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OPTIMIZATION OF SELECTIVITY, DETECTABILITY AND ANALYSIS TIME IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The resolution requirements in pharmaceutical analysis by high-performance liquid chromatograpy are frequently higher than those for other multicomponent separations where the components are present in similar proportion. Furthermore, it is often necessary to separate a large number of components most of which are present at very low levels and have unknown structures. This places extraordinary requirements in terms of selectivity of separation and resolution of minor components as they may not be detected because of variability of stationary phase, improper selectivity evaluations, or poor resolution at the tail end of the major peak. Discussion with respect to resolution, detectability/quantitation, and analysis time is included.

INTRODUCTION

It is possible to separate a variety of compounds even at ultratrace levels by high-performance liquid chromatography (HPLC)[1]. Unfortunately, it is not easy to determine *ab initio*, from the published literature, the specific condition for a given separation[2–5]. The difficulty stems from a narrow focus provided by most researchers, *i.e.* the publications tend to deal primarily with the separations of components of interest to them. The theory in many cases has been oversimplified to provide simple equations. When a significant number of compounds are present with different aromaticity, functional groups, and acidic, basic, or neutral character in a given sample, the development of a separation method becomes very difficult. These points will be addressed with regard to reversed-phase separations of pharmaceutical compounds by HPLC. The discussion should be useful for separation of pharmaceutical compounds as well as other multifunctional compounds.

EXPERIMENTAL

A variety of C_8 and C_{18} HPLC columns were investigated with different mobile phases. To evaluate various C_{18} columns, the mobile phase was composed of methanol–water–acetic acid (70:23:2) and the flow-rate ranged from 0.8 to 1.6 ml/min. Detailed molecular probe investigations were conducted on the selected C_8 Whatman column that proved to be most useful for a complex separation of acidic, basic, and

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neutral compounds after investigation of various manufacturer's columns (see case I under *Separation of peak pairs*). Details on mobile phases and columns used are provided in the text.

THEORY

The following resolution equation is familiar to most chromatographers[3]:

$$R_s = \tfrac{1}{4}(\alpha - 1)\sqrt{N}\,\frac{k'}{1 + k'} \tag{1}$$

Where R_s = resolution; N = number of theoretical plates; α = separation factor; k' = capacity factor. It should be noted that this equation has been oversimplified since the following assumptions are made for the two components whose resolution is to be determined: (a) k_1' is approximately equal to k_2' therefore their average value (k') is used; (b) peak width is equal; (c) N is same. This equation is useful when α value is ≈ 1; the resolution value is inflated by 1% at $\alpha = 1.01$ and $>50\%$ at $\alpha = 1.5$ as compared to eqn. 2. In general, the average of the R_s values predicted by these two equations is often better than with either equation.

If we assume N to be the same for both components but use the peak width of the second peak in calculations, the equation takes the following form:

$$R_s = \tfrac{1}{4}\,\frac{\alpha - 1}{\alpha}\sqrt{N}\,\frac{k'}{1 + k'} \tag{2}$$

In those cases where the asumptions stated above do not hold, appropriate equations should be derived and used[6]. In most practical work, however, the resolution is calculated by eqn. 3:

$$R_s = \frac{t_2 - t_1}{\tfrac{1}{2}(w_1 + w_2)} \tag{3}$$

Where t_2 and t_1 = retention times of the two components; w_2 and w_1 = peak widths of the same components.

For reliable quantitation, the goal should be to obtain a value of $R_s \geqslant 1.5$ for every peak pair[3]. However, this resolution is not always possible when a significant number of multifunctional compounds are present. For two bands of equal size one can use $R_s = 1.0$ (2% of one band overlaps the other), since most electronic integrators can easily calculate peak areas. However, for a given value of R_s, band overlap becomes more serious when one of the two bands is much smaller than the other. For example, this resolution is insufficient when the concentration of the minor component is 1 in 16. Furthermore, as the relative concentration of the minor band decreases, there is decreasing accuracy in the measured area of the minor band. This suggests that resolution requirements increase as the concentration of the minor component

decreases to trace or ultratrace levels. Discussed below are a few examples of separations with respect to resolution, detectability/quantitation, and analysis time.

RESULTS AND DISCUSSION

Impurities in pharmaceutical compounds originate mainly during the synthetic process from raw materials, solvents, intermediates, and by-products[7]. Degradation products and contaminants of various types make up some of the other sources of impurities. As a result, it is necessary to incorporate stringent tests to control impurities in pharmaceutical compounds. This fact is evident from the requirements of the United States Food, Drug & Cosmetic Act and various pharmacopeias which provide tests for control of specific impurities. It is interesting to note that specifications for impurities can vary between pharmacopeias. Because a pharmaceutical compound can be prepared by a variety of methods, the need for methodologies suitable for controlling low levels of impurities and rational limits becomes apparent. This is essential to assure that the observed toxicologic or pharmacologic effects are due to the compound of interest and not due to impurities.

Analytical methods that can control impurities to ultratrace levels are available[1,8–10]; however, the level to which any impurity should be controlled is primarily determined by its pharmacologic and toxicologic effects. This should include all impurities: those originating from synthesis and those originating from other sources such as degradation[11]. For example, penicillins and cephalosporins have been known to undergo facile cleavage of the β-lactam bond in aqueous solution. This is of special interest since some studies on penicillins have shown that their instability may effect possible reactions involved in penicillin allergy[12]. The control of low levels of impurities is extremely important when a drug is taken in large quantities for

TABLE I

STATIONARY PHASE EVALUATONS

Mobile phase, methanol–water–acetic acid (70:23:2); 0.8 ml/min, except flow-rate, with column E and F = 1 ml/min and with column G = 1.6 ml/min.

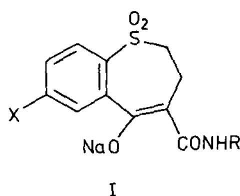
Column	*Manufacturer*	d_p (μm)	*N claimed (minimum)*	*L (cm)*	*N (found[a])*	*Impurities*
A	Nucleosil C_{18} (Chromapak)	5	10000	25	5384	5
B	Partisil ODS (Whatman)	5	10000	25	2800	6
C	Ultrasphere ODS (Altex)	5	25000	25	3900	9
D	Zorbax ODS	5	10000	25	1410	7
E	μBondapak C_{18} (Waters)	10	5000	30	864	7
F	μBondapak C_{18} (Waters)	10	5000	30	1369	7
G	Radial compression C_{18}	10	5000	10	2340 (calc.)	6

[a] For compound I.

therapeutic purposes or as a fad, such as the use of methotrexate (10–20 g) to treat neoplasia or faddist use of vitamins, especialy vitamin C.

Variabilities in stationary phase

Resolution of impurities or by-products in pharmaceutical compounds depends on appropriate selection of a mode of chromatography, column, mobile phase, and detector. Even when all other conditions are optimum, variability in columns from manufacturer to manufacturer or from the same manufacturer can affect separations. Table I lists evaluations of impurities in a potential anti-inflammatory compound (I) with the following structure[13]:



A review of the table reveal that almost a two-fold number of impurities (9 *vs.* 5) can be seen when column C is used as opposed to column A. The differences can be attributed to variability in selectivity offered by these columns rather than the theoretical plates, because *N* (found) for compound I was higher in column A than in column C. A comparison of columns from the same manufacturer, *i.e.*, columns E and F, shows that *N* (found) is significantly different; however, the number of impurities found is the same and lies between those of columns A and C. Again, there is no correlation with the number of theoretical plates. The radial compression column G from the same manufacturer actually shows one impurity less even though the calculated number of theoretical plates is higher than columns E and F. Smaller particle size (5 μm) alone is not responsible for higher resolution as is obvious from differences in results for columns A–D. Also columns E–G with twice the particle size show almost the same number of impurities as columns B and D. The result of this study shows that resolution of components is clearly a function of the selected stationary phase(which is variable from manufacturer to manufacturer and even from the same manufacturer) and the mobile phase. This investigation suggests the need of select probes to a asure that the selected stationary phase is providing the desired resolution and. detectability.

Studies with select probes

Columns from various manufacturers were evaluated for a separation that entailed mixture of acidic, basic, and neutral compounds[14]. The columns that were not end-capped were found unsuitable for further investigations. From the remaining, one manufacturer's column (Whatman) was selected for in-depth investigation. The type of data provided by this manufacturer is given in Table II. It is readily apparent that the probes given in Table II (benzene, naphthalene, and biphenyl) are similar in structure, *i.e.*, they are all aromatic hydrocarbons, and the effect of polar groups cannot be determined with this approach.

TABLE II

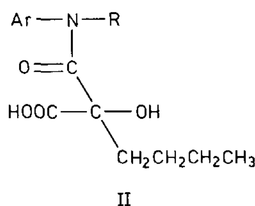
TYPICAL MANUFACTURER'S DATA FOR A C_8 COLUMN (10 μm)

Whatman column, 25 cm × 4.6 mm I.D.; methanol–water (80:20).

	Benzene	*Naphthalene*	*Biphenyl*
Retention time (t_R, min)	4.53	5.90	7.31
Peak width at 60% height (W60%, min)	0.102	0.139	0.173
Efficiency (N)	7890	7207	7142
Capacity factor (k')	0.69	1.20	1.73
Asymmetry ratio	1.41	1.33	1.29

Many workers[15–30] have discussed the types of silanols that can exist and the problems of separations of basic samples in the presence of free silanols. Frequently, mobile phase additives are used to circumvent problems resulting from silanols. A large number of solutes have been used for characterizing selectivity of the reversed-phase columns[17], however, no single test solute is useful for this purpose. Of the various solutes used for evaluating silanols, nitrobenzene is the most well-known and, perhaps, as useful as any. Therefore, it was selected to evaluate three columns from different batches with a mobile phase of n-heptane to determine the differences in terms of residual silanol sites in these columns. The results are given in Table III. The data do not permit clear differentiation, *e.g.*, column 1585 has the highest number of theoretical plates, a desirable characteristic for a column, but it gives the same peak width for nitrobenzene as column 1887 (the column with the lowest N).

The same three columns could be differentiated more clearly with a new molecular probe (II). It has the following characteristics: (1) aromatic character; (2) carbonyl group and substituted nitrogen in its structure; (3) hydroxyl and carboxyl groups on the same asymmetric C atom; (4) a short carbon chain (C_4).



II

The data with the new molecular probe are given in Table IV. With a mobile phase containing acetonitrile–0.02 M acetate buffer (41:59, v/v) (pH 4.1), significant differences can be observed among the three columns from the same manufacturer. At the 0.1 μg level, the new molecular probe was not detected with column 1887, whereas the other two columns gave a peak for it. With column 1646, the peak tails and has a width two times that of column 1585. It should be noted that the number of theoretical plates is also significantly higher ($\approx 3.5\times$) with column 1585 (the number of theoretical plates was calculated for the tailing peak with column 1646 to provide a relative value).

As can be seen from the data in Table IV, column 1887 behaves very differently

TABLE III

EVALUATION C_8 COLUMNS WITH NITROBENZENE[14]

Whatman column, 10 μm, 25 cm; mobile phase, *n*-heptane; flow-rate, 1 ml/min; amount injected, 0.04 μg (15 μl).

	Column number		
	IR 1585	*IR 1646*	*IR 1887*
Retention time (t_R, min)	13.7	14.5	11.4
Peak width (W, min)	1.2	1.4	1.2
Retention (t_R) benzene	8.5	8.4	8.0
Capacity factor (k')	0.6	0.7	0.4
Theoretical plates (N)	2027	1789	1447

from the other two in that 0.1 μg of the new probe is not detected (0.2 μg and upward were detected). A plot of this data, when extrapolated down to the 0.1 μg level, indicates a much broader peak width (2.3 min) for this column as compared to the other columns ($\leqslant$1 min). This shows that detectability of a component at low levels (0.1 μg or $\approx$0.1%) can vary from one batch of columns to the next. Detectability decreases because there is a concomitant loss of peak height due to peak broadening. As the peak broadens, the resolution for closely resolved peaks is also affected negatively; this can eventually result in the complete loss of resolution and detectability as observed at the 0.1 μg level for column 1887. Hence it is important to select a probe that is useful for monitoring selectivity and detectability of a given separation while recognizing no single probe is likely to be universal for this purpose.

Separation of peak pairs

Selectivity optimizations generally entail improving the separation of a pair of peaks where the observed resolution is minimum or more simply, the α value is low. However, this rule does not work well in pharmaceutical analysis for a very large number of cases because a significant number of compounds have to be simultaneously resolved that may vary in aromaticity, functional groups and their acidic, basic or neutral character. Two such cases are discussed below.

TABLE IV

EVALUATION OF THE COLUMNS WITH THE NEW PROBE (COMPOUND II)[14]

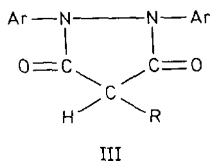
Whatman C_8 10 μm, 25 cm; 0.1 μg molecular probe injected; mobile phase, acetonitrile–0.02 M acetate buffer (pH 4.1) (41:59, v/v); flow-rate, 2.4 ml/min.

	1585	*1646*	*1887*
Retention time (min)	3.4	3.8	Not detected[b]
Peak width	0.5	1.0[a]	Not detected[b]
Capacity factor (k')	1.9	2.2	—
Theoretical plates (N)	740	214	—

[a] Tails.

[b] Sample of 1 μg gives t_R = 4.4 min; W = 6.0 (large tail); k' = 2.7.

Case 1. An optimum separation, both in time and resolution, was developed for a substituted diphenylhydrazino compound (III) on a 25 cm × 4.6 mm I.D. C_8 column with a mobile phase containing acetonitrile–acetate buffer pH 4.1 (44:56).



The HPLC method (Fig. 1) resolves various potential transformation products from compound III[31]. Of interest in this separation are the peak pairs 7, 8 and 8, 9. Peak 7 is well resolved from peak 8. The calculated resolution of this peak pair is ⩽ 1.0 and is more than adequate for quantitation of peak 7. However, this resolution is not enough for the component at the tail end of peak 8, *i.e.*, see peak 9. This is due to the fact that in pharmaceutical analysis it is necessary to inject sufficient amounts of the active ingredient to allow quantitation of low levels of impurities. In this case it is 7 μg per injection, an amount which allows quantitation of impurities down to a few hundredths of percent. This produces a very broad peak for the main component which can have a width ≈ 10 × that of a minor component. The calculated required

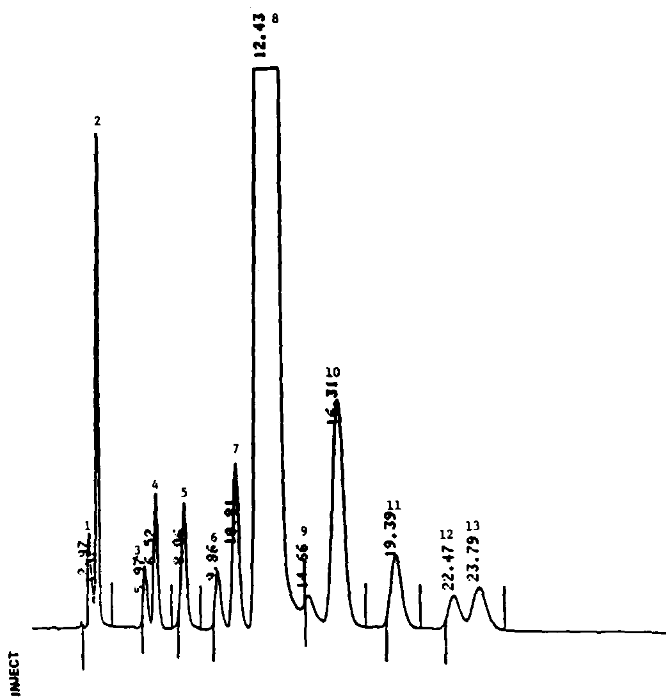
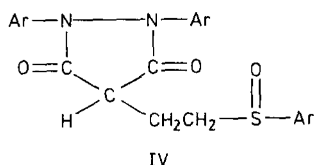


Fig. 1. Resolution of transformation products of a diphenylhydrazino compound[31].

resolution is $\geqslant 1.25$ for peaks 8 and 9. This puts an extra premium on mobile phase optimization and selectivity. Furthermore, it becomes necessary to assure that no component is eluting under the substantial area of the main peak. These requirements were met in the mobile phase optimization for this compound.

Case 2. Frequently separations are encountered where one of the components has unusually long retention time. For example, with separation of compound IV, the elution of the thioether impurity takes an unduly long time on a 25 cm × 4.6 mm I.D. C_8 column packed with 5- or 10-μm particles.



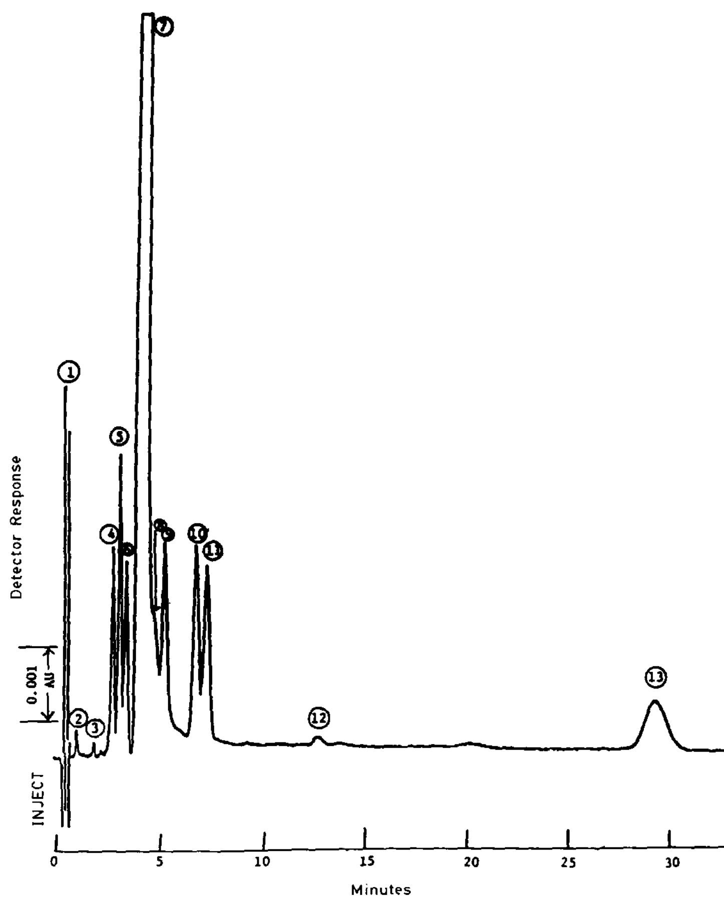


Fig. 2. Resolution of transformation products of Compound IV[32].

The problem primarily required a reduction in analysis time with a minimal loss of resolution. This was attained by selection of a smaller column length, *i.e.*, 10 cm with the mobile phase containing 0.3% phosphoric acid–(acetonitrile–tetrahydrofuran, 4:1) (65:35). Fig. 2 shows such a chromatogram[32]. It can be seen that most of the components, except component 8, are fairly well resolved and the retention time of the thioether impurity (peak 13) is $\approx$30 min, a reasonable analysis time for most practical purposes. A component, peak 8, eluting at the tail of the main peak is poorly resolved and requires much higher resolution. Poor resolution can affect quantitation significantly. For example, alteration of the aqueous content of the mobile phase to 70% can provide excellent separation with $R_s = 1.3$ and much better quantitation[33]. However, this improvement in resolution is possible at the expense of a significant increase in analysis time. Hence, it is important to assess various priorities in the optimization of mobile phase.

CONCLUSIONS

(1) Variabilities in the stationary phase, *i.e.*, differences in columns from different manufacturers or the same manufacturer can significantly influence the number of impurities resolved with a given mobile phase.

(2) Studies with the selectal probes can help select reliable columns and assure the same detectability for the observed components.

(3) It is important to work with R_s values greater than 1.25 for close peak pairs especially when the component of interest elutes at the tail end of the main peak. It is best to let the concentration of the minor component and the analysis time dictate the acceptable R_s value.

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