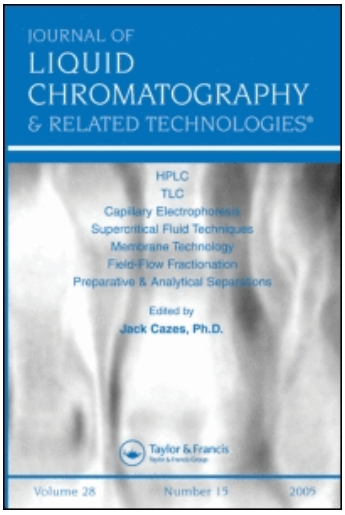

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AN EVALUATION OF COLUMN-TO-COLUMN RETENTION VARIABILITY FOR COMPOUNDS RELATED TO PANADIPLON (U-78,875) USING THREE DIFFERENT REVERSE-PHASE HPLC SEPARATION MODES

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ABSTRACT

In order to assess the potential for substituting one brand of column for another in the analysis of multiple analytes, panadiplon (U-78,875) and eight of its related compounds were examined with respect to relative retention and peak shape on six different reversed-phase C8-bonded silica columns in the isocratic and gradient separation modes, and on six different bare silica columns in the dynamically modified silica (DMS) mode. No two columns in any separation mode showed identical retention behavior for all nine analytes. Gradient chromatography demonstrated generally more uniform relative retention for all columns than either the isocratic or the DMS mode, which is largely attributable to a reduction in time available for partitioning as a consequence of increasing solvent strength. DMS may offer improvements over isocratic reversed-phase HPLC in reproducibility for multiple analytes containing either alkyl homology or single, strongly interactive groups. In general, gradient chromatography provides the greatest immunity from column-to-column variability for analysis of multiple analytes.

INTRODUCTION

An issue of prime importance in the development of rugged HPLC methodology is the consistent performance of columns. The establishment of specificity for all compounds of interest entails not only the purity of peaks represented by those compounds, but an examination of multiple lots of HPLC columns and column packing material. During the lifetime of an HPLC method, changes in a manufacturer's column production methodology can have a serious impact on the performance of the method (relative retention, peak shape). This provides a great incentive to develop methods that are less dependent on column variability, as the column is the most significant component of an HPLC system that is not under a developer's control.

There are three commonly used strategies that have been employed to reduce column-to-column variability for multiple analytes. In the case of basic compounds, addition of small amounts of an amine modifier to the mobile phase can improve poor retention time reproducibility and peak shape due to analyte interaction with residual silica silanols[1-4]. Sadek and Carr[4] demonstrated that certain amines are much more efficient at covering residual silanols than others; improvement in analyte retention characteristics is also very analyte-dependent.

A second strategy that could be employed to obtain improved analyte retention behavior is dynamically modified silica (DMS). By adding a long-chain quaternary amine salt to an organo-aqueous mobile phase near neutral pH (see Reference 5), underivatized silica is dynamically coated. This coating is effective at shielding many of the partially ionized silanol sites on the silica surface, producing a hydrophobic layer similar to the bonded-phase chains of alkyl bonded silica. Several workers have investigated the effects of dynamic modifier identity and concentration[5-11], buffer pH[8-9,11], organic modifier identity and concentration[8-11], and stationary phase parameters[5] for DMS. Reference 12 reviews the use of DMS as a general technique for improving the reproducibility of column selectivity in RP-HPLC. It was anticipated that the use of dynamically modified silica

could also improve the fidelity of retention for compounds related to U-78,875.

For gradient HPLC, it is likely that the change in solvent strength during a chromatographic run might be effective in controlling the time of elution, and, indirectly, could mitigate differences in retention caused by differences in column chemistries. Since an increase in solvent strength "forces" the analyte from the column, the net effect might be a diminished dependence on column-specific retention mechanisms. This was the rationale for examining different bonded-phase packings in the gradient elution mode.

The objective of this work was to examine three different reversed-phase HPLC separation modes for impurities and degradation products of a developmental drug candidate (panadiplon, U-78,875) to determine which, if any, is less dependent upon variation of the chromatographic stationary phase. The modes examined were: 1) isocratic; 2) dynamically modified silica (DMS); and 3) gradient.

MATERIALS

Instrumentation

The chromatographic equipment consisted of a Hewlett-Packard 1090L HPLC system, configured with a Waters WAVS® Box to switch 3 columns during one run. An external LDC UVMonitor D detector with cadmium lamp and 229 nm filter was used. Mixtures of U-78,875 and eight of its related compounds (either two or three individual compounds per vial; Figure 1) were prepared in concentrations sufficient to give peak responses of at least 0.1 AU, in either mobile phase (isocratic or DMS) or dimethylformamide (gradient).

Columns

For the isocratic and gradient separation modes, the same C8-bonded, 250 X 4.6 mm i.d columns were compared. These were: Zorbax R_x-C8 (serial

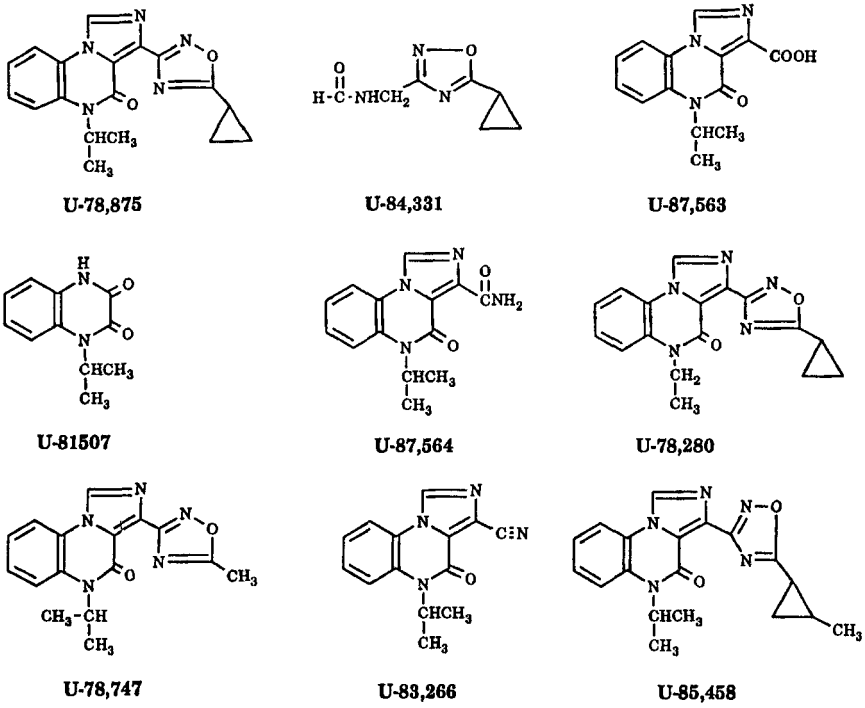

Figure 1. Structures of Panadiplon (U-78,875) and related compounds.

number AU2932); YMC A-203-5 C8 (s/n 4252672); Spherisorb C8 (s/n C2478); Zorbax C8 (s/n L15786); Nucleosil C8 (s/n 051789R-P); and Jones Apex C8 (s/n 4M25325F/38703). For the DMS mode, the columns compared (250 X 4.6 mm i.d.) were: Zorbax SIL (s/n B6898); YMC Silica (s/n 4255489); Spherisorb Silica (s/n 07240KP); Nucleosil Silica (s/n 07240MP); Jones Apex Silica (s/n 4M25300/44152); and Lichrosorb Silica (s/n 070985-C). All columns were packed with 5 micron particle size, silica-based material. Table 1 gives pore size, surface area, and carbon load (where applicable) for the column types used. In all cases, the same batch of mobile phase was used for comparison of six columns under each separation mode, and duplicate injections of compound solutions were made.

TABLE 1
Characteristics of 5 Micron Bare and C8-Bonded Silicas Used in this Work (from Manufacturers' Literature). All Columns were 250 mm long X 4.6 mm i.d.

Column Type	Pore Size (Å)	Surface Area (m^2/g)	Carbon Load (%)
Zorbax Rx-C8	80	180	5.5
Zorbax C8	70	300	12
Zorbax SIL	70	300	--
YMC C8	120	*	10
YMC Silica	120	290	--
Spherisorb C8	80	220	6
Spherisorb Silica	80	220	--
Nucleosil C8	100	350	9
Nucleosil Silica	100	350	--
Jones Apex C8	100	170	7
Jones Apex Silica	100	170	--
Lichrosorb Silica	60	550	--

*Surface Area for Bonded-Phase material not given

METHODS

Isocratic

For comparison of columns in the isocratic mode, the mobile phase was prepared by mixing 350 ml of acetonitrile (Baxter Burdick and Jackson) and 650 ml of DI water, adding 2.0 ml of N,N-dimethyloctylamine (DMOA; Ames Laboratories) and 20 mg of 1,4,8,11-tetraazacyclotetradecane (CYCLAM; Aldrich Chemical). While stirring magnetically, the pH to is adjusted to 7.5 $\pm$ 0.1 with 85% phosphoric acid. The flowrate was 1.0 ml/minute and injection volume 10 µl for the isocratic system.

Dynamically Modified Silica

For the DMS system, the mobile phase consisted of 100 mM KH_2PO_4 (Mallinckrodt), pH 6.5 buffer:Acetonitrile:n-Butanol (Baxter Burdick and

Jackson), 830:140:30, containing 10 mM Dodecyltrimethylammonium Bromide (Aldrich, 99%). A flowrate of 1.0 ml/minute was used with the DMS system, with 20 µl injections.

Gradient

Gradient mobile phase A consisted of 950 ml of water, 50 ml acetonitrile, and 20 mg CYCLAM, with pH adjusted to 7.0 with a dilute (1:20) aqueous solution of phosphoric acid, while mobile phase B consisted of 950 ml of acetonitrile and 50 ml of water. The gradient method injection volume was 20 µl and flowrate 0.8 ml/minute, with the following program:

Time (min.)	%A	%B
0.0	95	5 (isocratic)
4.5	95	5 (begin initial gradient)
11.5	65	35 (begin shallow gradient)
47.5	50	50 (begin final gradient)
59.5	5	95 (isocratic)
64.5	5	95 (begin down ramp)
66.5	95	5 (begin equilibration)
76.5	95	5 (isocratic equilibration)

Data Collection

Void time was deemed to be the first full inflection (maximum or minimum) of the baseline. Peak integration, relative retention, and tailing factor were calculated using the in-house software developed by Upjohn Control; USP definitions were used.

RESULTS AND DISCUSSION

Isocratic Chromatography

Figure 2 shows the retention behavior of these compounds on the six C-8 bonded phase columns investigated, using isocratic conditions. It is apparent that there is little similarity in retention behavior for all nine

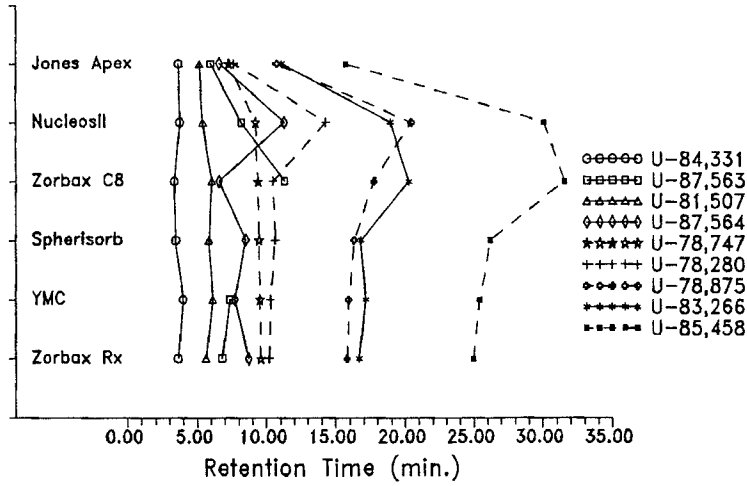

Figure 2. Isocratic retention times for six columns.

compounds for the column set as a whole; only the Zorbax Rx, YMC, and Jones materials maintain the same elution order. There is a total of nine retention order reversals within this set of columns; U-87,563 (a carboxylic acid) did not give a well-formed peak on the Spherisorb column. The variability (percent RSD for retention relative to U-78,875) of retention ranges from 7.5% for U-85,458 (most retained) to 48.4% for U-84,331 (least retained); these values lie in the same approximate range as observed by Hansen et al.[13] for comparison of bonded-phase columns. Peak shape (Table 2) was acceptable for most compounds except those having either strong hydrogen-bonding character (the primary amide U-87,564) or ionic character (carboxylic acid U-87,563, and enolizable amide U-81,507). These three analytes could be expected to chromatograph poorly for some column packings, at the relative high pH (7.5) of this mobile phase, due to a partial ion exchange retention mechanism on unshielded silanols.

Dynamically Modified Silica

Figure 3 shows the relative retention variability and asymmetry for DMS chromatography. The overall precision in relative retention for these

TABLE 2
Asymmetry Expressed as USP Tail Factor for U-78,875 and Eight Related Compounds Using Three Different Reverse-Phase Separation Modes

COMPOUND	Isocratic		DMS		Gradient	
	range	mean	range	mean	range	mean
U-78,875	1.1-1.3	1.2	1.0-1.5	1.3	0.9-1.5	1.2
U-84,331	0.8-2.1	1.6	1.2-1.4	1.3	0.9-1.5	1.3
U-81,507	1.6-6.1	3.3	1.2-1.7	1.4	1.2-4.5	2.3
U-87,563	1.7-4.0*	2.5	1.2-2.8	2.0	0.7-3.8	1.7
U-87,564	1.4-5.0	2.3	0.9-1.4	1.1	1.2-2.0*	1.6
U-78,747	1.2-3.2	1.6	1.1-1.4	1.2	0.9-1.7	1.4
U-78,280	1.1-1.4	1.3	0.9-1.3	1.1	0.9-1.8	1.3
U-83,266	0.8-1.3	1.1	0.9-1.4	1.2	0.8-1.2	1.0
U-85,458	1.0-1.2	1.1	1.0-1.7	1.3	1.0-1.6	1.3

*(n=5) - No data for Spherisorb due to poor peak shape

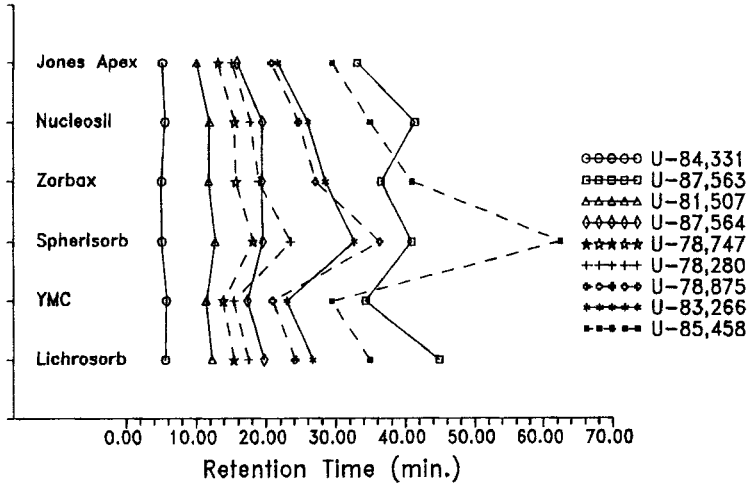

Figure 3. DMS retention times for six columns.

compounds was somewhat better in this separation mode than with bonded-phase chromatography, but there are six retention order reversals: four for the Spherisorb silica; and for both the Zorbax and Spherisorb silicas in the case of the acidic U-87,563.

Hansen et al[13] used DMS to show much improved peak symmetry and a much improved fidelity of relative retention for eleven tricyclic amine drugs. In that study, the percent RSD for retention of ten such compounds (relative to imipramine) was uniformly less than ten percent, across an eleven-column set of silica materials. While all of the compounds of the present work contain nitrogen, none of them are expected to be cationizable, as were the nitrogens of the compounds compared in Reference 13. While it is difficult to rationalize the difference in DMS retention behavior between the tricyclic amine drugs and U-78,875-related compounds, one hypothesis might be that the ionizable nitrogens confer an electron-rich center on the tricyclic compounds that is not present (at least, to the same degree) in U-78,875-related compounds. If these centers allow the analytes to partition in a manner different from U-78,875-related compounds, one might explain

the difference in column-to-column retention variability between Reference 13 and the present work.

The one aspect of chromatography that the DMS separation mode controlled quite well for these compounds was peak shape. All 9 compounds provided peaks with USP tailing factors of at most 2.8 (shown by U-87,563 on Spherisorb silica); likewise, there was no instance of peak shape so poor that a retention time was not assignable, as was the case for the other two separation modes examined. The improvement in peak shape for DMS confirms the observations of Hansen et al[5,12].

Gradient Chromatography

Figure 4 shows that gradient elution, just as the other two elution modes studied, does not prevent retention order reversals; however, it reduces the number of reversals to a minimum of four (without the information of amide U-87,564 retention, which gave no recognizable peak in this mode for the Spherisorb column). It also reduces the variability in retention across all columns to a level below that observed for either of the other two separation modes. U-87,563 was the only compound investigated for which the percentage RSD for retention relative to U-78,875 was greater than 10%. As this compound is a carboxylic acid (expected to be ionized at the pH of the mobile phase), there is the possibility of mixed retention mechanisms (ionic as well as hydrophobic).

Comparison of the Three Separation Modes

The dashed lines in Figures 2-4 show the subset of compounds differing only in aliphatic substitution. These seem to "track" very well across the column set for DMS mode (Figure 3), and the percentages RSD for retention relative to U-78,875 for these compounds (U-78,747, U-78,280, and U-85,458) are all below 10%. This suggests that alkyl homology does little to change the retention mechanism, in this separation mode. There are undoubtedly differences in adsorption capacity of the silicas for long-chain quaternary compound[14], which is a likely determinant of the general

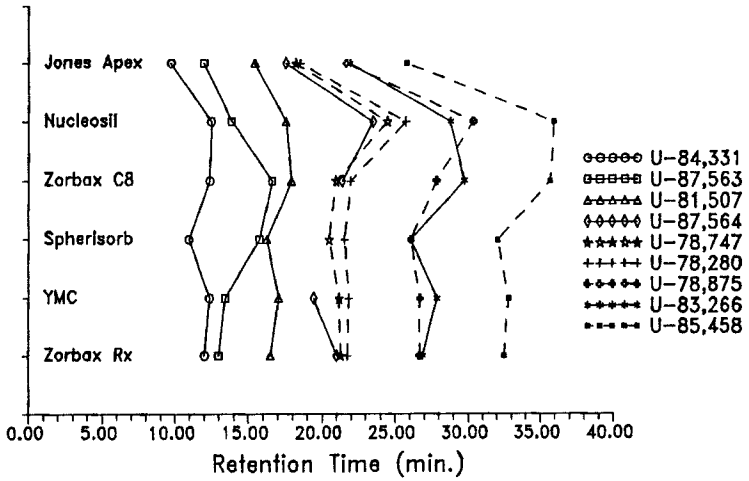

Figure 4. Gradient retention times for six columns.

differences in retention of the alkyl homologues across the column set. The "tracking" of retention for the alkyl homologues across the six columns is not so strong for bonded phase chromatography (Figures 2 and 4) as for DMS (Figure 3), possibly due to differences in the nature of hydrophobic interaction of the alkyl homologues with alkyl chains on the C8-bonded stationary phases. That the alkyl homologues retain more predictably in the DMS mode than in the C8-bonded modes suggests that greater control of hydrophobic aspects of retention may be possible for DMS chromatography than for C8-bonded chromatography. These bonded phases are known to have a variety of carbon loads; overall geometric differences between them are likely.

The better precision of relative retention for gradient chromatography than for the other two separation modes is probably due largely to "compression" of the time available for partitioning as the mobile phase strength is raised. Figure 5 plots the variation in retention relative to U-78,875 on each set of six columns. There is less variation in the case of each compound in the gradient mode than in the DMS mode, while the DMS

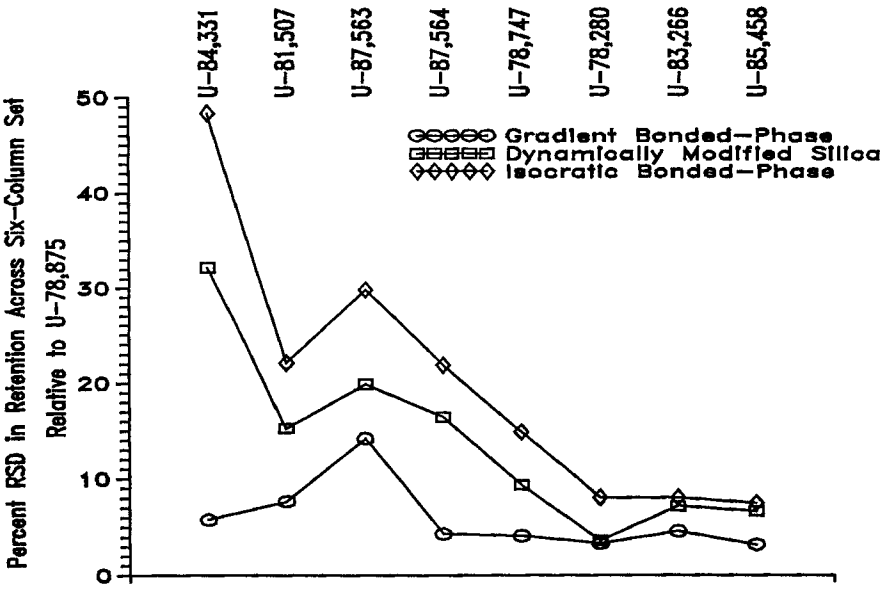

Figure 5. Variability of retention for eight compounds relative to U-78,875 in three different HPLC separation modes.

mode is more uniform in relative retention than the bonded-phase isocratic mode. The reduced variation between gradient and isocratic chromatography should be attributable in part to the influence of increasing elution strength in mitigating bonded-phase packing differences. The variation in DMS isocratic chromatography is intermediate between the bonded-phase isocratic and bonded-phase gradient modes, which suggests that differences in silica are still very important.

There is only a slight general improvement in peak shape for gradient chromatography over that observed isocratically, most likely also due to the "compression" effect of a gradient. While the DMS mobile phase pH was somewhat lower than that of the isocratic mobile phase pH (6.5, rather than 7.5) much better symmetry was observed for the hydrogen-bonding U-87,564 and acidic U-81,507 and U-87,563 in the dynamically modified silica mode than either of the bonded-phase modes.

CONCLUSIONS

An investigation of the elution behavior of U-78,875 and eight related compounds with isocratic and gradient reversed-phase, and dynamically modified silica HPLC has shown that none of these separation modes show identical retention behavior for all compounds investigated on any pair of the six packings investigated for any mode. The spectrum of compounds that one might encounter in an arbitrary impurities or metabolite mixture for any drug substance will probably have a wide range of acidity and/or oxidation state, and will be chemically more heterogeneous than the majority of compounds investigated for DMS in previous studies[5-12]. DMS appears to be most useful for standardization of separations where only alkyl homologues are examined; or for mixtures of compounds exhibiting single, strong retention mechanisms.

Gradient chromatography did not prevent retention order reversals for the present study, but it reduced the overall variability between packings due to its ability to control the time of elution via increasing solvent strength, and may be an improvement over either isocratic reversed phase or DMS, for an arbitrarily chosen compound mixture. If resolution of multiple components is not critical (as in single-analyte assays), it may be possible to replace any of a number of columns for the intended column, if the assay has been designed to be sufficiently rugged. This work suggests that, for resolution of multiple analytes of varying structure, gradient chromatography promises greater immunity from unforeseen changes in HPLC column production methodology than either isocratic reversed-phase or dynamically modified silica chromatography.

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