CHROM. 21 110

DEVELOPMENT AND OPTIMISATION OF A HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR TIOCONAZOLE AND ITS POTENTIAL IMPURITIES

I. SELECTION OF SEPARATION CONDITIONS

A. G. WRIGHT and A. F. FELL

Department of Pharmaceutical Chemistry, University of Bradford, Bradford, West Yorkshire BD7 1DP (U.K.)

and

J. C. BERRIDGE*

Pfizer Central Research, Sandwich, Kent CT13 9NJ (U.K.)

(First received June 7th, 1988; revised manuscript received November 14th, 1988)
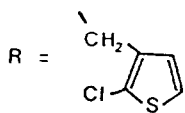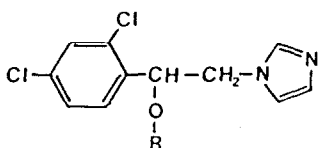
SUMMARY

Statistical mixture design techniques have been utilized to develop an isocratic high-performance liquid chromatographic separation of 1-{2-[(2-chloro-3-thienyl)-methoxy]-2-(2,4-dichlorophenyl)ethyl}-1H-imidazole (tioconazole) and its potential impurities. By using suitable quality criteria such as reduced plate height, peak asymmetry and selectivity for the interrogation of chromatograms the variables producing best column efficiency and greatest selectivity have been identified. The optimum separation for these variables has been located by the sequential simplex approach and confirmed by response surface mapping.
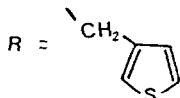
INTRODUCTION

Tioconazole, 1-{2-[(2-chloro-3-thienyl)methoxy]-2-(2,4-dichlorophenyl)ethyl}-1H-imidazole, is an imidazole drug used as a broad-spectrum anti-fungal agent. Three related impurities and one degradation product (Fig. 1) are found in trace amounts[1,2]. Reversed-phase chromatographic separation of related compounds B and C has proved extremely difficult. Indeed, resolution is incomplete for the chromatographic method currently prescribed in the United States Pharmacopeia (U.S.P.)[1]. The resolution enhancement due to peak sharpening, achieved by taking the second derivative of the chromatogram, was previously used to improve quantitation of compounds B and C[3].

The U.S.P. method[1] specifies an eluent containing 0.20% ammonia (sp.gr. 0.88) which results in a high pH. Consequently the column lifetime is short due to dissolution of the silica support. To overcome this problem a pre-column was incorporated to pre-saturate the mobile phase with silica. The 25-cm analytical column employed in this assay gives rise to a long analysis time, while still not providing good resolution of compounds B and C. The U.S.P. method was not developed with the
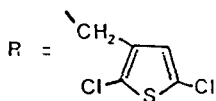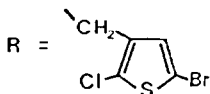
Fig. 1. Structures of tioconazole, related impurities and hydrolytic breakdown product.

quantitation of the hydrolysis product in view. The present work reports the application of systematic optimisation procedures for the development of a high-performance liquid chromatography (HPLC) method to overcome these limitations. A large number of variables are involved in the development of a reversed-phase HPLC separation[4]. The principal chromatographic parameters governing the separation behaviour include: organic modifier, column stationary phase chemistry, pH, buffer and ion-pairing agent. A simultaneous optimisation of all these parameters is beyond any single optimisation procedure. Thus it was decided to explore the rational use of statistical mixture designs and partial factorial designs, to identify the most significant variables in method development for this problem. A sequential search procedure was then employed to optimise the method.

The limitations of the existing assay are that the mobile phase is aggressive towards the packing material, and that the analysis time is long, due to the length of column. Thus the key aims were to develop an assay which would operate at lower pH values and with shorter columns. The analytes in this assay are basic (pK_a for tioconazole is around 6) and exist in two pH-dependent forms. At pH 8 and above, the unionized form is the major species; at pH 4 and below, the protonated form is predominant. The U.S.P. method separates the unionized species, leading to the requirement for an eluent of high pH. Lowering the pH to extend column lifetime involved making a study of the protonated species. For chromatography of these

ionized species an anionic ion-pairing agent would normally be added. This technique therefore formed an essential part of the method development.

EXPERIMENTAL

Measurements were made on two systems. Initial studies used an LKB 2150 pump, a 2151 variable-wavelength detector and a 2210 chart recorder (LKB Instruments, Croydon, U.K.). The sample injection valve was a Rheodyne 7010 fitted with a 20 μl loop (Alltech Asoc., Carnforth, U.K.). Systematic development was based on a Hewlett-Packard 1090A chromatograph incorporating a DR5 ternary pumping system, an autosampler with a Rheodyne 7010 injection valve, an integral 1040A diode array detector (with the 220-nm signal being recorded[1], an integral DPU integrator an HP 7470A plotter and an HP85 microcomputer (Hewlett-Packard, Wokingham, U.K.). The columns used were an Ultrasphere 5-μm ODS (45 × 4.6 mm I.D., packed in-house); a Nucleosil 5-μm cyanopropylsilica (50 × 4.6 mm I.D. Technicol, Stockport, U.K.) a Hypersil 5-μm phenyl (50 × 4.6 mm I.D., Technicol) and a Hypersil 5-μm phenyl (150 × 4.6 mm I.D., Technicol). A summary of these columns is given in Table I.

TABLE I

COLUMNS USED IN THE STUDIES

Particle size, 5 μm; I.D., 4.6 mm.

Column packing	Chemistry	Length (mm)
Ultrasphere	ODS	45
Nucleosil	Cyanopropyl	50
Hypersil	Phenyl	50
Hypersil	Phenyl	150

Mobile phases were prepared using HPLC grade solvents (Rathburn Chemicals, Peebles, U.K. and Fisons, Loughborough, U.K.). Sodium octylsulphonate was HPLC grade (Fisons). Potassium dihydrogenphosphate was AnalaR grade (BDH, Poole, U.K.) and triethylamine was reagent grade (Hopkins and Williams, Chadwell Heath, U.K.). Buffer pH was adjusted with reagent-grade phosphoric acid (BDH). Tioconazole, the three related impurities and the degradation product were supplied by Pfizer Central Research, Sandwich, U.K.

RESULTS AND DISCUSSION

Selection of columns
The influence of column stationary phase has received only limited attention with regard to separation optimisation. Previous work[5] has suggested that using different column packing materials may provide enhanced selectivity and lead to improved separation. Thus three column types exhibiting differing retention charac-teristics were investigated during this study. The column packing materials selected

were ODS, phenyl and cyanopropyl, with ODS being the most retentive and cyanopropyl the most polar and thus the least retentive.

The columns utilised for this investigation were all short (5 cm or below) to reduce development time to a minimum. The elution time and re-equilibration time could be significantly reduced, this being an important consideration, as many different mobile phases were to be employed.

Selection of significant mobile phase parameters

After having selected the columns, other variables were considered. The factors most frequently varied to effect a separation are the proportions of organic modifiers. For reversed-phase chromatography, methanol, acetonitrile and tetrahydrofuran (THF) offer the widest range of modifier selectivities and an experimental design aiming to achieve optimum separation should incorporate all three. However, there are many other variables which also affect chromatographic behaviour and they should also be considered.

Preliminary studies with the Ultrasphere 5-μm ODS column (45 $\times$ 4.6 mm I.D.) and simple modifier–water eluents, revealed baseline disturbances on injection of a sample. These perturbations were attributable to changes in local column pH caused by the sample solvent. The inclusion of potassium dihydrogenphosphate buffer in the mobile phase was found to rectify the problems of baseline disturbance. Thus buffered mobile phases were used throughout these studies.

Buffers serve several functions in HPLC; they prevent excessive changes in pH and they may also block the interfering effects of residual silanol groups on the stationary phase[6,7]. Potassium dihydrogenphosphate controls the pH effectively but has only limited action against residual silanols. Another buffer system, triethylamine (TEA) adjusted to the appropriate pH with phosphoric acid, is effective both in controlling pH and in blocking silanol interactions. One of these buffers was included in this study in turn to assess the influence of silanol interaction on the separation.

As discussed above, tioconazole and the potential impurities can exist in two pH-dependent forms. At pH values above 8 the solutes exist as neutral species, while at pH values below 4 the solutes are protonated. These protonated solutes may be eluted as such or, by the addition of an anionic ion-pairing agent, as neutral ion complexes. Thus two other variables are introduced, pH and the presence of an ion-pairing agent.

Once the appropriate variables were selected it was necessary to devise an experimental scheme allowing a rational investigation of the key parameters involved. The experimental scheme proposed was based upon a combination of partial factorial and lattice mixture designs[5,9–12] for each of the three columns (Fig. 2). Many variables were assessed at only two levels (*i.e.*, an upper and lower level, or frequently the presence or absence of the variable). The optimum separation conditions were predicted from the lattice mixture designs for each set of experimental variables. These optimum separation conditions were run in each case and the chromatogram recorded. The importance of each set of variables was determined by measuring a number of parameters for the optimum chromatogram. Column efficiency was described by the reduced plate height for tioconazole and the selectivity was determined by calculating the selectivity factor between compounds B and C. The silanol interaction was assessed by the asymmetry factor.

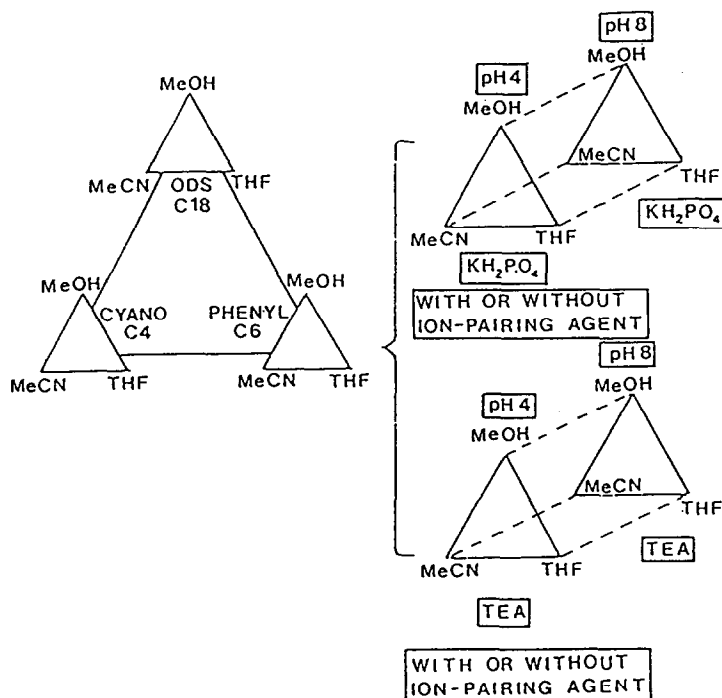Throughout these studies the detection wavelength employed was 220 nm, the

Fig. 2. Experimental scheme employed in the method development. MeOH = Methanol; MeCN = acetonitrile.

flow-rate 1.5 ml min^{-1} and column temperature 40°C. The concentrations of buffer and ion-pairing agent used were 0.05 M and 0.025 M, respectively. The lattice mixture designs employed for this study all required nominally isoelutropic eluents. The retention time for the last peak yielded by these eluents was approximately 7 min in all cases.

The Hypersil 5-μm phenyl column (50 × 4.6 mm) was investigated using the experimental scheme shown in Fig. 2. The results are recorded in Table II.

The same procedures were followed for the Ultrasphere 5-μm ODS column (45 × 4.6 mm I.D.). Lattice mixture designs were initiated for TEA buffer both with and without ion-pairing agent. However, significant retention time variations occurred on mixing equal proportions of the nominally isoeluotropic binary eluents of methanol–buffer and acetonitrile–buffer. Typically the retention time for tioconazole was 3 min with the methanol binary and 2.7 min with the acetonitrile binary. Mixing these two eluents (1:1, v/v) led to a retention time of 6.9 min. Since the mixed eluents prepared from the isoeluotropic binary eluents yielded retention times for the last peak in excess of those required, the experimental scheme was abandoned for the ODS column. Adjustment of solvent strength for the mixed eluents was not attempted as the lattice mixture designs were run as automated procedures through the method sequence facility of the LC system. It should be noted that this problem was not encountered with the phenyl column.

TABLE II

REDUCED PLATE HEIGHT, BEST PEAK ASYMMETRY AND SELECTIVITY VALUES FROM THE EXPERIMENTAL SCHEME

Column	pH	Buffer	Ion-pairing	Optimum eluent (%)			Minimum reduced plate height	Best peak asymmetry	α_{max} for B and C
				Methanol	Acetonitrile	THF			
Phenyl	4	KH_2PO_4	+	25	16	9	7.2	1.53	1.130
Phenyl	4	KH_2PO_4	−	−	46	−	5.8	1.20	1.202
Phenyl	4	TEA	+	16	39	−	4.9	1.06	1.128
Phenyl	4	TEA	−	55	−	−	8.3	1.20	1.129
Phenyl	8	TEA	−	62	17	−	3.5	1.05	1.153
Phenyl	8	KH_2PO_4	−	47	11	−	6.4	1.35	1.088
Cyanopropyl	4	TEA	+	−	40	−	−	−	1.153

A mixture design was followed for the Nucleosil 5-μm cyanopropyl column (50 × 4.6 mm I.D.) for TEA buffer containing ion-pairing agent. The peak shapes were strongly tailed and this produced high peak asymmetry values and poor resolution. The poor column performance did not warrant continuation of the experimental scheme, thus it was abandoned at this point.

The Hypersil phenyl column results (Table II) showed TEA to be the more appropriate buffer in this separation as compared to potassium dihydrogenphosphate. It yielded better resolution due to the improved peak shape; this was attributable to the effectiveness of TEA in blocking silanol interactions. The three cases where TEA was used as buffer all provided good selectivity, acceptable efficiency and some degree of resolution between peaks B and C. It was felt that consideration of the three sets of variables would benefit, if the best separations possible for each set of conditions were compared. Thus optimum conditions were determined using another strategy. In each lattice mixture design it was observed that THF had no effect on improving selectivity or resolution between compounds B and C. Thus only methanol and acetonitrile were considered. The optimisation strategy selected was the sequential simplex procedure. This optimisation strategy was also applied to confirm that the phenyl column did give the best separations. The optimum separation conditions were determined for both the ODS and cyanopropyl columns for comparison. The variables studied were methanol, acetonitrile and pH 4 TEA buffer containing ion-pairing agent.

Location of the optimum separation

With the variables of interest thus defined, the optimum separation conditions were located using the modified sequential simplex search method developed by Berridge[8]. This is an entirely automated and unattended procedure which does not require retention behaviour to be well defined. For these optimisations a sequential simplex was run with the maximum retention time for the last peak specified as 10 min, to ensure that analysis times were all comparable. The modifiers used were constrained to the following ranges: methanol 0–60%, acetonitrile 0–50%. Minimum retention time = 1 min, maximum retention time = 10 min.

The quality of the chromatographic separations were assessed using the chromatographic response function (CRF) developed by Berridge[8]

$$CRF = \sum_{i=1}^{n-1} R_i + n^2 - |T_A - T_n| - (T_0 - T_1)$$

where R_i is the resolution between adjacent peak pairs, n is the number of peaks observed in the chromatogram, T_1 is the retention time of first detected peak, T_0 is a specified minimum retention time for the first peak, T_A is the maximum desired retention time for the last peak, and T_n is the retention time for the last peak.

Resolution between adjacent peaks is determined from the data generated by the LC integrator using the following equation:

$$R_s = \frac{2(t_{R,2} - t_{R,1})}{W_1 + W_2}$$

where $t_{R,1}$ and $t_{R,2}$ are retention times of peaks 1 and 2, respectively and W_1 and W_2 are base-widths for peaks 1 and 2, respectively.

TABLE III

SEQUENTIAL SIMPLEX OPTIMA WITH TEA BUFFER

Column	pH	Ion-pairing	Optimum eluent		Minimum reduced plate height	Resolution between B and C	CRF
			Methanol	Acetonitrile			
Phenyl	4	+	31	19	5.3	0.45	28.2
Phenyl	4	−	23	33	6.1	0.40	28.5
Phenyl	8	−	29	21	6.6	0.36	28.6
ODS	4	+	20	40	6.2	0.27	28.0
Cyanopropyl	4	+	21	9	21	0.25	28.3

The sequential simplex results (Table III) showed that the phenyl column did yield the best separations, confirming the early decision to abandon studies on the ODS and cyanopropyl columns. The best resolved chromatogram located for the phenyl column corresponded to methanol–acetonitrile–pH 4 TEA buffer containing ion-pairing agent (31:19:50, v/v/v). The movement of this sequential simplex procedure is shown in Fig. 3. Confirmation that this was the global optimum was achieved by using a sequential surface-mapping experiment. Chromatograms were run using conditions representative of the whole response surface, defined by the limits for acetonitrile and methanol levels in the range 0–70%. Both modifier ranges were searched simultaneously in 5% increments, necessitating 120 chromatograms to cover the complete surface. The quality of each separation was assessed using the CRF defined above. The response contours are plotted in Fig. 4. The global optimum separation conditions were located as methanol–acetonitrile–pH 4 TEA containing ion-pairing agent (35:15:50, v/v/v), giving a CRF value of 28.5. A representative chromatogram is shown in Fig. 5.
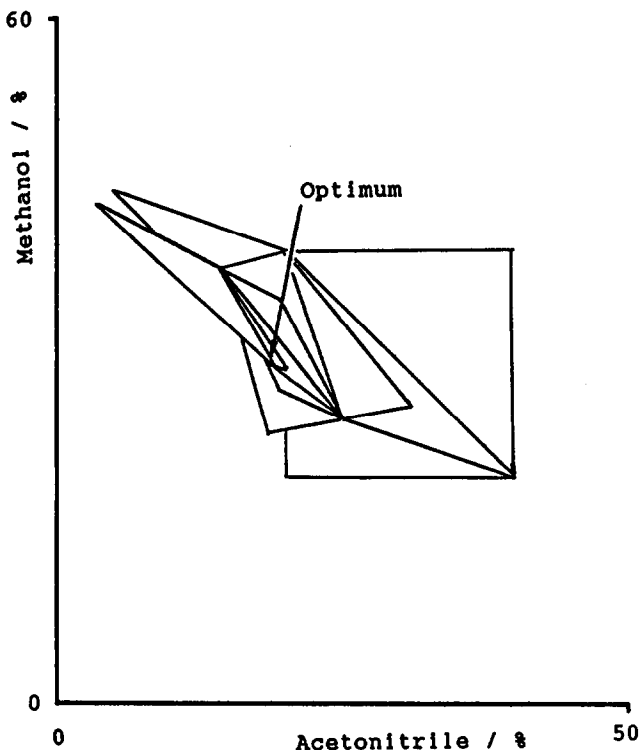
Fig. 3. Movements of the sequential simplex procedure for the phenyl column. Mobile phase consists of methanol, acetonitrile and triethylamine (0.05 M) at pH 4 containing ion-pairing agent (0.025 M).

Prediction of column dimensions necessary for complete separation

The optimum mobile phase was determined for a short Hypersil 5-μm phenyl column (50 × 4.6 mm I.D.) and, while some resolution of B and C was achieved, the performance of the system was not sufficient for complete separation. Thus, as selectivity had already been optimised, it was necessary to increase column plate count to achieve complete resolution. It was therefore necessary to increase column length. Since capacity factor (k') for the last peak and column length are among the parameters which govern analysis time, minimum analysis time can be attained by using the shortest possible column which provides sufficient theoretical plates for the separation. It was assumed that flow-rate was fixed and need not be considered.

The desired capacity factor for the last peak in this assay was specified as 10. The optimum separation conditions located by sequential simplex and grid mapping procedures both yielded separations where the capacity factor for the last peak was in excess of 10. It was therefore of interest to calculate the reduction in the proportion of buffer in the mobile phase, needed to yield a k' value of 10. It was assumed that selectivity was dependent upon the relative proportions of the modifiers to each other and independent of buffer level. Thus the ratio of methanol to acetonitrile was kept constant (*i.e.*, 70:30, v/v).

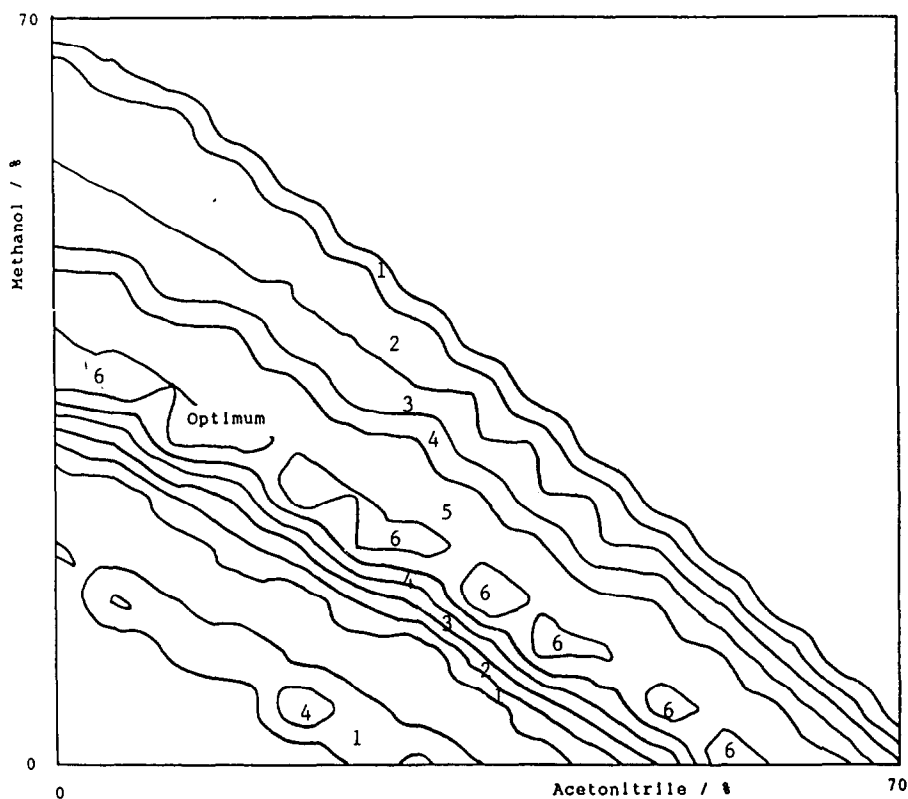As the temperature employed throughout was 40°C, it was also of interest to

Fig. 4. Contour plot of the response surface. CRF values for contours: (1) 0–5; (2) 5–10; (3) 10–15; (4) 15–20; (5) 20–25; (6) 25–30.
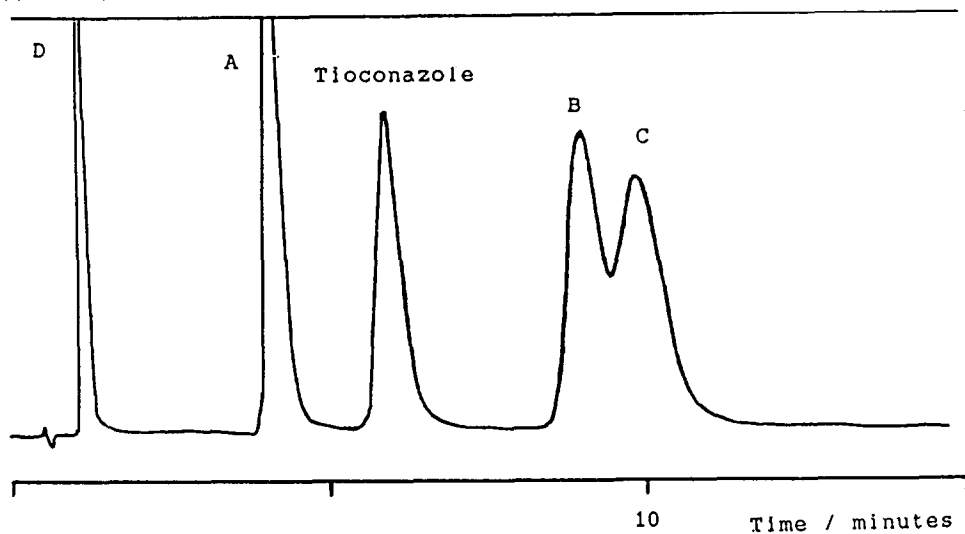

Fig. 5. Separation of tioconazole and related impurities for the 5-cm column. Mobile phase, methanol–acetonitrile–50 mM pH 4 TEA buffer (containing 25 mM 1-octanesulphonic acid) (31:19:50; v/v/v). Detection wavelength, 220 nm. Flow-rate, 1.5 ml min^{-1}.

TABLE IV

TEMPERATURE AND BUFFER LEVELS FOR INVESTIGATIONS OF INFLUENCE OF TEM-
PERATURE ON SEPARATION

Temperature (°C)	Proportion of buffer (%)	Capacity factors	
		B	C
50	55	20.0	22.1
40	55	27.4	30.6
30	55	40.5	46.0
50	50	10.9	11.9
40	50	13.9	15.4
30	50	19.7	21.9
50	45	6.4	6.9
40	45	8.0	8.8
30	45	10.4	11.5

calculate the eluent composition required to produce a capacity factor of 10 if the
separations were carried out at room temperature (25°C). To calculate these eluent
compositions, nine chromatograms with different buffer levels were run at different
temperatures to provide the necessary data. Second order equations of the form $y = ax^2 + bx + c$ (where y was equivalent to $\log k'$) were found to fit data both for buffer
level at a set temperature and for temperature at a set buffer level. To determine the
eluent composition required to produce a capacity factor of 10 for the last peak at
40°C, the equation for compound C linking $\log k'$ and buffer level at 40°C was
calculated. Substituting 10 for k' and solving the quadratic for the buffer level showed
the proportion of buffer required to be 46% (v/v).

Calculation of the eluent composition required at 25°C was more complicated.
The relationship between $\log k'$ for compound C and temperature was determined for
each buffer level. A temperature of 25°C was substituted into each function to calculate
a $\log k'$ value for that buffer level. Three $\log k'$ values (one for each buffer level) were
determined in this way. These data were fitted to a second order equation to describe
the dependence of $\log k'$ with buffer level at 25°C. Substitution of 10 for k' was again
used to determine the eluent composition required. It was found to be 44% (v/v).

These capacity factor data enabled a prediction of the column length required
for complete resolution. An analogous process to that described above was applied to
capacity factor data for compound B. The equations linking $\log k'$ and temperature for
each buffer level were determined and the $\log k'$ values at 25°C calculated for each one.
Fitting these data to a second order equation generated a function relating $\log k'$ to
buffer level at 25°C. At this point instead of substituting 10 for k', the proportion of
buffer producing $k' = 10$ for compound C was substituted into the equation and k' for
compound B calculated. The value of k' determined for compound B was 9.

In this way the k' values for both compounds B and C were known (9 and 10.
respectively) and allowed the prediction of the number of theoretical plates necessary
for complete separation of all components. The following resolution equation was
used for this prediction:

$$R_s = \frac{1}{4} \cdot \frac{(\alpha - 1)}{\alpha} \cdot N^{0.5} \cdot \frac{k'}{(1 + k')}$$

where R_s is the resolution between B and C, α is the ratio of k' between C and B, N is the number of theoretical plates, and k' is the average of k' for B and C. Complete resolution was assumed to correspond to an R_s value of 1.5.

Substituting the calculated values into the equation produced a value for N of 4400 plates. The length of column required was predicted, based on chromatograms where the 5-cm column generated 360 plates per cm, and found to be *ca.* 12 cm. To allow for a suitable excess the column length of 15 cm was selected.
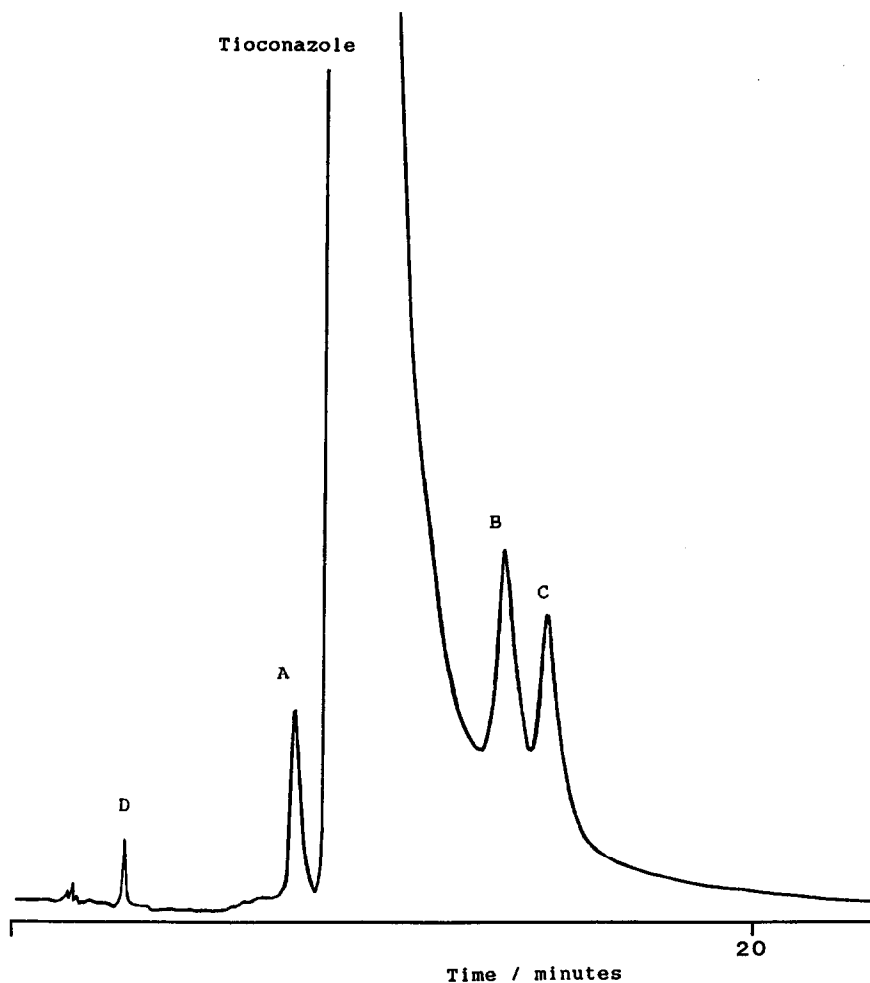

Fig. 6. Separation for the 15-cm column. Mobile phase, [methanol–acetonitrile (70:30, v/v)]–50 mM pH 4 TEA buffer (containing 25 mM 1-octanesulphonic acid) (54:46, v/v). Detection wavelength, 220 nm. Flow-rate. 1.5 ml min^{-1}.

Validation of method

On reducing the concentration of the related impurities to realistic levels, the question of peak tailing of the major component arises. The extent of this peak tailing prevents accurate determinations of compounds B and C (Fig. 6). This will be considered in a subsequent publication[13].

CONCLUSIONS

In the present work, the initial development criteria were fulfilled as the analysis time was reduced from 25 to less than 15 min, compounds B and C were adequately resolved, the mobile phase did not cause column damage, and the hydrolysis product could also be assayed.

The experimental scheme adopted, incorporating partial factorial designs, lattice mixture designs and sequential simplex has been demonstrated as being suitable for developing a HPLC separation for tioconazole and its related impurities.

ACKNOWLEDGEMENT

REFERENCES

1 *United States Pharmacopeia XXI*, Supplement 2, 1895, United States Pharmacopeial Convention, Rockville, MD, 1985.
2 J. C. Berridge, P. E. Last and R. V. Platt, *J. Pharm. Biol. Anal.*, 3 (1985) 391.
3 J. C. Berridge and K. S. Andrews, *Analyst (London)*, 109 (1984) 287.
4 J. P. Bounine, G. Guiochon and H. Colin, *J. Chromatogr.*, 298 (1984) 1.
5 J. L. Glajch, J. C. Gluckman, J. G. Charikofsky, J. M. Minor and J. J. Kirkland, *J. Chromatogr.*, 318 (1985) 23–39.
6 J. S. Kiel, S. L. Morgan and R. K. Abramson, *J. Chromatogr.*, 320 (1985) 313–323.
7 W. R. Melander, J. Stoveken and Cs. Horváth, *J. Chromatogr.*, 185 (1979) 111–127.
8 J. C. Berridge, *J. Chromatogr.*, 244 (1982) 1–14.
9 R. D. Snee, *CHEMTECH*, 9 (1979) 702–710.
10 J. L. Glajch, J. J. Kirkland, K. M. Squire and J. M. Minor, *J. Chromatogr.*, 199 (1980) 57–79.
11 J. L. Glajch, J. J. Kirkland and L. R. Snyder, *J. Chromatogr.*, 238 (1982) 269–280.
12 J. L. Glajch and J. J. Kirkland, *Anal. Chem.*, 55 (1983) 319A–336A.
13 A. G. Wright, A. F. Fell and J. C. Berridge, *Analyst (London)*, in press.