complex mobile phase is already available, it can be optimized by dividing it into two simpler systems (A and B), which, when combined, cover the composition of interest. Again, only three data points are needed to find a relationship between mobile phase composition and capacity factors.

Estimation of spot widths

Solute spot widths are more difficult to determine than capacity factors. The exact width for each analyte will depend upon several factors that control the plate efficiency. For TLC, the solvent migration rate of the mobile phase varies as it travels up the plate. In contrast, HPLC systems have a relatively constant flow rate. The net result is that TLC systems involve different separation efficiencies for each solute, depending upon how far

Table I. Separation of Benzoic Acid Compounds Using Three Initial Systems

	Mobile phase A†		Mobile phase B‡		Mobile phase C§	
Compound*	R_f	Spot width (mm)	R_f	Spot width (mm)	R_f	Spot width (mm)
1	0.11	3.2	0.01	1.9	0.03	2.4
2	0.52	2.2	0.28	3.0	0.41	3.0
3	0.45	2.4	0.38	3.6	0.44	3.0
4	0.36	2.8	0.05	1.8	0.13	2.4
5	0.55	1.0	0.50	0.8	0.45	0.8

* One microgram each of 4-nitrobenzoic acid; 4-aminobenzoic acid, 4-hydroxybenzoic acid, 3-chlorobenzoic acid; and 3-butoxybenzoic acid (compounds 1–5, respectively).
† Mobile phase A = chloroform–methanol–acetic acid, 90:10:1, v/v.
‡ Mobile phase B = hexane–ethyl ether–acetic acid, 50:50:1, v/v.
§ Mobile phase C = hexane–ethyl acetate–acetic acid, 50:50:1, v/v.

Table II. Retention Time Data for Benzoic Acid Compounds

	Mobile phase A*		Mobile phase A:B (50:50)		Mobile phase B*	
Compound*	R_f	ln k	R_f	ln k	R_f	ln k
1	0.11	2.09	0.05	2.94	0.01	4.60
2	0.52	–0.08	0.38	0.41	0.28	0.94
3	0.45	0.20	0.40	0.36	0.38	0.49
4	0.36	0.58	0.19	1.45	0.05	2.94
5	0.55	–0.20	0.55	–0.20	0.50	0.00

*See Table I for identification of compounds and description of mobile phases.

the solute travels up the plate. In addition, spot sizes are strongly influenced by mobile phase viscosity and solute diffusion coefficients. Given these obstacles, prediction of spot size versus mobile phase composition is difficult at best. However, this information can be estimated empirically by using the same three systems that have already been run to estimate capacity factors. For example, if components have similar-size spots in solvents A and B, then it is safe to assume that mixtures of these solvents will yield comparable-size spots. If these same solvents produce different spot sizes, combinations of A and B will yield intermediate-size spots. However, often one mobile phase will dominate the other in controlling spot size. A 50:50 mixture of A and B will indicate which one has the strongest influence.

Table III. Correlation Coefficients from the Regression of R_f or ln k Versus Mobile Phase Composition (B) or ln of Mobile Phase Composition (B)

Compound	R_f vs. X_B	ln k vs. X_B	ln k vs. ln X_B
1	0.9868	0.9664	0.7123
2	0.9908	0.9995	0.8451
3	0.9423	0.9964	0.8997
4	0.9969	0.9775	0.7423
5	0.7500	0.7500	0.3810

* See Table II for data.

Table IV. Estimates of ln k from Regression of Data in Table II Plus Original Data

	log k						
Compound	0% B	20% B*	40% B*	50% B	60% B*	80% B*	100% B
1	2.09	2.59	3.09	3.34	3.60	4.10	4.60
2	–0.08	0.12	0.32	0.42	0.53	0.74	0.94
3	0.20	0.26	0.32	0.35	0.37	0.43	0.49
4	0.58	1.05	1.52	1.76	2.00	2.47	2.94
5	–0.20	–0.16	–0.12	–0.10	–0.08	–0.04	0.00

* Estimates from regression of data in Table II.

Table V. Spot Widths, Measurements, and Estimates Using Mobile Phases A and B

	Width (mm)						
Compound	0% B	20% B*	40% B*	50%	60% B*	80% B*	100% B
1	3.2	(2.7)	(2.3)	2.1	(2.1)	(2.0)	1.9
2	2.2	(2.5)	(2.7)	2.8	(2.8)	(2.9)	3.0
3	2.4	(2.8)	(3.0)	3.1	(3.3)	(3.4)	3.6
4	2.8	(2.5)	(2.3)	2.2	(2.1)	(2.0)	1.8
5	1.0	(1.0)	(1.0)	1.0	(1.0)	(0.9)	0.8

* Estimate

These three determinations provide the necessary information to make accurate intermediate estimates. The same three plates used to estimate retention data also provide spot width data. The final step involves construction of three-point window diagrams and the estimation of optimal conditions.

Experimental

All solvents were Fisher HPLC-grade solvents (Fisher Scientific, Pittsburgh, PA). Benzoic acid compounds were purchased commercially and used as received (Aldrich Chemical, Milwaukee, WI). Sample solutions were prepared in methanol at a concentration of approximately 1 mg/mL. Sample application was performed using 5-µm Microcap capillaries (Drummond Scientific). Standard silica gel plates were used (silica gel 60F, 20 × 20 cm, 250-µm thick) (Merck, Darmstadt, Germany). Plates were developed in a Vario KS chamber using the metal partition to produce a sandwich configuration (Camag Scientific, Wilmington, NC). Initial humidity studies were run at room conditions, approximately 35% relative humidity. Higher humidity studies were accomplished by conditioning the spotted plate face-down over a saturated solution of sodium chloride, using a second Vario KS chamber. Each plate was developed approximately 12 cm from the origin. Visualization was done by short-wavelength ultraviolet (UV) quenching in a UV view cabinet (Camag Scientific). Spot shapes were marked with a heavy lead pencil. Spot widths, measured in the direction of solvent flow, were estimated with the aid of a small magnifier (7x) with scale, 0–10 mm, with 0.1-mm divisions (Spectronics, Rochester, NY). Regression analysis calculations and window diagram plots were achieved using Windows Excel programs.

Results and Discussion

Selection of initial method conditions

Optimization of TLC mobile phases using three-point window diagrams was illustrated by the separation of five benzoic acid derivatives (see Table I). For many TLC methods, the vapor phase influence is usually the major contributor to analytical variability. To keep these factors under control, the present data were generated by using a Vario KS chamber in a sandwich configuration. This approach limits the interaction between the stationary phase and the vapor, which contributes to method ruggedness. Each plate was spotted at ambient humidity, approximately 35%. Table I summarizes the data obtained by using three binary mobile phases (modified with acetic acid to minimize tailing on the

silica plate) to separate these compounds. Mobile phase A was composed of methanol diluted with chloroform, mobile phase B was composed of ethyl ether diluted with hexane, and mobile phase C was composed of ethyl acetate diluted with hexane. Examination of the R_f values listed in Table I shows that each of these mobile phases by itself was not capable of resolving all solutes. Spot shape was reasonable with all three systems, and each provided a resonable starting point for further optimization. For example, mobile phase A did not separate compounds 2 and 5. However, mobile phase B did a good job of separating these two but had trouble separating compounds 1 and 4. Based on visual examination of the data in Table I, it appears likely that some combination of these mobile phases would yield a satisfactory system.

Optimization of initial conditions using three-point window diagrams

As indicated in the optimization strategy section, a relationship must be established between mobile phase composition and solute capacity factors. It is also necessary to correlate spot widths and mobile phase composition. The first three-point window diagram was constructed from mobile phase A, mobile phase B, and a mixture of the two. For each benzoic acid derivative, Table II summarizes R_f and ln k values for A, B, and a 50:50 mixture of A and B. This was the only data that was needed to get a first-cut estimation of intermediate retention values. Because the functionality between mobile phase composition and retention values was unknown, simple but likely relationships were evaluated. Table III shows correlation coefficients for each benzoic acid compound using 0, 50, and 100% B regressed against R_f or ln k (columns 1 and 2) and ln k versus ln of the mobile phase composition (column 3). From the three-point regression data, mobile phase composition correlated well with R_f or ln k. There was a much weaker relationship between ln k and ln X_B. By using the three-point regression lines, it was easy to estimate intermediate points on the line (see Table IV). In addition, the same three plates provided the necessary raw data to estimate solute spot widths. TLC spot widths usually have a high degree of variability from run to run. Also, spots are difficult to measure accurately. However, after the spot widths were measured experimentally, it was possible to obtain a good estimate of intermediate values by assuming a linear relationship between solvent A or B and the 50:50 mixture of A and B. Table V shows the spot widths measured from these three plates. This table also contains estimates of spot widths at 20, 40, 60, and 80% mobile phase B.

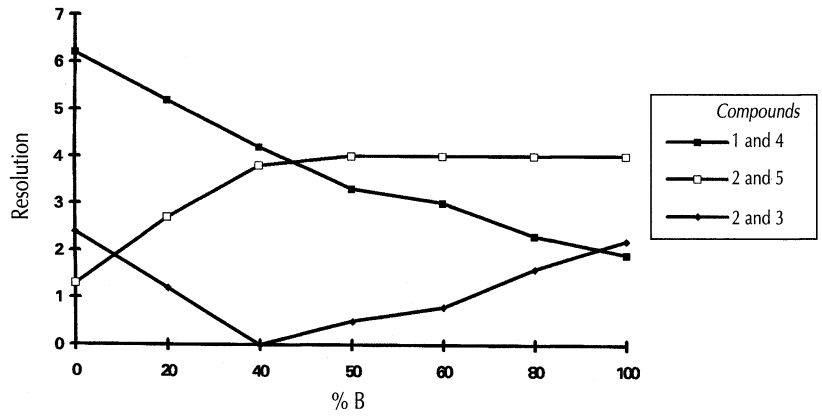

Figure 1. Three-point window diagram constructed from mobile phases A and B.

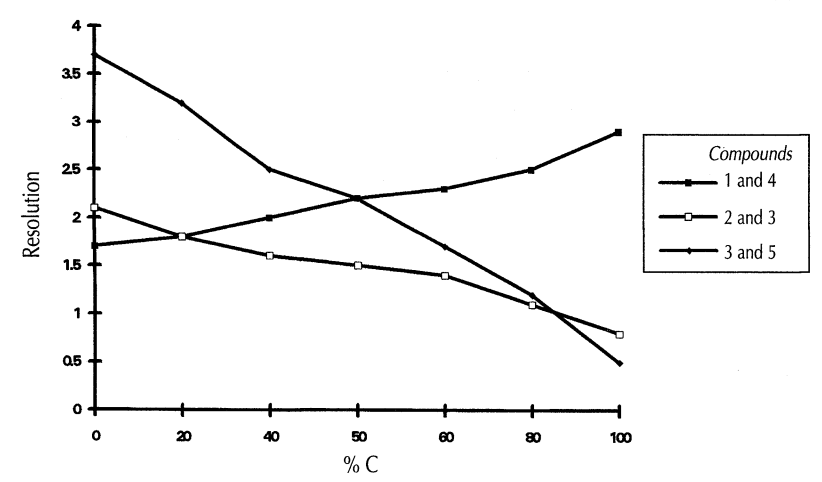

Figure 2. Three-point window diagram constructed from mobile phases B and C.

Examination of the spot width data shows that, for a 50:50 mixture of A and B, compound 4 had an intermediate width, halfway between the extremes of A and B (2.2 mm, which is the average between 2.8 and 1.8 mm). In contrast, for the same 50:50 mixture of A and B, the spot size of compound 1 was more affected by mobile phase B. This fact needs to be considered in estimating intermediate mobile phase combinations. The spot width of compound 1 was about 2.1 mm in a 50% B solution, which is about the same as the 1.9-mm width in the 100% B solution. By using the third point (A and B, 50:50), it was possible to obtain much more accurate estimates of other intermediate points. Similarly, by using the data from Tables IV and V, each benzoic acid derivative could be evaluated, and intermediate capacity factors and spot widths could be estimated. By focusing on the pairs that are difficult to resolve, resolution values could be calculated and plotted versus mobile phase composition. The resulting window diagrams provided a reasonably accurate picture of optimum resolution for the specific method conditions being evaluated (see Figure 1). In a similar manner, mobile phases B and C could be evaluated, as well as mobile phases A and C. The resulting three-point window diagrams are shown in Figures 2 and 3.

All three window diagrams were constructed by using minimal data and were consistent with the precision limitations of TLC. However, from these three simple

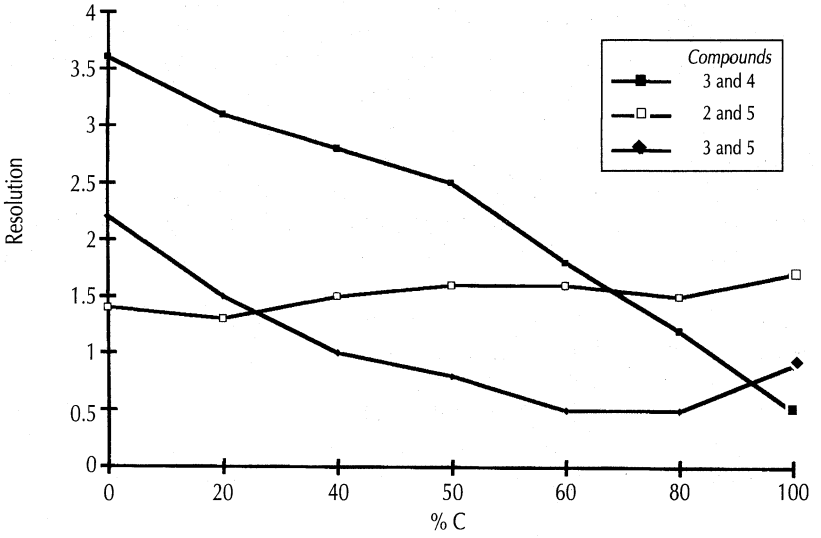

Figure 3. Three-point window diagram constructed from mobile phases A and C.

experiments, a wealth of information was obtained in regard to an optimum mobile phase. Figure 1 shows that 80–95% B yielded a robust system with a minimum resolution of 2.0. Figure 2 shows that mobile phases B and C could be combined to provide adequate resolution between 0 and 50% C (50–100% B). Combinations of A and C (Figure 3) were less productive because at least one solute pair had a resolution of 1.5 or less, which is only marginal for a robust TLC method. Resolution appeared best in the 10:90 mixture of A and B, which should provide the most robust system. Even with mobile phase variations as large as ± 5%, three-point window diagrams should yield acceptable results. Alternatively, the mobile phase of the 90:10 mixture of A and B provided adequate resolution, but acceptable method robustness was limited to a narrow range of mobile phase composition.

Factors such as humidity and plate conditioning can change resolution values, and not always in the same direction. At this point in the method development process, it was important to determine the effect of these factors on method performance. The three-point regression study was repeated at a humidity of approximately 70%. These results compared well with the original system. Since plate conditioning was controlled by using a Vario KS sandwich chamber, no additional optimization was needed for chamber conditioning. If conventional developing chambers had been used, it would have been necessary to optimize the mobile phase composition under different plate conditioning parameters, such as saturation time and chamber volume. In effect, each three-point diagram was itself a "data point," describing a fixed set of chromatographic parameters. Three-point diagrams allow mobile phase optimization to proceed quickly, and they build on the typical characteristics of TLC. A wide range of conditions are easily covered with this technique, which contributes to its effectiveness as an optimization technique. After optimum conditions are selected from the three-point window diagrams, the system should be fine-tuned by experimentally running several plates, focusing on the range around the optimum. At this point in the development process, an in-depth optimization effort is worth pursuing because an approximation of optimum conditions is known.

Conclusion

Three-point window diagrams provide a simple and effective approach for optimizing resolution by controlling mobile phase composition. As part of the method development process, mobile phase optimization can be accomplished by constructing window diagrams and using estimates of data for capacity factors and spot widths. The data can be generated using relatively few experiments and are consistent with the low efficiency of TLC. By providing data that focus on resolution under conditions typically encountered in most laboratories, an empirically derived optimization is possible, yielding a final method that is more likely to generate acceptable results in its day-to-day use.

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Manuscript accepted January 14, 1997.