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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF SULINDAC AND RELATED COMPOUNDS USING A COMPUTER SIMULATION

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SUMMARY

The reversed-phase chromatography of sulindac and its structurally related compounds was investigated. Several mobile phase parameters were examined. The best resolution was obtained using a mixed organic modifier consisting of methanol and acetonitrile. Employing a factorial experimental design, the effect of four primary operating variables was determined and a computer simulation of the chromatographic process was carried out. This adequately accounted for the interactive effects, accurately predicted optimum conditions and indicated corrective changes in the operating parameters to obtain a desired separation.

With the simulated model, further information concerning column performance was available. The enthalpy of binding of each component at various mobile phase conditions was calculated and the effect of temperature on column efficiency documented. The predicted optimum in column efficiency at 35–45°C could be explained, in part, by the kinetic and diffusional processes occurring within the microparticulate high-performance liquid chromatographic column.

INTRODUCTION

This paper describes the use of a computer simulation to study reversed-phase chromatography of sulindac, (*Z*)-5-fluoro-2-methyl-1-{[4-(methylsulfinyl)phenyl] methylene}-1H-indene-3-acetic acid, and several structurally related compounds (Fig. 1). Other workers have employed high-performance liquid chromatographic (HPLC) methods to analyze sulindac[1–3]. With the aid of a computer, we have attempted to document chromatographic behaviour and describe a practical technique to define a complex interactive system.

As part of an investigation to develop an assay method to monitor drug stability, a cursory search for a suitable mobile phase with which to resolve these components indicated that a factorial experimental design was appropriate. Several mathematical techniques, some employing factorial designs, have been used previously to investigate and optimize HPLC systems[4–13]. Rather than use a computer method only as a tool to find the optimum, a factorial experimental design was employed to de-

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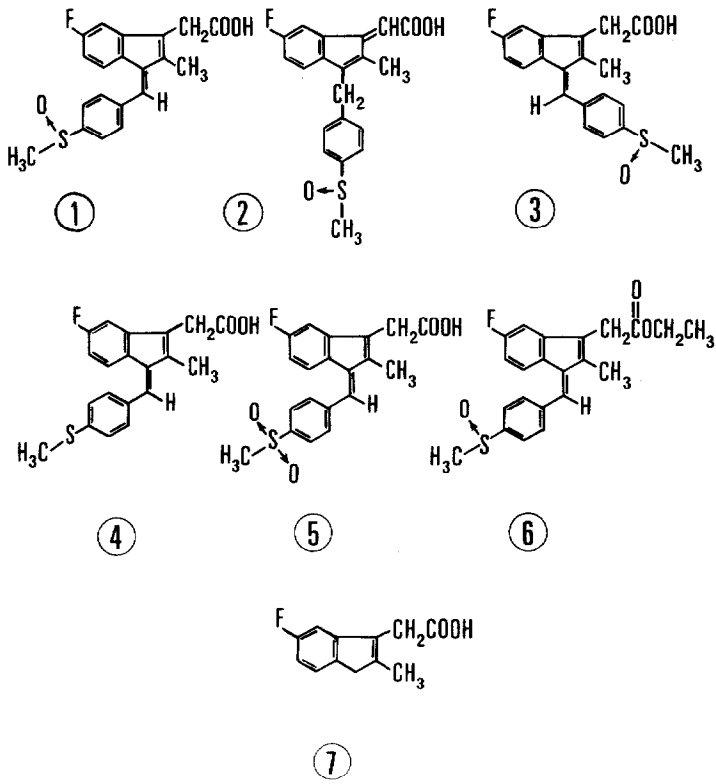

Fig. 1. The molecular structures of *cis*-sulindac (1) and its related compounds *exo*-sulindac (2), *trans*-sulindac (3), *cis*-desoxysulindac (4), *cis*-sulindac sulfone (5), *cis*-sulindac ethyl ester (6) and 5-fluoro-2-methylindene-3-acetic acid (7).

scribe the response surfaces surrounding a known optimum condition. Preliminary experiments examined retention behaviour by varying one parameter at a time to locate the approximate optimum and establish the primary variables affecting resolution. Then, a factorial design was used to assess the effect of four primary operating variables on the chromatographic response in the vicinity of the apparent optimum. Finally, a computer simulation was developed to illustrate graphically the effect of mobile phase conditions on component separations and column efficiency. The simulation helped not only to define optimum conditions more precisely but also to provide a detailed understanding of the chromatographic process.

EXPERIMENTAL

Apparatus

A Hewlett-Packard Model 1084B high-pressure liquid chromatograph equipped with a UV detector at 254 nm and a variable volume syringe injector was used. For all experimental work an Ultrasphere ODS column (250 × 4.6 mm I.D., Beckman Instruments) was employed without a guard column or precolumn but with

zero dead-volume fittings and 0.025 cm I.D. capillary tubing. Three Ultrasphere ODS columns were employed during the study to ensure acceptable column-to-column reproducibility.

Reagents

The methanol used was reagent grade from American Chemicals. Acetonitrile was glass-distilled quality from BDH. Ammonium hydrogen phosphate, concentrated ammonium hydroxide (14.7 *M*), phosphoric acid (85%) and glacial acetic acid were reagent grade from American Chemicals. The water used in making up the mobile phase was distilled twice and filtered through a 0.45-μm Metricel GN-6 filter. The isomers of sulindac, *cis*-sulindac ethyl ester, *cis*-desoxysulindac, *cis*-sulindac sulfone and indene-3-acetic acid derivative (FMIAA) were obtained from Merck Sharp & Dohme (West Point, PA, U.S.A.). Laboratory grade methyl *p*-hydroxybenzoate (MPHB) was obtained from Fisher Scientific.

Mobile phase preparation

Buffer solutions were prepared by weighing an amount of ammonium hydrogen phosphate granular solid equivalent to 1.0 mole and dissolving it in 2 l of distilled water. The pH of the solution was adjusted with concentrated ammonium hydroxide to pH 6.0 using a pH meter. This stock solution (0.5 *M*) was diluted appropriately in distilled water to obtain the required final buffer concentration (0.01–0.1 *M*). The mixed organic modifier was prepared by measuring the required volume of acetonitrile into a 1- or 2-l volumetric flask and diluting to volume in methanol. Although the organic modifier and ammonium phosphate solution were pumped separately to the column for many of the preliminary experiments, all chromatograms obtained for the factorial design used premixed mobile phases. These were prepared by adding the required volume of the organic modifier to a volumetric flask and diluting to volume in the ammonium phosphate solution.

Sample preparation

For preliminary work, samples were prepared in a solvent similar to the mobile phase. For the factorial experimental design, the injected sample contained six components: *cis*-sulindac, its *trans* and *exo* isomers, the sulfone, FMIAA and the preservative MPHB. This mixed sample, prepared in a solvent similar to the mobile phase at approximately optimum conditions, contained about 1 mg/ml of *cis*-sulindac and 0.02–0.04 mg/ml of each of the remaining five components. If resolution of the sample was incomplete, the individual components were chromatographed separately to obtain retention times and peak widths. Component retention appeared to be largely independent of sample volume.

Preliminary experiments

To study the effect of buffer concentration, pH and the nature of the organic modifier on the capacity factors, the instrument was operated at a mobile phase flow of 1.5 ml/min, a column temperature of 40°C and a column pressure of about 200 bar. The premixed sample was injected (10 μl) in duplicate and the capacity factors calculated. The dead volume was determined from the initial response obtained when methanol was injected with a mobile phase containing only acetonitrile.

To investigate the effect of buffer concentration, 0.01–0.1 *M* ammonium phosphate stock solutions were prepared from the 0.5 *M* stock solution. Methanol was pumped from a separate reservoir at 45% of the total liquid volume flow.

The effects of pH and organic modifier were examined using three buffer solutions. The pH 6.0 ammonium phosphate buffer solution was prepared at 0.05 *M* concentration as described previously. A pH 3.0 ammonium phosphate solution was prepared similarly, however, the pH of the stock solution was adjusted with concentrated phosphoric acid. The third solution (pH 3) was prepared by adding 10 ml of glacial acetic acid to a 1-l volumetric flask and diluting to volume in distilled water. The organic modifier was pumped isocratically from a separate reservoir at various percentages of the total mobile phase flow. Duplicate chromatograms were obtained for each condition and the capacity factors calculated.

The factorial experimental design

The simulated sample was prepared containing the known potential degradation products. *Cis*-sulindac was present as the preponderant drug component. The remaining compounds were present at much lower concentrations as they might be expected to occur if present as degradation products. The MPHB was included because it was a preservative in some liquid dosage forms to be analyzed.

Because the best HPLC resolution was obtained with a mixed organic modifier consisting of methanol and acetonitrile, the complexity of the mobile phase suggested reducing the chromatographic system to a mathematical model where the interactions of the system's four major variables were considered. A four-factor orthogonal, composite experimental design was employed [14] centered near an estimated optimum set of conditions. The simulated sample was chromatographed with 25 different mobile phase conditions and the retention times and peak widths for each component were obtained. This technique is discussed in detail elsewhere[14,15]. The region of the response surfaces examined is indicated in Table I. Sixteen experiments defined the boundaries of the response surfaces, eight experiments were the axial values and one experiment defined the central condition[14]. A previously published multiple linear regression technique[16], using a program written for an HP 9825A desktop computer, was employed to analyze the data. The mathematical form of the response functions, φ, relating the observed response, Y (the capacity factors and peak widths), to the chromatographic variables, x_n, was not known:

$$Y = \varphi\,(x_n) \tag{1}$$

Therefore, the response surfaces were approximated by second degree polynomials of the form

$$Y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_4x_4 + b_5x_1^2 + b_6x_2^2 + b_7x_3^2 + b_8x_4^2 + b_9x_1x_2 + b_{10}x_1x_3 + b_{11}x_1x_4 + b_{12}x_2x_3 + b_{13}x_2x_4 + b_{14}x_3x_4 \tag{2}$$

where x_1 is the volume per cent acetonitrile in the organic modifier in the mobile phase, x_2 is the molar concentration of the ammonium phosphate buffer solution, x_3 is the volume per cent of the mixed organic modifier in the mobile phase and x_4 is the column operating temperature in centigrades. The significance of all terms in the

TABLE I

FOUR-VARIABLE FULL FACTORIAL DESIGN INDICATING THE LOCATION OF THE HALF-FACTORIAL TEST POINTS

Variable	*Factor level**	*Value of the variable at the indicated factor level*
Organic modifier concentration (%)**	1.41	37.7
	1.00	36.0
	0.50	34.0
	0.00	32.0
	−0.50	30.0
	−1.00	28.0
	−1.41	26.3
Acetonitrile concentration in organic Modifier (%)***	1.41	2.6
	1.00	15.0
	0.50	30.0
	0.00	45.0
	−0.50	60.0
	−1.00	75.0
	−1.41	87.4
Buffer concentration (*M*)§	1.41	0.0974
	1.00	0.085
	0.50	0.070
	0.00	0.055
	−0.50	0.040
	−1.00	0.025
	−1.41	0.0126
Temperature (°C)§§	1.41	59
	1.00	55
	0.50	50
	0.00	45
	−0.50	40
	−1.00	35
	−1.41	31

* The factor levels 1.41, 1.00, 0, −1.00 and −1.41 define the full factorial design consisting of 25 experiments. The levels 0.50 and −0.50 define the 16 half-factorial test points.

** The amount of organic modifier in the mobile phase prepared as described in the text and expressed as volume per cent.

*** The amount of acetonitrile in the organic modifier prepared as described in the text and expressed as a volume per cent.

§ The amount of ammonium phosphate buffer prepared as a 0.5 *M* aqueous stock solution and adjusted to pH 6.0 with concentrated ammonium hydroxide, then diluted to the indicated molar concentration in distilled, filtered water.

§§ The operating temperature of the Ultrasphere ODS HPLC column (250 × 4.6 mm I.D.).

fitted equations was established by a standard analysis of variance[17]. Three- and four-factor interactions were not included because of their lack of statistical significance. The various response surfaces of interest were plotted on an HP 9871A printer by solving the appropriate fitted equations at incremental values of the independent variables. All responses of interest were determined from the capacity factor and peak width values.

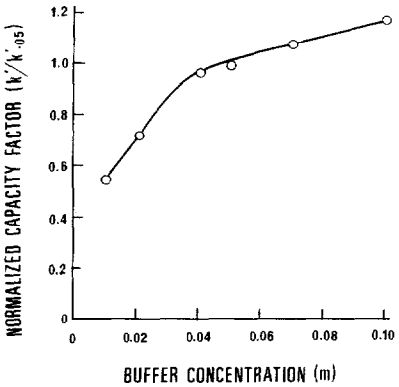

Fig. 2. The capacity factors normalized for each component to that value obtained at 0.05 *M* buffer and plotted against the concentration of aqueous ammonium phosphate buffer pH 6.0 with 45% methanol in the mobile phase.

Because of its demonstrated substantial effect on component retention (Fig. 2), buffer concentration was a factor chosen as a primary variable. Changing the buffer concentration changes many parameters of the system, as does organic modifier concentration, including ionic strength and pH. Nevertheless, it is an important variable in mobile phase preparation. The principal concern in a practical chromatographic model must always be pragmatic and not necessarily rigorous theoretical treatment.

RESULTS AND DISCUSSION

Initial trials examined chromatographic conditions that would resolve the sulindac-related components including the desoxysulindac and the *cis*-sulindac ethyl ester. Suitable resolution of these last two compounds was obtained with a mobile phase in which 60% methanol by volume was added as the organic modifier to the aqueous 0.05 *M* ammonium phosphate solution buffered at pH 6.0. However, the *cis*-sulindac and the sulfone eluted in less than 3 min as poorly resolved components. Similar results were obtained with 40% acetonitrile in the mobile phase instead of methanol. With less organic modifier the retention of the ethyl ester and desoxy derivatives increased substantially. Reversed-phase isocratic HPLC appeared unable to resolve all components without unacceptably long retention of the less polar compounds. For stability assessment, the primary criterion was the resolution of the polar compounds. With very low concentrations of ethanol present in the dosage forms the ethyl ester is not likely to be a significant transformation product. The *cis*-desoxysulindac, formed reversibly from sulindac, is present in very small quantities in actual samples. It is believed to be the physiologically active form of the drug[18]. Since its presence was not considered critical to the routine monitoring of drug stability, isocratic resolution of the five most polar components was examined rather than a more complex gradient elution system which may have resolved all components with acceptable retention times.

Effect of buffer concentration

The capacity factors, obtained at various phosphate buffer concentrations (pH 6.0) from 0.01 to 0.1 *M*, were normalized to the value obtained for each component at 0.05 *M* buffer. The resulting normalized correlations were superimposable. The final data, treated as a single correlation, are shown in Fig. 2. Normalization about a well-defined central point tersely summarized the data and demonstrated the similar response of each component to changing ionic strength. Similar response curves for organic acids have been reported elsewhere[19].

There appeared to be two distinctly different regions in the correlation, one at low ionic strength and another at higher ionic strength. The linear correlation obtained at higher buffer concentration has been explained by the solvophobic theory[20]. This theory predicts a linear increase in the capacity factor with increasing eluent surface tension, a parameter of the mobile phase which increases with the buffer concentration. However, at low buffer concentrations (<0.2 *M*) for other systems examined elsewhere[20], the capacity factor was observed to decrease with increasing ionic strength. The solvophobic theory was used to explain this phenomenon by suggesting that the ionic character of the solvation environments of the partitioning solutes (eluites) increased[20]. Using a similar argument, the observed increase in capacity factors for the sulindac-related compounds with increasing buffer concentrations could be explained by ionic accumulation in the solution environment of the stationary phase enhancing retention of the organic eluites. Since a hydrocarbon-bonded stationary phase should have a weak propensity for the retention of inorganic ions at its surface, the effect would be observable only at very low buffer concentrations (in this case <0.04 *M*). As the buffer concentration increases beyond some critical value the abundance of ions saturate all available sites. No further enhancement is observed and the underlying effect of buffer concentration on surface tension is the only remaining important influence.

Effect of pH

Chromatographic behaviour was investigated with mobile phases of pH 3.0 and 6.0 modified with methanol and acetonitrile. At pH 3.0 the sulindac-related components are protonated. At pH 6.0 these components are predominantly in their unprotonated conjugate base form. In the phosphate buffers, with the exception of FMIAA, the order of elution at pH 3.0 was found to be the same as at pH 6.0 but retention was greater because of the increase in non-polar character with protonation. FMIAA was retained more strongly at pH 3.0 than at pH 6.0 relative to the other components. Apparently, protonation of this smaller molecular structure had a greater effect on its hydrophobicity than protonation of the larger structures containing the additional hydrophobic surface of the benzyl sulfoxide group.

Effect of organic modifier

The chromatographic system was examined at pH 3.0 and 6.0 using 0.05 *M* ammonium phosphate buffers and 1% aqueous acetic acid solutions (pH = 3) at various concentrations of methanol or acetonitrile as the organic modifier. The capacity factors for each component were plotted logarithmically against the organic modifier concentration. Typical data are shown in Fig. 3. Linear correlations from semilogarithmic plots have been reported for several chromatographic systems[21,22].

TABLE II

A SUMMARY OF COMPONENT SEPARATION AT SEVERAL MOBILE PHASE CONDITIONS

The selectivity factor, α, is tabulated as an average of the selectivity factors calculated from pairs of capacity factors at several organic modifier concentrations. The exact number of these pairs of capacity factors used is indicated in the last column. The subscripts associated with each α identify the pairs of components: *exo*-sulindac (1), *cis*-sulindac (3), *cis*-sulindac sulfone (4) and *trans*-sulindac (5). The relative standard deviation (R.S.D.) is expressed as a percentage of the α value calculated from the data used to obtain the tabulated average selectivity factor.

Mobile phase conditions			*Average selectivity factors ± R.S.D.*						*No. of data points*
Organic modifier	*Buffer concentration**	*pH*	$\alpha_{1,3}$	$\alpha_{1,4}$	$\alpha_{1,5}$	$\alpha_{3,4}$	$\alpha_{3,5}$	$\alpha_{4,5}$	
Methanol	0.05 *M* Phosphate	6	1.52 ± 6.0	1.69 ± 8.4	2.64 ± 11.4	1.11 ± 2.7	1.74 ± 5.9	1.56 ± 3.5	5
Methanol	0.05 *M* Phosphate	3	1.23 ± 3.1	1.27 ± 0.5	1.51 ± 1.2	1.04 ± 3.6	1.23 ± 2.4	1.19 ± 1.6	4
Methanol	1% Acetic acid	3	1.04 ± 0.9	1.00	1.26 ± 1.6	1.04 ± 0.9	1.21 ± 1.0	1.26 ± 0.6	6
Acetonitrile	0.05 *M* Phosphate	6	1.41 ± 8.2	3.26 ± 6.4	2.13 ± 12.8	2.32 ± 8.9	1.52 ± 15.1	1.55 ± 13.3	6
Acetonitrile	0.05 *M* Phosphate	3	1.09 ± 5.1	1.80 ± 5.5	1.26 ± 4.3	1.94 ± 11.8	1.36 ± 2.4	1.43 ± 9.8	4
Acetonitrile	1% Acetic acid	3	1.26 ± 3.8	1.57 ± 1.3	1.00	1.98 ± 3.0	1.26 ± 3.8	1.57 ± 1.3	5

* Ammonium phosphate buffer was made up as described in the text to give a 0.05 *M* concentration at either pH 3.0 or 6.0. Glacial acetic acid was diluted to give a 1% aqueous solution; the measured pH was about 3.

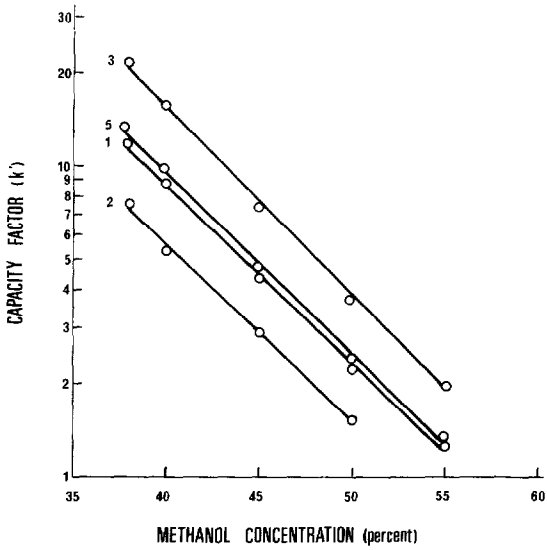

Fig. 3. The capacity factors plotted logarithmically against the percent methanol in the mobile phase containing 0.05 *M* ammonium phosphate buffer pH 6.0. The column was operated at 40°C with 10-μl sample injection. See Fig. 1 for the identity of components.

In the present case, small deviations from linearity in the semilogarithmic plots could be eliminated by treating the data as a Freundlich isotherm[23,24]. The primary parameter determining the slopes of these correlations appeared to be the available hydrophobic surface of each molecular species. Geometric isomerism or the oxidation state of the sulfur group had little influence. The correlations obtained for FMIAA were linear but of lesser slope than for the other components.

Because the linear correlations obtained for the three sulindac isomers and the sulfone were parallel, the selectivity factors must be independent of the organic modifier concentration. Therefore, the selectivity factor served as a convenient parameter to summarize the effect of pH and the nature of the organic modifier on the resolution of these four compounds (see Table II). For each pair of components the selectivity factors were calculated from the data at each organic modifier concentration. These calculated factors were averaged for each set of conditions and a relative standard deviation determined (Table II).

In this system, the resolution of *cis*-sulindac from its sulfone is critical. The sulfone, eluting immediately after the *cis*-sulindac, must be well separated because of the preponderance of the primary component, *cis*-sulindac, in the sample. Using a selectivity factor of 1.2 as the minimum criterion for adequate resolution, it is clear from Table II that mobile phases containing methanol did not resolve these two compounds. The best separation was obtained with acetonitrile at pH 6.0. However, in this case peak asymmetry was unacceptable. Adequate peak symmetry and suitable separation of the sulfone from the *cis*-sulindac were obtained with a mobile phase containing both methanol and acetonitrile. Selectivity could be controlled by changing the volume ratio of the two organic solvents. A chromatogram obtained at conditions believed to be the approximate optimum is shown in Fig. 4.

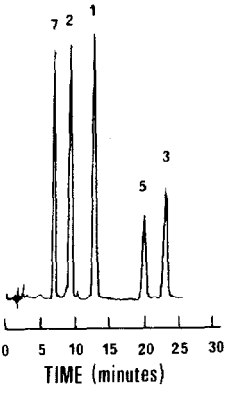

Fig. 4. A chromatogram obtained under conditions believed to be an approximate optimum. The column was operated at 35°C and 200 bar. The mobile phase consisted of 32% organic modifier (425 ml of acetonitrile made up to 1 l with methanol) in 0.05 *M* aqueous ammonium phosphate pH 6.0. This condition became the midpoint of the factorial experimental design. See Fig. 1 for the identity of components.

The computer model

Initially, the goodness-of-fit of the response surfaces for capacity factors and peak widths was established by calculating the coefficients of determination, that is, the R^2 values. Because these values do not always provide a meaningful indication of fit or predictive ability, the capability of the model to provide accurate predictions within the boundary limits of the factorial design was investigated. A set of sixteen experiments was performed under conditions indicated in Table I such that they

TABLE III

DATA OBTAINED FROM THE LINEAR REGRESSION ANALYSIS FOR THE PREDICTED *vs.* ACTUAL PARAMETERS USING THE ORIGINAL FACTORIAL EXPERIMENTS AND THE HALF-FACTORIAL TEST POINTS

Modelled parameter	*Modified response*	*Original factorial experiments**			*Half-factorial experiments***		
		Slope	*Intercept*	*Correlation coefficient*	*Slope*	*Intercept*	*Correlation coefficient*
Capacity factor	Y	0.97	0.36	0.98	1.04	−0.95	0.99
	log Y	1.00	0.02	1.00	0.92	0.51	1.00
	log $(Y-n)$	0.95	0.48	1.00***	0.98	0.19	1.00§
Peak widths	Y	0.98	0.02	0.99	1.04	−0.07	0.97
	log Y	1.00	−0.001	1.00	0.86	0.06	0.98
	log $(Y-n)$	0.99	0.08	0.98	0.95	0.01	0.98

* Values from all six components under the 25 different experimental conditions giving a total of 150 data points.

** Values from all six components under the 16 different experimental conditions giving a total of 96 data points.

*** See Fig. 5 for the linear regression of the original factorial experiments with the modelled response log $(Y-n)$.

§ See Fig. 6 for the linear regression of the half-factorial test points with the modelled response log $(Y-n)$.

consisted of an orthogonal set centered about the midpoint of the original factorial design (half-factorial points). Predicted values from the model were compared with the actual experimental values to assess the predictive ability of the simulated system.

Employing a modelled response, Y, in the predictor equation, poor predictability and fit were observed for numerically small response values in spite of high R^2 values (see Table III). This poor fit was partially remedied by modelling the logarithm of the responses, log Y. A good fit was obtained but the predictive ability for numerically high response values was unacceptable. This bias was corrected by introducing empirically selected values for n, the modelled responses becoming log $(Y-n)$. Although the predictions for the sixteen half-factorial experiments were improved there was a proportionate increase in the errors for the fit to the original factorial set. A summary of the results is shown in Table III. The predicted values were plotted against the actual values for both the original factorial design and the half-factorial points (Figs. 5, 6). A linear correlation was obtained for each modelled response. A comparison of the slopes of the regression lines and their intercepts was a good indication of the fit to the original factorial data set (Fig. 5) in the first case and the predictability of the half-factorial points (Fig. 6) in the second case. A perfect fit should have a slope of one and a zero intercept.

Application of the computer model

Employing the computer simulation, the effect of operating conditions on the three parameters affecting resolution, namely, the capacity factor, the selectivity factor and the number of theoretical plates, could be examined to assess satisfactory component separation and adequate column efficiency. Four aspects of the chromatographic behaviour were investigated and are discussed to illustrate, in part, the

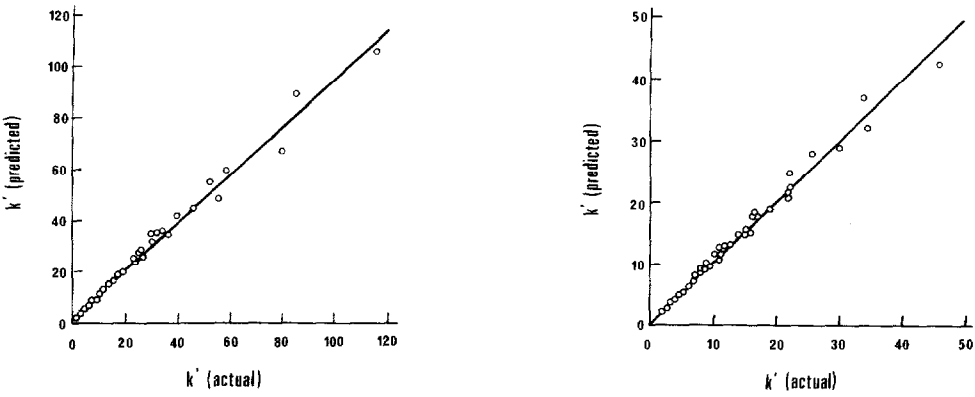

Fig. 5. The actual capacity factors obtained from the original factorial design consisting of 25 experiments plotted against the predicted capacity factors obtained from the computer simulation using log $(Y-n)$ as the modelled response. The capacity factors for all six components are included for a total of 150 data points. Because of overlapping data, for clarity a plotted point may represent several data points.

Fig. 6. The actual capacity factors obtained for the sixteen half-factorial points plotted against the predicted values obtained from the computer simulation as in Fig. 5 for the modelled response log $(Y-n)$. As in Fig. 5, a plotted point may represent several data points.

general utility of a computer model:

(1) The enthalpy of binding for each component was determined from the effect of temperature on the capacity factor.

(2) The effect of mobile phase conditions on the selectivity factors was used to document the separation process and to predict possible operating changes which could be made to improve chromatographic conditions as the nature of the column changes with use.

(3) The effect of conditions on the theoretical plate count was investigated to assess the variation in column efficiency.

(4) Finally, a grid search examined the conditions which would provide optimum resolution.

(1) *Enthalpy of binding.* The enthalpy change due to binding of the eluite at the surface of the stationary phase can be determined from the slope of the Van't Hoff plot, a plot of the logarithm of the capacity factors *vs.* the reciprocal of the temperature[25]. For the sulindac-related components, Van't Hoff plots obtained from the computer model at different mobile phase conditions showed good linear correlations. The computed enthalpy of binding for each component was plotted against the per cent acetonitrile and the buffer concentration in the mobile phase. Binding to the stationary phase was weakly dependent on the buffer concentrations, but decreased with increasing acetonitrile concentration reflecting its greater solvent strength. The enthalpy of binding for the six components at two acetonitrile concentrations is shown in Table IV.

(2) *Selectivity factors.* Most decisions concerning improved resolution could be made by simply examining the selectivity factors. Separations in two regions of the chromatogram were studied. Initially, the separation of the sulfone from the *cis* and *trans* isomers of sulindac was monitored to assess the effect of acetonitrile concentration. Then, the separations of FMIAA, MPHB and the sulindac *exo* isomer were examined. Although many of the detailed aspects of the chromatographic response might be determined by trial and error, the computer model allowed the component separations to be analyzed systematically.

Because of the linear response obtained for the Van't Hoff plots, the selectivity factor plotted logarithmically against the reciprocal of temperature also must be

TABLE IV

ENTHALPY OF BINDING FOR EACH COMPONENT AT TWO ACETONITRILE CONCENTRATIONS IN THE ORGANIC MODIFIER

The total per cent organic modifier in the mobile phase was 32%.

Component	*Enthalpy of binding (kcal/mole)*	
	15% Acetonitrile in organic modifier	*75% Acetonitrile in organic modifier*
FMIAA	5.7	3.4
MPHB	5.3	3.7
exo-sulindac	7.6	4.7
cis-sulindac	7.9	4.1
cis-sulindac sulfone	9.0	6.2
trans-sulindac	8.7	4.5

linear. Good linear correlations were obtained from the computer simulation. The resulting planar responses obtained when the selectivity factors were plotted in three-dimensional graphics against the reciprocal of temperature and the acetonitrile concentration in the mobile phase were used to monitor the relative separations of the sulfone, *cis* and *trans* isomers.

The separations of the components eluting early in the chromatogram are sensitive to minor variations in the conditions of the HPLC column. Adjustments to provide satisfactory resolution in this region of the chromatogram may be required when initially employing a new column or re-using an old one. The separation of FMIAA and MPHB showed a weak shallow response with temperature but showed marked changes with increasing buffer concentration. Decreasing the buffer concentration altered the resolution of these two components without substantially affecting the separation of the compounds eluting later in the chromatogram, providing a means of positioning the MPHB between the *exo* isomer and FMIAA.

The separation of the two components MPHB and *exo*-sulindac was more temperature dependent than the separation of FMIAA and MPHB. If separation problems involved the first two components then an improvement could be achieved by decreasing the temperature without substantially affecting the separation of the MPHB and FMIAA.

(3) *Number of theoretical plates.* The effect of temperature on the observed number of theoretical plates for sulindac-related compounds was characterized by an optimum occurring at about 35–45°C (see Figs. 7–9). Generally, a significant decrease was observed above 50°C. This phenomenon could be explained by considering intracolumn and extracolumn contributions to efficiency. The observed number of theoretical plates, N, may be viewed as consisting of four major contributing factors:

$$N = N_{DIS} + N_{DIF} + N_{KIN} + N_{INSTR} \tag{3}$$

The first two terms represent the contribution of those factors affecting molecular diffusion. These include the rate of axial diffusion in the interstitial space, N_{DIS}, and the combined effects of the resistance to diffusion at the particle boundary and intraparticular diffusion resistance, N_{DIF}. With increasing temperature, the contribution from these terms to the system's efficiency is positive. The third term, N_{KIN}, represents the effect of kinetically related factors on column efficiency. Kinetically controlled mass transfer in liquid chromatography has been discussed in two recent papers[26,27]. If the eluite (E) interaction with the hydrocarbon stationary phase is analogous to the weak binding at an active site (B) on the surface of the stationary phase to form a complex (EB), then the process may be described as

$$E + B \underset{}{\overset{K}{\leftrightarrows}} EB \tag{4}$$

where the equilibrium constant can be expressed either in terms of the rate constants k_a and k_d for association and dissociation, respectively, or as proportional to the eluite capacity factor, k'. An expression for the kinetic contribution to column efficiency, derived from a general equation of plate height[26], indicates the dependence of the kinetic contribution on the dissociation rate constant of the complex EB

$$N_{KIN} = L(1 + k_0)(1 + k')^2 k_d / 2k'\mu_e \quad (5)$$

where L is the column length, k_0 the ratio of intraparticular void volume to interstitial void volume and μ_e the interstitial mobile phase velocity. Increasing temperature will not only enhance diffusion and increase the number of productive encounters of the eluite with the active site (k_a) but also increase the rate at which the complex will dissociate (k_d). Whether the number of theoretical plates due to intracolumn effects increases or decreases with temperature depends upon the relative contributions of diffusion and kinetic factors. Predominantly diffusion-controlled interactions result in an increased intracolumn plate count at higher temperatures. Kinetically controlled interactions result in a decreased intracolumn plate count at elevated temperatures.

The final term, N_{INSTR}, in eqn. 3 represents the contribution of extracolumn effects to the observed efficiency. These instrument-related effects include contributions to band broadening from the injector, the capillary tubing and the detector. It is generally accepted that this contribution is significant if it represents greater than 10% of the observed theoretical plate count. This results when extracolumn band broadening exceeds 50% of the intracolumn band broadening, a circumstance which may occur for components with small k' values. With these components, increasing temperature will reduce retention and increase the contribution of N_{INSTR}. Under the instrument operating conditions employed for the factorial experimental design, the extracolumn contribution to band broadening was 0.16 min. Its effect on efficiency was calculated under a variety of experimental conditions. Under conditions where one or more components were weakly retained ($k' < 3$; peak width < 0.3 min) increasing temperature significantly reduced efficiency because of extracolumn effects. Therefore, there were three operating regimes which could be defined by the response of the system's observed efficiency to changing temperature. There was a

TABLE V

CONSTRAINTS EMPLOYED IN THE GRID SEARCH FOR THE OPTIMUM CHROMATOGRAPHIC CONDITION

The selectivity factor constraints do not refer to component numbers as identified in Table II or VI but rather to adjacent pairs in the order in which the components elute under any specific condition. The last two adjacent pairs usually comprise *cis*-sulindac, *cis*-sulindac sulfone and *trans*-sulindac where greater separation is required. Therefore, these last two selectivity factors were less constrained.

Constraints on chromatographic conditions			*Constraints on selectivity factors*		
	Max.	*Min.*		*Max.*	*Min.*
Organic modifier concentration (%)	36	28	$\alpha_{1,2}$	1.4	1.1
Acetonitrile concn. in organic modifier (%)	75	15	$\alpha_{2,3}$	1.4	1.1
			$\alpha_{3,4}$	1.4	1.1
Buffer concn. (M)	0.085	0.025	$\alpha_{4,5}$	2.0	1.3
Column temperature (°C)	55	35	$\alpha_{5,6}$	2.0	1.1

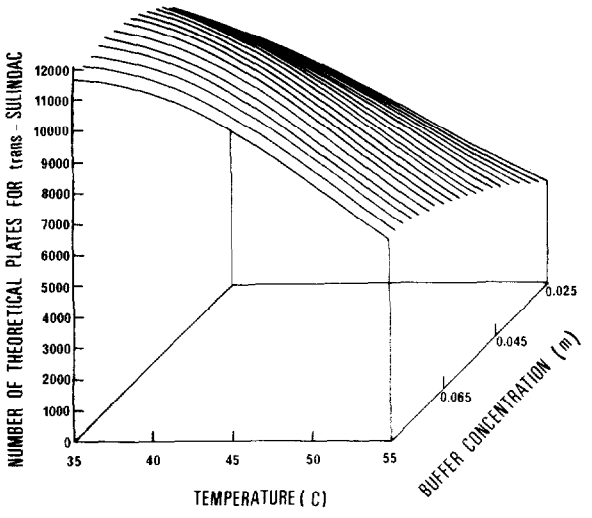

Fig. 7. The number of theoretical plates for *trans*-sulindac plotted as a function of temperature and buffer concentration in the mobile phase with 45% acetonitrile in the organic modifier and 32% organic modifier in the mobile phase .

diffusion-controlled region, a kinetically controlled region and an instrument-limited region.

The more efficient contemporary columns containing packings of small particle sizes (3–5 μm) have larger available surface areas and reduced interstitial spaces. As a result, there is less resistance to diffusion and a kinetically controlled condition is more likely. An example of kinetic control is illustrated in Fig. 7. The *trans*-sulindac under the conditions of Fig. 7 is strongly retained by the column. At 0.085 *M* buffer the contribution of N_{INSTR} at 35°C was insignificant. It was less than 10% at 55°C. At 35°C, an optimum appeared to have formed where contributions from the diffusion and kinetic processes were about equal. Above 40°C the decrease in plate count with

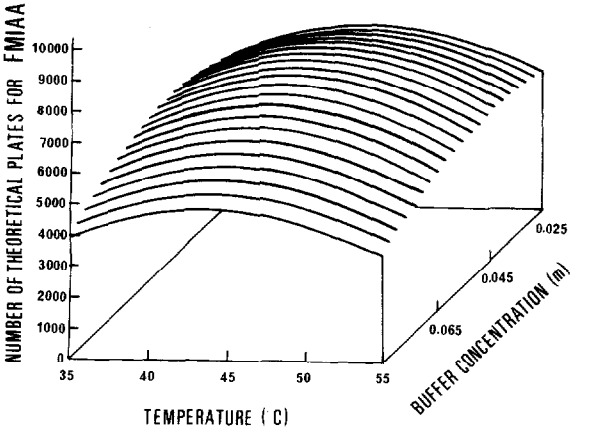

Fig. 8. The number of theoretical plates for FMIAA plotted as a function of temperature and buffer concentration in the mobile phase with 45% acetonitrile in the organic modifier and 32% organic modifier in the mobile phase.

TABLE VI

THREE PREDICTED ELUTION ORDERS FOR THE SULINDAC-RELATED COMPONENTS OBTAINED FROM A GRID SEARCH EMPLOYING THE COMPUTER-SIMULATED MODEL OF CHROMATOGRAPHIC BEHAVIOUR

Two of these predicted chromatograms were optima. Components: 1 = FMIAA; 2 = MPHB; 3 = *exo*-sulindac; 4 = *cis*-sulindac; 5 = *cis*-sulindac sulfone; 6 = *trans*-sulindac.

Component	*Predicted parameters*		*Actual parameters*		*Chromatographic conditions*			
	Capacity factor, k'	*Selectivity factor, α*	*Capacity factor, k'*	*Selectivity factor, α*	*Organic modifier concn. (%)*	*Acetonitrile in mobile phase (%)*	*Buffer concn. (M)*	*Column Temperature (°C)*
1*	3.67		3.64		34.3	32.5	0.031	45
		1.13		1.16				
2	4.13		4.24					
		1.14		1.07				
3	4.71		4.52					
		1.29		1.33				
4	6.06		6.00					
		1.52		1.49				
5	9.24		8.98					
		1.32		1.29				
6	12.18		11.60					

2*	3.17		3.38		36.0	21.0	0.085	55
		1.23		1.28				
1	3.89		4.33					
		1.20		1.18				
3	4.65		5.11					
		1.39		1.37				
4	6.93		6.99					
		1.26		1.27				
5	8.76		8.89					
		1.49		1.49				
6	13.05		13.22					
1**	2.98				28.0	75.0	0.072	35
		1.15						
3	3.44							
		1.24						
4	4.28							
		1.11						
2	4.74							
		1.80						
6	8.52							
		1.11						
5	9.42							

* Optimum conditions.

** This order of elution was unable sufficiently to resolve component 2 from component 4 to be considered an acceptable optimum.

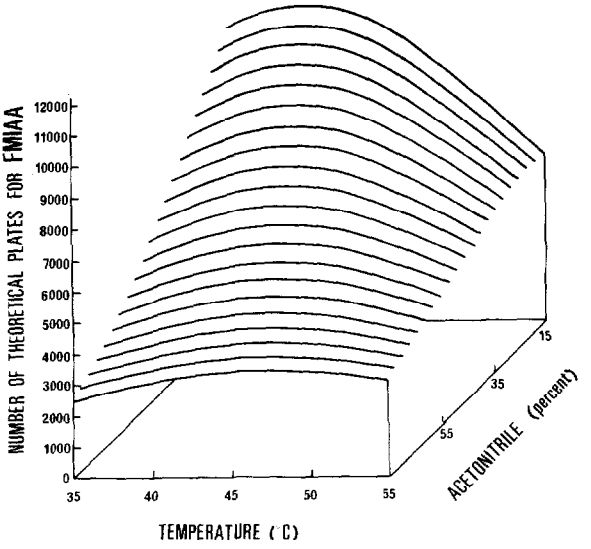

Fig. 9. The number of theoretical plates for FMIAA plotted as a function of temperature and per cent acetonitrile in the organic modifier with an organic modifier concentration of 32% and a buffer concentration of 0.055 *M* in the mobile phase.

increasing temperature appeared to be attributable to kinetically controlled mass transfer.

The retention of FMIAA under the conditions of Fig. 8 was considerably weaker than for the *trans*-sulindac. The contribution of N_{INSTR} to efficiency was significant from 35 to 55°C and accounted for the shallow optimum observed at 40–45°C. Subtracting the extracolumn effects from the observed number of theoretical plates indicated that column efficiency gradually increased to about 45°C with very little change in the region of 45–55°C. It appeared that the mass transfer phenomenon for FMIAA was diffusion-controlled at lower temperatures. An optimum was reached at 45°C above which extracolumn effects began to dominate and a decrease in efficiency was observed with increasing temperature.

By monitoring the effect of temperature on the system's efficiency at different acetonitrile concentrations in the mobile phase, the three factors controlling mass transfer were defined (Fig. 9). At high concentrations of acetonitrile, FMIAA retention was small and extracolumn effects predominated. The system's efficiency is instrument-limited. At 15% acetonitrile and 35°C, the contribution of N_{INSTR} was not significant and the efficiency increased with increasing temperature influenced by diffusion-controlled mass transfer. Above 45°C, the kinetic processes began to dominate. The number of theoretical plates decreased with increasing temperature.

(4) *A grid search for the optimum.* A computer search for the system's optima was conducted within the boundary limits of the factorial design. The method employed is described in detail elsewhere[16]. The model equations for the capacity factors were solved for each set of conditions on the grid and the selectivity factors for each pair of adjacent peaks were calculated. These selectivity factors were compared with the estimated maximum and minimum values between which the factor should lie in order to obtain a practical chromatogram (see Table V). Two optima were predicted

differing in the elution order of FMIAA and MPHB. The predicted capacity factors and operating conditions are shown in Table VI. Actual chromatograms obtained on a new Ultrasphere ODS column, whose efficiency was as the manufacturer had specified, indicated good agreement with these predictions. The total scan times to complete the chromatograms were about 17 and 19 min, an improvement from the approximate optimum condition where the total scan time was about 24 min (Fig. 4). A third possible elution order with the MPHB eluting after the *cis*-sulindac (Table VI) was indicated but resolution was not sufficient to provide a third optimum.

CONCLUSIONS

Sulindac and its structurally related compounds have been resolved employing a reversed-phase HPLC system. A mixed organic modifier consisting of methanol and acetonitrile produced the best separations. Using a factorial experimental design to establish a computer-simulated model, the influence of four primary variables on chromatographic separation and column efficiency was examined. The enthalpies of binding for each component were calculated. The effect of the chromatographic conditions on the selectivity factors was determined indicating those variables best suited to modifying a given chromatogram to obtain a required change in resolution. The effect of .column temperature on the number of theoretical plates was monitored and an optimum efficiency defined at 35–45°C. Finally, a grid search was used to explore the response surfaces surrounding the selected approximate optimum condition. This defined two separate conditions in the region where an acceptable resolution was possible. Therefore, the simulated model provided not only a method to optimize resolution but also a detailed documentation of chromatographic behaviour influenced by several interacting variables.

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