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Application of a comparative evaluation of several reversed-phase columns to the automated analysis of candidate pharmaceuticals

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Abstract

This report discusses the evaluation of several modern reversed-phase chromatographic packing materials, and describes the application of a selected column in a method used to screen the purities of pharmaceutical candidates and intermediates. Columns are first evaluated with an acetonitrile gradient using a test mixture containing acids, bases and compounds of widely differing polarities. Selected phases are further assessed by analysing many typical samples. Finally, the use of the chosen column and method are described and discussed.

1. Introduction

Reversed-phase HPLC analysis is subject to well-known problems with unwanted retention mechanisms due to reactions of certain analytes with the siliceous support [1]. Ion exchange and hydrogen bonding with residual silanols can occur, as well as participation from residual trace metals in the base silica. Our laboratory has employed a large number of methods for analysis of acids, bases and other highly polar compounds. Many different mobile phases were necessary to chromatograph these diverse compounds on commonly used columns such as Spherisorb ODS-2. Method selection was time-consuming and results often disappointing. Recently, several deactivated reversed-phase packing materials have become commercially available. A comparative study of some of these materials was made so that a single method for this work could be adopted.

2. Experimental

2.1. Instrumentation

Hewlett-Packard (Bracknell, UK) 1090 Series M liquid chromatographs were used. They were equipped with 90-position autosamplers, DR5 pumps, ovens operated at 40°C, and diode array detectors. HP 79988A Revision 5.3 foreground/background operating software (Pascal Series) or HPLC^{3D} ChemStation (DOS Series) software was used. One autosampler was cooled to 8°C by air blown from a heat-exchange unit through which ethylene glycol coolant at 4°C was passed using a Julabo (Leighton Buzzard, UK) F10 recirculating bath.

2.2. Columns

Columns evaluated had dimensions of 150 × 4.6 mm and were packed with 5-μm particle size

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reversed-phase silicas. The columns were: Spherisorb ODS-2 (Phase Separations, Deeside, UK), Hypersil BDS C_{18} (Shandon, Runcorn, UK), Inertsil ODS-2 and 250 mm Kromasil C_{18} (Capital HPLC, Broxburn, UK); Suplex pKb 100 and Supelcosil ABZ (Supelco, Saffron Walden, UK); Ultracarb 5 ODS 20 and Ultracarb 5 ODS 30 (Phenomenex, Macclesfield, UK); and Zorbax SB-C_{18} (Hichrom, Reading, UK).

2.3. Test mixture

The test mix used for initial column screening was prepared in acetonitrile–deionised water (1:1). Its composition is shown in Table 1. The components of this mix were purchased from BDH (Poole, UK) (Component Nos. 1, 2, 4 and 5) and Aldrich (Gillingham, UK) (Components Nos. 3, 6, 8 and 9) and were of at least general-purpose grade. 2-Hydroxy-5-methylbenzaldehyde was available within Glaxo Research and Development.

2.4. Compound library for detailed column screening

A collection was made of 86 RP-HPLC candidates providing a representative range of polarities, pK values and chemistries. These were all proprietary compounds prepared within Glaxo Research and Development during various research programmes.

Table 1
Composition of test mixes

No.	Component	Concentration (μg/ml)
1	Pyridine	690
2	Benzylamine	110
3	N-Acetylprocainamide · HCl	85
4	Benzyl alcohol	155
5	Phenol	120
6	4-Nitrobenzoic acid	220
7	2-Hydroxy-5-methylbenzaldehyde	65
8	4-Chlorocinnamic acid	105
9	Phenyl ether	100

2.5. Analysis conditions

The mobile phases were: *system 1*: A = 0.1% (v/v) phosphoric acid, B = 95% (v/v) acetonitrile + 0.1% (v/v) phosphoric acid; *system 2*: A = water, B = 95% (v/v) acetonitrile; *system 3*: A = 50 mM, pH 2.3 ammonium dihydrogenphosphate, B = 95% (v/v) acetonitrile.

These were prepared with deionised water, HPLC-grade acetonitrile, and analytical-reagent grade phosphoric acid. System 3 was filtered to remove salt particulates. To avoid the possibility of introducing contaminants, systems 1 and 2 were not filtered. All mobile phases were thoroughly degassed with high-grade helium before use.

The test gradients employed were: B = 0% (2 min) to 100% over either 20 or 40 min, then held at 100% for 10 min before returning to B = 0% over 2 min. A post time of 6 min was added, giving a total cycle time of either 40 or 60 min. Measurements were made at 215 nm and 40°C, using a flow-rate of 1.0 ml/min. Aliquots of 10 μl were injected. UV spectra were used to confirm peak identities.

2.6. Peak evaluation criteria

Peak width values at half-height (w_h) and symmetry (SYM) values reported by the HP1090 data system [2,3] were recorded for each of the main component peaks. A perfectly symmetrical peak has a SYM value of unity. Successful chromatography (a "hit") was arbitrarily defined by a w_h value of < 0.25 min.

2.7. Analytical protocol

Research compounds were analysed as $\leq 0.1\%$ (m/v) solutions in acetonitrile–water mixtures. Inertsil columns, protected with 20 mm guard cartridges, were used with the longer gradient to generate purity profiles. Many compounds only absorb at low wavelengths; thus for screening purposes the detector is set at 215 nm with a bandwidth of 10 nm to provide rapid initial purity assessments. This approach requires that the acid chosen (phosphoric) is UV trans-

parent and that all mobile phase components are of the highest quality. Quality control is maintained by running blanks and test mixes so that artefacts or column deterioration may be flagged rapidly.

3. Results and discussion

3.1. Initial column screening

Each column was evaluated by analysing the test mix using system 1 with the longer gradient. The results are shown in Fig. 1. The columns exhibit different activities towards bases, acids and metal chelators. Three columns having generally lower activities in this test (Inertsil, Kromasil C_{18} and ABZ) were selected for a more rigorous comparative evaluation with Spherisorb ODS-2.

3.2. Detailed column screening

The three selected columns were compared by analysing each of the 86 library compounds using 20-min gradients with mobile phase systems 1–3. The results are summarised in Table 2.

The w_h and SYM figures were only included for those compounds recorded as "hits" according to the peak evaluation criteria. The three selected materials each performed significantly better than Spherisorb ODS-2, which failed to satisfactorily chromatograph approximately 40% of the test compounds. In contrast, provided the mobile phase was acidified (system 1), Inertsil and Kromasil had a 99% success rate, and ABZ also performed very well. The single failure on Inertsil was due solely to the lipophilicity of the compound concerned and a slight extension of the isocratic period at 100% B resulted in satisfactory elution. It therefore does not indicate activity of the column towards the analyte. Compounds failing on Spherisorb ODS-2 generally had highly basic, acidic, ionic or polar natures; this observation agrees with our long-term experience of this material used with systems 2 and 3. The poor symmetry values of peaks recorded for "hits" are a further indication of its activity. Some residual activity is still apparent on the other three phases, and for each column an acidic mobile phase (system 1) is essential to maximise the chance of successfully running a novel compound. Inertsil provided significantly better w_h and SYM values than Kromasil and was therefore selected as the analytical packing material of choice from amongst those tested.

An independent comparison of several modern reversed-phase columns was published by McCalley [4] after completion of this work. In this case too, Inertsil was the best of the materials evaluated.

3.3. Selection of gradient conditions

We required a rugged, simple, high-throughput single procedure giving high information content and high-quality information. These criteria can be best met by a gradient approach if the method is to be capable of addressing the wide polarity range of samples and their (unknown) impurities [5]. This approach maximises the proportion of novel samples that can be successfully analysed.

A brief examination was made of the effect of different gradients on the resolution. Formal optimisation using computer simulation [6] was not attempted since each sample represents a potential set of unknown compounds. The test mix was run on an Inertsil ODS-2 column with system 1 using different gradient (GT) and cycle (CT) times, and the apparent resolution (R_s) between benzyl alcohol and phenyl ether was noted:

$$R_s = 1.177(t_{R1} - t_{R2})/(w_{h1} + w_{h2})$$

where t_{R1} and t_{R2} are the retention times in minutes of benzyl alcohol and phenyl ether, respectively, and w_{h1} and w_{h2} the corresponding peak widths at half height.

Table 3 demonstrates the loss of resolution with decreasing gradient time. Long gradient times produce a 4-fold increase in resolution, although less resolution per unit GT. However, because it is necessary to maintain realistic isocratic and re-equilibration periods, a more

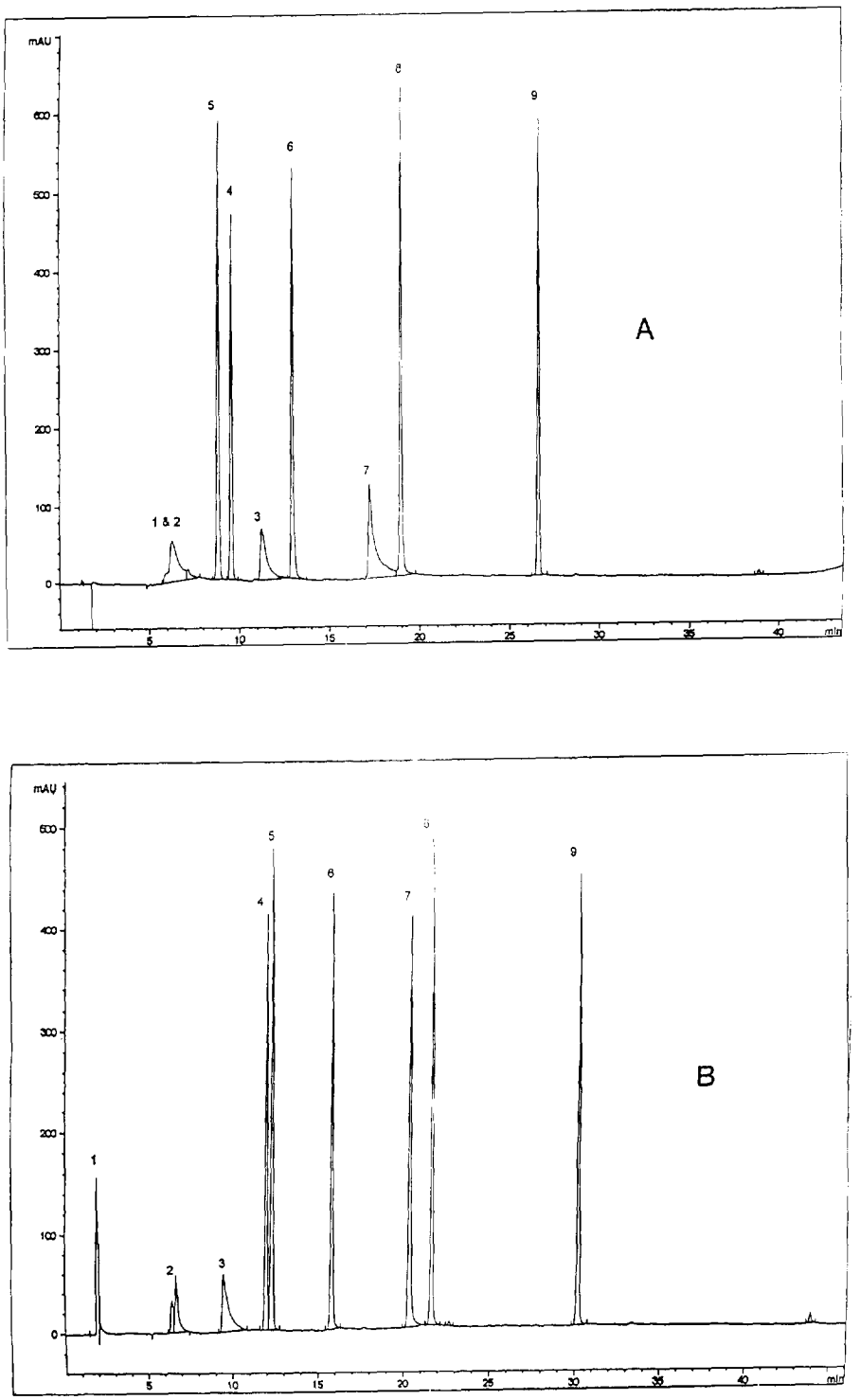
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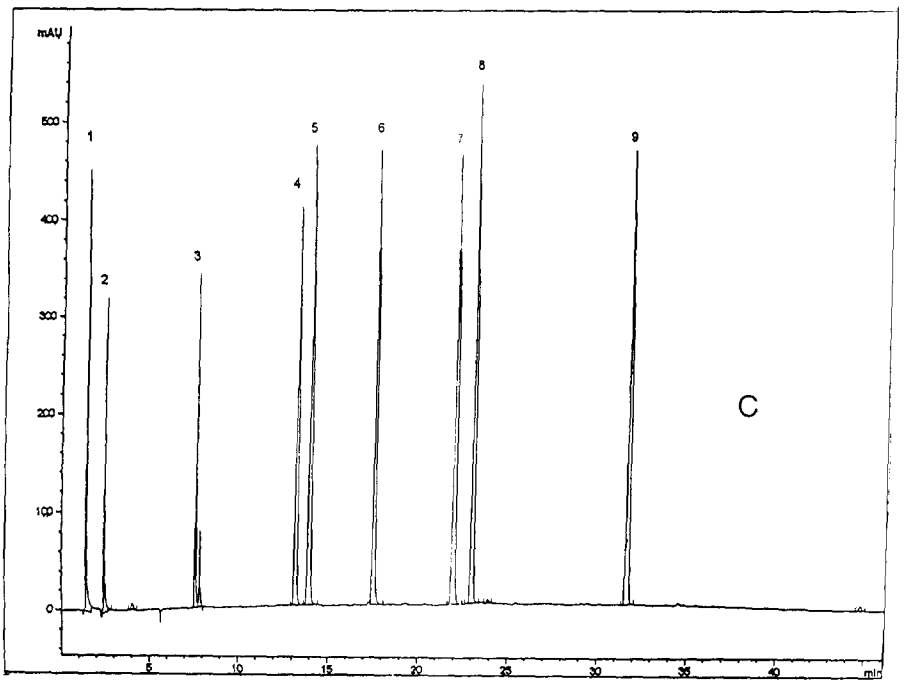
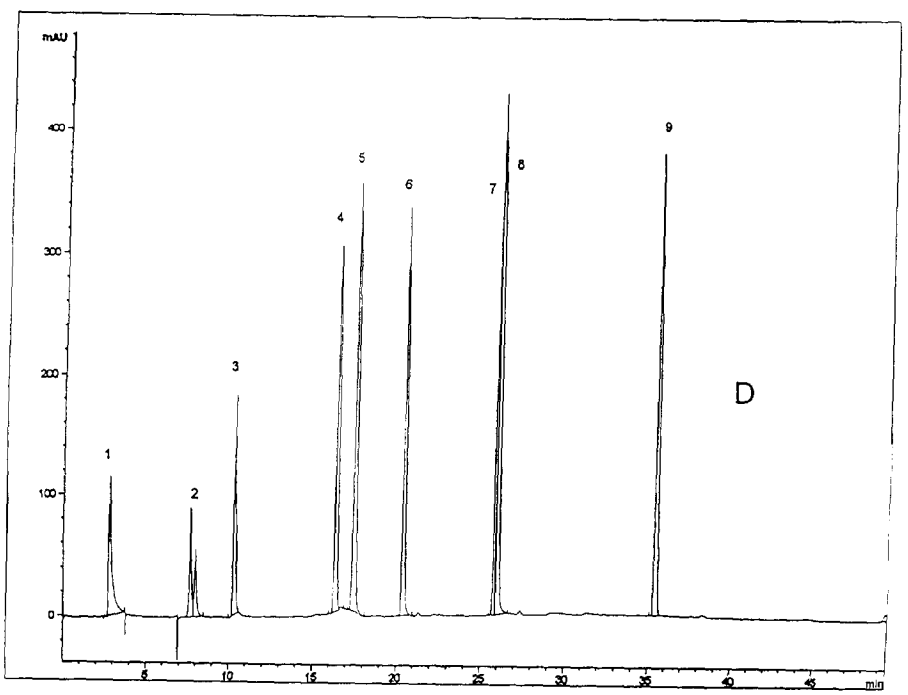
Fig. 1.





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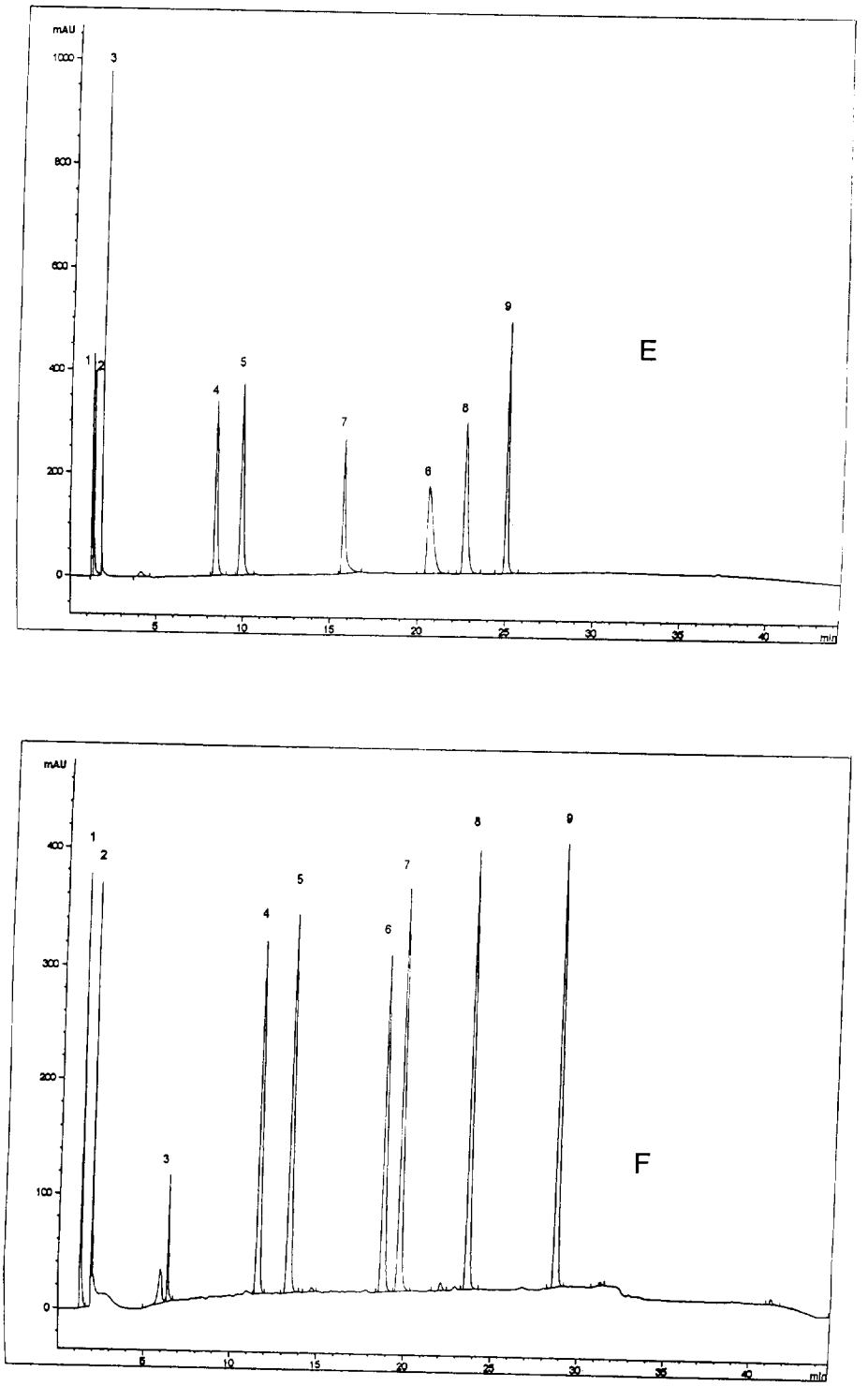
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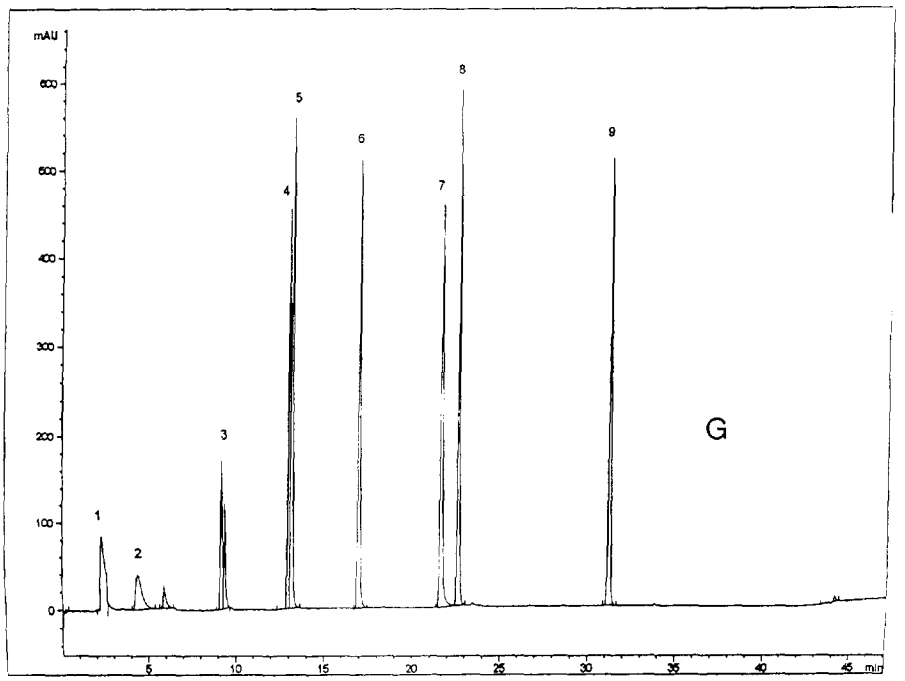

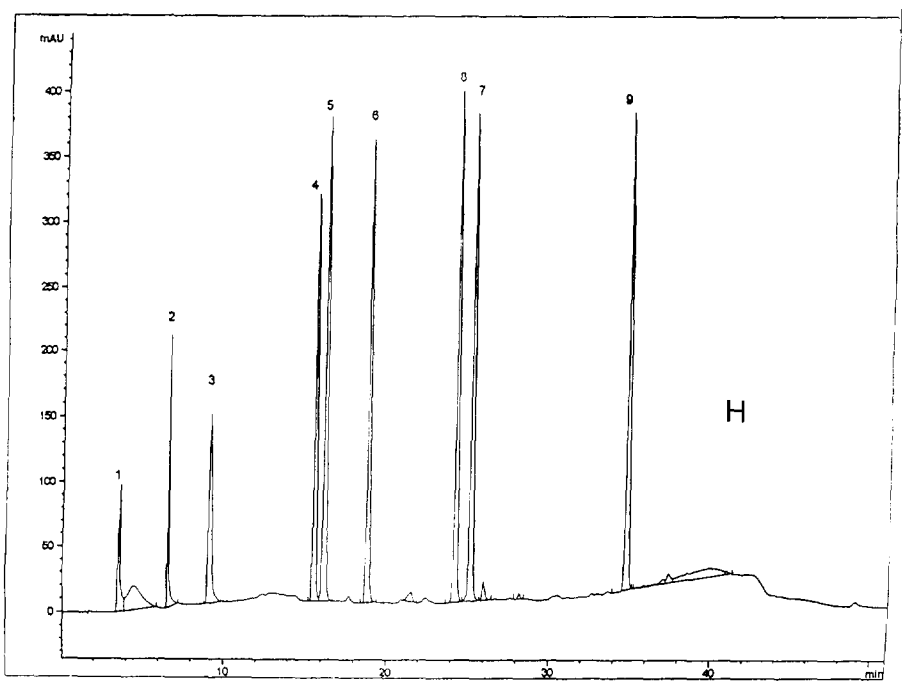

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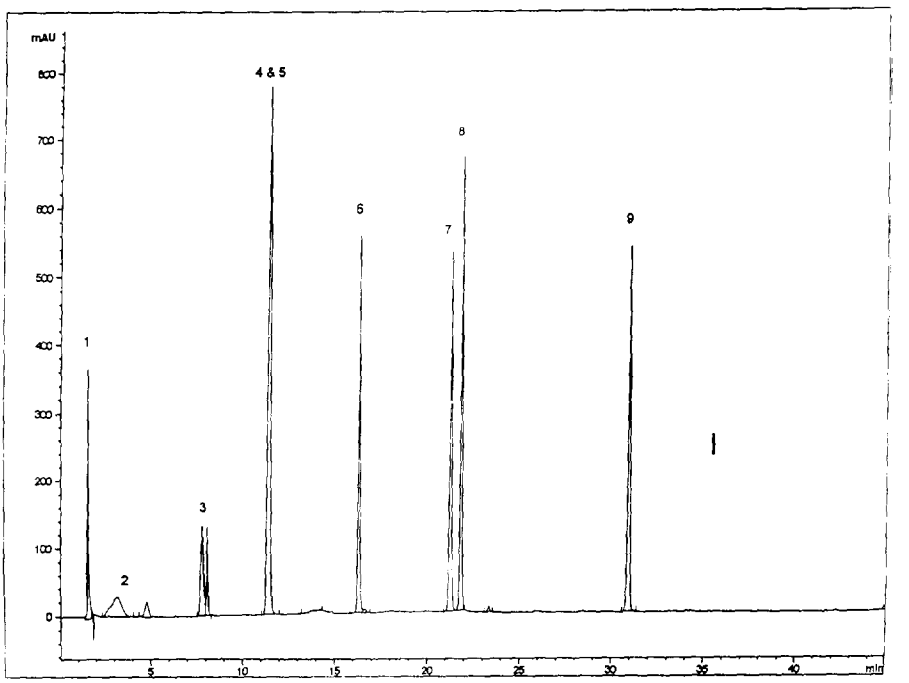

Fig. 1. Chromatograms of the test mix obtained on candidate columns. For conditions see Experimental section. Peak Nos. refer to identities given in Table 1. (A) Spherisorb ODS-2; (B) Hypersil BDS C_{18}; (C) Inertsil ODS-2; (D) Kromasil C_{18}; (E) Suplex pKb-100; (F) Supelcosil ABZ; (G) Ultracarb 5 ODS 20; (H) Ultracarb 5 ODS 30; (I) Zorbax SB-C_{18}. Usually 2 to 4 injections were made to ensure consistent chromatography. Note that generally only one (new) column of each type was tested and that it would be incorrect to infer from these results that any one commercial packing material is necessarily superior to any other.

relevant comparator is resolution per unit CT, and this is almost constant for gradients of 15 to 45 min. The choice of gradient then depends on a subjective judgement of how much resolution can be sacrificed. Fig. 2A and B demonstrate the loss of information content incurred around the major peak when using a 20-min gradient instead of one of 40 min. Comparison with isocratic conditions (Fig. 2C), selected such that the capacity factors exceeded 10, shows that loss of resolution of materials having closely similar retentions is acceptable when GT = 40 min,

Table 2
Comparison of candidate column performances

Column	System	Hits	Mean w_h (±R.S.D., %)	Mean SYM (±R.S.D., %)
Spherisorb ODS-2	1	58	0.089 (± 45)	0.77 (± 71)
Spherisorb ODS-2	2	47	0.101 (± 47)	1.29 (± 145)
Spherisorb ODS-2	3	55	0.094 (± 33)	0.81 (± 56)
Inertsil ODS-2	1	85	0.088 (± 29)	0.89 (± 38)
Inertsil ODS-2	2	45	0.109 (± 31)	0.89 (± 45)
ABZ	1	77	0.081 (± 29)	0.78 (± 32)
ABZ	2	50	0.091 (± 34)	0.97 (± 71)
Kromasil C_{18}	1	85	0.106 (± 21)	0.68 (± 21)

Table 3
Effect of gradient and cycle times on resolution (R_s)

GT	CT	R_s	R_s/GT	R_s/CT
5	18	20	4.1	1.2
15	28	51	3.3	1.8
25	38	68	2.7	1.8
35	48	79	2.2	1.6
45	58	86	1.9	1.5

GT is the time in minutes between 0 and 100% B. CT is the time between injections, allowing 2- and 4-min isocratic periods at the respective extremes of the gradient to ensure representative chromatography of both hydrophilic and lipophilic materials. CT also includes 2 min to return to 0% B, and a 5-min re-equilibration time before each injection.

whereas when GT = 20 min it is not. This point is the more relevant when one considers that the impurities most likely to be present will often be isomers of the parent compound or other closely related compounds.

A GT of 40 min was therefore selected, and isocratic and equilibration times adjusted as detailed in the Experimental section to ensure elution of most lipophiles and to obtain a CT of 1 h.

3.4. Selection of mobile phase system

It is clear from the comparisons made using the compound library that an acidic mobile phase, or possibly one of high ionic content, is necessary to ensure successful chromatography of most routine samples. The mobile phase advocated (system 1) has the advantage over our previously employed standard acidic system (system 3) of allowing high acetonitrile concentrations to be used without causing precipitation of buffer salts. Experience with about 1250 different samples indicates that the vast majority of acidic analytes are ion-suppressed in this buffer system, and that most bases are effectively converted to their conjugate acids.

3.5. Sample stability

A possible source of failure is the stability of the sample, either during storage in the autosampler before the analysis or during the run. These matters are treated separately below.

An extensive evaluation of solution stability was made with a collection of about 200 different samples that represented several distinct synthetic lines and chemical classes. Sets of about 10–15 individual samples were each run twice by immediate repetition of the sequence. No evidence of significant solution instability was found. Currently each sample is analysed once only and the added precaution is taken of cooling the sample solutions to 8°C whilst they await analysis. Potentially unstable solutions are given priority and/or re-analysed after a defined time in solution. No serious problems have occurred; but on three or four occasions samples have precipitated before injection.

Samples with acid-labile protecting groups are quite common. For most compounds studied to date, on-column acid hydrolysis does not seem to represent a problem. Although the mobile phase pH is quite low (around 2), sample solutions are typically 0.1% (m/v) or less, and contact time at 40°C with acid is limited to the retention time since samples are dissolved in non-acidified water–acetonitrile mixtures. If the stability of a particular sample is in doubt, a run using system 2 can be informative. It is also possible to check for acid lability by use of micro-preparative chromatography. This entails injection of a suitable aliquot of a concentrated sample solution and collection of the fraction containing the major component. Re-injection of this fraction then reveals any artefacts due to on-column decomposition. In practice, very few examples of serious incompatibility with system 1 have been identified in cases where there was good a priori reason to question stability towards the conditions used. However, an instrument employing system 2 is available for acid-labiles and to screen potential preparative applications for omission of buffer additives. More rigorous criteria would be required to test candidate pharmaceuticals against specifications applied in, for example, clinical trial work. The system reported here is quite adequate to provide initial impurity profiles of research compounds.

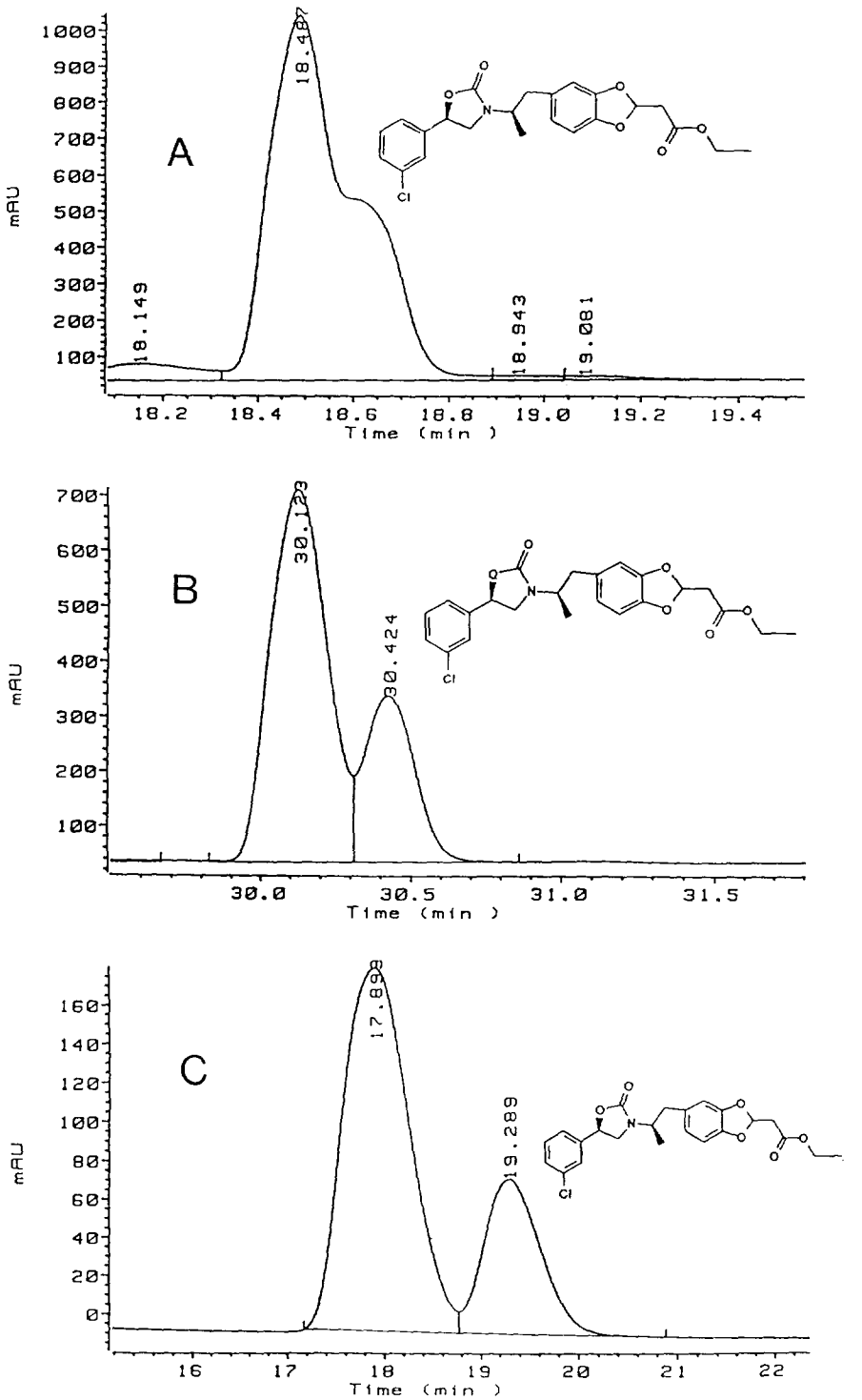

Fig. 2. Analysis of an approximately 2:1 mixture of diastereomers on an Inertsil column. Mobile phase: system 1 with (A) a 20-min gradient; (B) a 40-min gradient; and (C) isocratically with 50% (v/v) acetonitrile.

3.6. Protocol

Current practice is to run two blanks and a sample of the test mix each day. This provides a visual check for artefactual, "system" peaks, and a record of column performance. Monitoring the blank runs helps evaluate different sources of supply of the mobile phase components and hence to further reduce background signals. Mean retention times of benzyl alcohol and phenyl ether had respective R.S.D.s of ± 0.3 and $\pm 0.5\%$ when monitored for a month. Trends in parameters such as retention times, peak areas, w_h and SYM advise the operator to change the either the test mix, precolumn or main column. All our ODS columns, whether Inertsil stock or alternatives under evaluation, are assessed with the test mix and the reported method. It is also intended to use the test mix in a study of minituarised systems.

4. Conclusions

There are many excellent deactivated C_{18} materials commercially available. Examination of several of these materials shows significant differences in their selectivities and activities towards selected probes. Further evaluation of three columns resulted in identification of one that was particularly suitable for routine analysis of many RP-amenable candidate pharmaceuticals and synthetic intermediates.

The selected column has been used in an automated procedure that incorporates performance checks. Successful analysis of about 95% of 1250 compounds was obtained during the preceding year. Rapid provision of high quality impurity profiles enables immediate assessment of the suitability of samples for purposes such as submission to biological screens or use in synthetic schemes. Benefits to the analyst include method simplification, ease of data storage and retrieval, and significant savings of time, solvents and instrumental requirements.

Deactivated, high-purity ODS-silica packing materials are recommended as the initial choice for new reversed-phase applications.

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