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COMPARISON OF THE REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATIONS OF FLUOROPYRIMIDINES, PYRIMIDINES, AND PURINES

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SUMMARY

The reversed-phase, high-performance liquid chromatographic separation of fluoropyrimidines, pyrimidines, and purines was investigated under isocratic conditions at ambient temperature. The performance of nine analytical, commercially available columns with five mobile phases is compared, and capacity and resolution factors are reported. The variables determining resolution are discussed, and the systems accomplishing the desired separation of fluoropyrimidine and pyrimidine bases and nucleosides are described. The best chromatographic results are achieved by using Spherisorb ODS-2 as stationary phase and 0.05 *M* monobasic ammonium phosphate (pH 3.5) as mobile phase.

INTRODUCTION

The pyrimidine antimetabolite 5-fluorouracil (5-FU) has been in clinical use for many years. Recently, interest has grown in the use of the naturally occurring nucleoside thymidine (TdR) to modulate the effects of 5-FU [1]. To determine biochemical and pharmacological changes in the concentrations of naturally occurring pyrimidines and fluoropyrimidines, it was necessary to develop a

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rapid and specific method of separating and quantifying these compounds in biological fluids and tissue specimens.

High-performance liquid chromatography (HPLC) has been widely applied to the separation of nucleic acid bases and nucleosides. Originally, ion-exchange HPLC [2—6] was used, but subsequently reversed-phase HPLC [7—16] has proven to be particularly suitable. The reversed-phase techniques employed paired-ion chromatography [11], gradient elution [6, 7, 9, 12, 14,16], and isocratic conditions [8, 10, 13, 15], but none of these methods attempted to separate fluoropyrimidines at the same time. The separation of halogenated uracil derivatives from other bases and nucleosides was achieved by thin-layer chromatography on silica gel [17]. Reversed-phase HPLC allowed the separation of 5-FU and its nucleosides [18—21]; however, the resolution of uracil (U) and 5-FU has remained problematic.

We investigated the HPLC characteristics of nine analytical, reversed-phase columns with five different solvents under isocratic conditions in order to achieve optimal resolution. A rapid, isocratic method was developed that separates all major pyrimidine and fluoropyrimidine bases and nucleosides.

EXPERIMENTAL

Chromatography

All reversed-phase analyses were performed with a Waters Assoc. (Milford, MA, U.S.A.) Model 204 liquid chromatograph equipped with a Model M6000A pump, a variable-wavelength UV detector (Varian Varichrom) set at 260 nm and a Varian recorder (Model 9176). Peak areas were determined by electronic integration (Varian Model CDS-111). The following analytical reversed-phase columns from Waters Assoc. were evaluated for separation: μBondapak C_{18} high efficiency (300 mm × 3.9 mm I.D., 10 μm particle size), μBondapak Phenyl (300 mm × 3.9 mm, 10 μm particle size), Radial-Pak C_{18} cartridge (100 mm × 8 mm I.D., 10 μm particle size), Radial-Pak C_{18} (100 mm × 8 mm, 5 μm particle size), and Radial-Pak C_8 (100 mm × 8 mm, 10 μm particle size). A Waters radial compression module (RCM-100) was used for all Radial-Pak liquid chromatography cartridges. Four reversed-phase Spherisorb columns from Custom LC (Houston, TX, U.S.A.) were tested: Spherisorb Hexyl (150 mm × 4.6 mm I.D., 5 μm particle size), Spherisorb ODS-1 (250 mm × 4.6 mm, 5 μm particle size), Spherisorb ODS-2 (250 mm × 4.6 mm, 5 μm particle size), and Spherisorb ODS-2 (100 mm × 6 mm, 3 μm particle size). Several solvents were tried with each reversed-phase column: distilled water, 0.05 *M* monobasic ammonium phosphate adjusted to pH 5.0 with ammonium hydroxide, 0.05 *M* monobasic ammonium phosphate adjusted to pH 3.5 with phosphoric acid, 0.05 *M* ammonium acetate adjusted to pH 4.0 with acetic acid, and 0.05 *M* sodium acetate buffer (pH 3.5).

Only reagents of analytical grade were used, and all solvents were filtered and vacuum degassed before use. In all experiments the flow-rate was 1.5 ml/min at ambient temperature.

Preparation of standards

All naturally occurring pyrimidine and purine bases, nucleosides, and nucleo-

tides were purchased from Sigma (St. Louis, MO, U.S.A.). 5-FU was obtained from Roche Labs. (Nutley, NJ, U.S.A.). 5-Fluorouridine (FUR) and 5-fluorodeoxyuridine (FUdR) were provided by the Drug Development Branch of the National Cancer Institute (Bethesda, MD, U.S.A.). Standard solutions were made with high purity water (Burdick and Jackson Labs., Muskegon, MI, U.S.A.). For column testing 2—5 nmol of each standard were injected (Waters U6K injector) to obtain nearly full-scale deflections at 0.1 a.u.f.s.

Preparation of biological samples

The sample of a patient undergoing treatment with N-(phosphonacetyl)-L-aspartate (PALA) 5-FU, and TdR was analysed. The patient received 1 g/m^2 of PALA as 1-h infusion and 24 h later 30 g of TdR as 3-h infusion followed by 200 mg/m^2 of 5-FU as 1-h infusion. A blood sample was drawn immediately after the 5-FU administration and placed in a tube containing heparin as anticoagulant. The plasma was separated by centrifugation and deproteinized by ultrafiltration using Amicon CF 25 membrane cones (Amicon Corp., Lexington, MA, U.S.A.). A 100-μl aliquot of the ultrafiltrate was injected using the Spherisorb ODS-2 (5 μm) column and 0.05 *M* monobasic ammonium phosphate (pH 3.5) as solvent.

Calculations

The following equation was used to express the retention or capacity factor (k): $k = (V_p - V_v)/V_v$, V_v being the void volume and V_p the volume to elute an individual peak [22]. The resolution factor (R) was calculated by the equation: $R = 2(V_{p_2} - V_{p_1})/(W_{p_1} + W_{p_2})$, V_p and W_p being the elution volumes and base widths of the peaks. The two peaks were regarded as reasonably well separated when $R = 1.0$, since at this value only 2% of peak overlap occurs [23]. Larger values of R reflect better separation.

RESULTS

Table I describes the columns and solvents used, and shows the resolution factors for U and 5-FU as an estimate of the performance of each system. The results with each column are described below.

μBondapak C_{18} (10 μm)

This column can be used for the separation of U and 5-FU with ammonium phosphate solutions of pH 5.0 ($R = 1.5$) and pH 3.5 ($R = 1.6$) or with sodium acetate buffer (pH 3.5) ($R = 1.4$) as mobile phases. The solutions with ammonium phosphate (pH 3.5) ($R \geqslant 1.6$) and sodium acetate (pH 3.5) ($R \geqslant 1.2$) allowed the separation of 5-FU, FUR, and FUdR from other bases and nucleosides (Table II). The capacity factors of this column with ammonium phosphate (pH 3.5) as solvent are shown in Table III. In the separation of all bases and nucleosides the pH of the ammonium phosphate solution was critical. At pH 3.5, adenine (A) and CdR coeluted. However, under identical conditions except at pH 3.2 the fluoropyrimidines and pyrimidines were separated (Fig. 1) without interference from purine bases and nucleosides [A, guanine (G), adenosine (AR), guanosine (GR), deoxyadenosine (AdR), deoxyguanosine (GdR)]; all resolution factors were greater than 1.3, except for AdR and GdR.

TABLE I

RESOLUTION FACTORS FOR URACIL AND 5-FLUOROURACIL

Column	Bonded phase	Particle size (μm)	Column size (mm)	0.05 *M* $NH_4H_2PO_4$ (pH 3.5)	0.05 *M* $NH_4H_2PO_4$ (pH 5.0)	0.05 *M* Sodium acetate buffer (pH 3.5)	0.05 *M* Ammonium acetate (pH 4.0)	Distilled water
μBondapak	C_{18}	10	300 × 3.9	1.6	1.5	1.4	<1.0	<1.0
μBondapak	Phenyl	10	300 × 3.9	<1.0	<1.0	<1.0	<1.0	<1.0
Radial-Pak	C_{18}	10	100 × 8.0	<1.0	<1.0	<1.0	<1.0	<1.0
Radial-Pak	C_{18}	5	100 × 8.0	1.9	1.4	1.6	<1.0	<1.0
Radial-Pak	C_8	10	100 × 8.0	1.1	1.0	1.0	<1.0	1.1
Spherisorb Hexyl	C_6	5	150 × 4.6	<1.0	<1.0	<1.0	<1.0	<1.0
Spherisorb ODS-1	C_{18}	5	250 × 4.6	<1.0	<1.0	<1.0	<1.0	<1.0
Spherisorb ODS-2	C_{18}	3	100 × 6.0	1.8	1.8	1.5	1.3	<1.0
Spherisorb ODS-2	C_{18}	5	250 × 4.6	2.2	2.1	1.6	N.D.*	<1.0

*N.D. = not done.

TABLE II

RESOLUTION FACTORS FOR FLUOROPYRIMIDINES AND PYRIMIDINES ON THE BEST THREE COLUMNS

Mean of three analyses with 0.05 *M* monobasic ammonium phosphate (pH 3.5) as mobile phase.

Compounds*	Spherisorb ODS-2 (5 μm)	Spherisorb ODS-2 (3 μm)	μBondapak C_{18} (10 μm)
C/U	6.0	5.9	7.1
U/FU	2.2	1.8	1.6
FU/CR	5.1	4.5	1.7
CR/CdR	7.3	8.4	5.3
CdR/UR	1.8	1.8	2.5
UR/T	2.6	2.7	3.0
T/FUR	4.9	5.2	2.9
FUR/UdR	3.6	3.8	2.9
UdR/FUdR	8.1	7.6	4.8
FUdR/TdR	21.8	20.6	14.3

*Abbreviations: C, cytosine; CR, cytidine; CdR, deoxycytidine; UR, uridine; T, thymine; UdR, deoxyuridine.

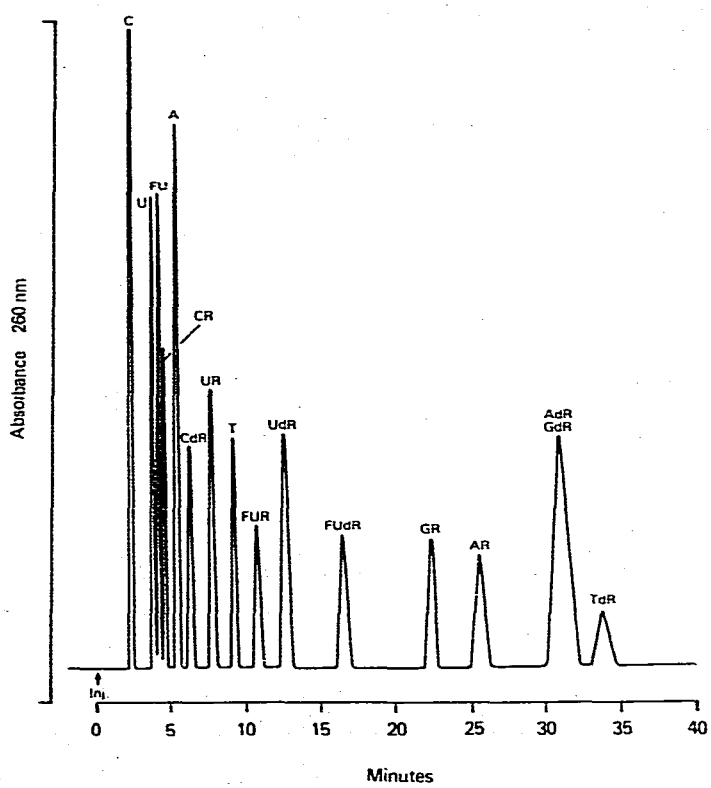

Fig. 1. Reversed-phase HPLC of nucleic acid bases and nucleosides (standards, 3 nmol each) on a μBondapak C_{18} high efficiency column with 0.05 *M* monobasic ammonium phosphate (pH 3.2) as mobile phase (isocratic conditions, flow-rate 1.5 ml/min, detector set at 0.1 a.u.f.s.).

TABLE III

CAPACITY FACTORS FOR FLUOROPYRIMIDINES AND PYRIMIDINES

Systems are arranged according to decreasing resolution from left to right.

Compound	Spherisorb ODS-2 (5 μm), 0.05 *M* $NH_4H_2PO_4$ (pH 3.5)	Spherisorb ODS-2 (5 μm), 0.05 *M* $NH_4H_2PO_4$ (pH 5.0)	Spherisorb ODS-2 (3 μm), 0.05 *M* $NH_4H_2PO_4$ (pH 3.5)	Spherisorb ODS-2 (3 μm), 0.05 *M* $NH_4H_2PO_4$ (pH 5.0)	μBondapak C_{18} (10 μm), 0.05 *M* $NH_4H_2PO_4$ (pH 3.5)	Radial-Pak C_8 (10 μm), 0.05 *M* $NH_4H_2PO_4$ (pH 3.5)	Radial-Pak C_8 (10 μm), 0.05 *M* $NH_4H_2PO_4$ (pH 5.0)	Spherisorb ODS-1 (5 μm), 0.05 *M* $NH_4H_2PO_4$ (pH 3.5)
C	0.4	0.5	0.5	0.7	0.4	1.2	1.3	0.8
U	1.2	1.0	1.5	1.4	1.2	1.6	1.6	1.5
FU	1.4	1.3	1.8	1.7	1.4	1.8	1.8	1.5
CR	2.2	2.0	2.7	2.6	1.8	2.6	2.4	2.1
CdR	3.6	3.9	4.4	4.9	2.9	5.0	5.0	4.0
UR	4.0	3.7	4.8	4.4	3.6	3.6	3.6	3.0
T	4.7	4.3	5.7	5.3	4.4	5.6	5.6	5.2
FUR	6.1	5.7	7.3	6.7	5.3	5.1	4.8	4.1
UdR	7.2	6.7	8.8	8.0	6.4	6.8	6.8	6.2
FUdR	10.0	9.3	12.1	11.0	8.6	8.7	8.5	7.3
TdR	22.5	20.8	26.8	24.5	18.4	19.7	19.2	17.8

Radial-Pak C_{18} cartridge (5 μm)

For the separation of U and 5-FU this cartridge can be used with ammonium phosphate solutions of pH 5.0 (R = 1.4) and pH 3.5 (R = 1.9) or with sodium acetate buffer of pH 3.5 (R = 1.6) as mobile phases. Furthermore, the separation of FUR and FUdR from other nucleosides ($R \geq 2.1$) was possible with an ammonium phosphate solution of pH 3.5. The capacity factors of this column for C, CR, CdR, T, and TdR were so high that it is not suitable for the separation of these compounds. This finding is most likely attributed to the fact that, in contrast to μBondapak C_{18} or Spherisorb ODS, the hydroxyl groups on the silica particles are not capped in Radial-Pak cartridges.

Radial-Pak C_8 cartridge (10 μm)

The separation of U and 5-FU ($R \geq 1.0$) could be achieved with all the described solvents except for the ammonium acetate solution. The naturally occurring pyrimidines ($R \geq 1.9$) were separated on this column with ammonium phosphate solutions at either pH 5.0 or pH 3.5; the capacity factors are given in Table III. The presence of an ammonium salt in the mobile phase was essential in this system, since the sodium acetate buffer allowed only the separation of U, 5-FU, and their nucleosides ($R \geq 1.0$), but retained C, CR, and CdR. In addition, ammonium acetate should not be used with this cartridge; it not only led to unsatisfactory separation, but also impaired the performance of the column in subsequent trials with other solvents.

Spherisorb ODS-1 (5 μm)

It was not possible to separate U and 5-FU ($R < 1.0$) with any of the described solvents on this column. However, a good separation of the naturally occurring pyrimidine bases and nucleosides ($R \geq 3.0$) could be achieved using the ammonium phosphate solution (pH 3.5) as mobile phase. The capacity factors for this system are listed in Table III.

Spherisorb ODS-2 (3 μm)

U and 5-FU as well as their nucleosides ($R \geq 1.5$) could be separated with ammonium phosphate (pH 3.5 or 5.0) or sodium acetate buffer of pH 3.5 as solvents. Ammonium phosphate solution of pH 3.5 was especially effective in separating all pyrimidine and fluoropyrimidine bases and nucleosides. The resolution and capacity factors for these compounds are shown in Tables II and III. Adenine and its nucleosides did not elute from the column within 40 min, and GR and GdR did not interfere with other compounds.

Standard curves for U, UR, UdR, FU, FUR, FUdR, C, CR, CdR, T, and TdR in concentrations from 0.05 to 1.00 nmol each were obtained by using this column and ammonium phosphate at pH 3.5 as solvent. The correlation coefficients were excellent (r^2 = 1.00). The lower limit of detection was 0.05 nmol of each of the eleven compounds at 0.02 a.u.f.s.

The ammonium constituent in the mobile phase seemed to be essential for the separation on all Spherisorb ODS columns. With sodium acetate buffer as solvent the C peak was delayed and broadened, while CR and CdR were retained on the column for over 60 min. Again, ammonium acetate should not be used as solvent; it not only decreased the retention times of all compounds but

also impaired the separation of CdR and UR in subsequent trials with ammonium phosphate solutions.

Spherisorb ODS-2 (5 μm)

This column and the Spherisorb ODS-2 (3 μm) performed similarly. U and 5-FU were separated on this column with the highest resolution factors of all columns tested (Table I); especially with ammonium phosphate as the mobile phase, R values of at least 2.1 could be achieved. Moreover, this column not only separated the pyrimidines and fluoropyrimidines well (Table II), but was also able to resolve purine bases and nucleosides. Of the purines, ammonium phosphate at pH 5.0 eluted only GR and GdR within 40 min, separating them from all pyrimidines (minimum R = 1.0). Ammonium phosphate at pH 3.5 as mobile phase eluted A and AR also without impairing resolution; the minimum resolution factor in the chromatogram was 1.7 (Fig. 2).

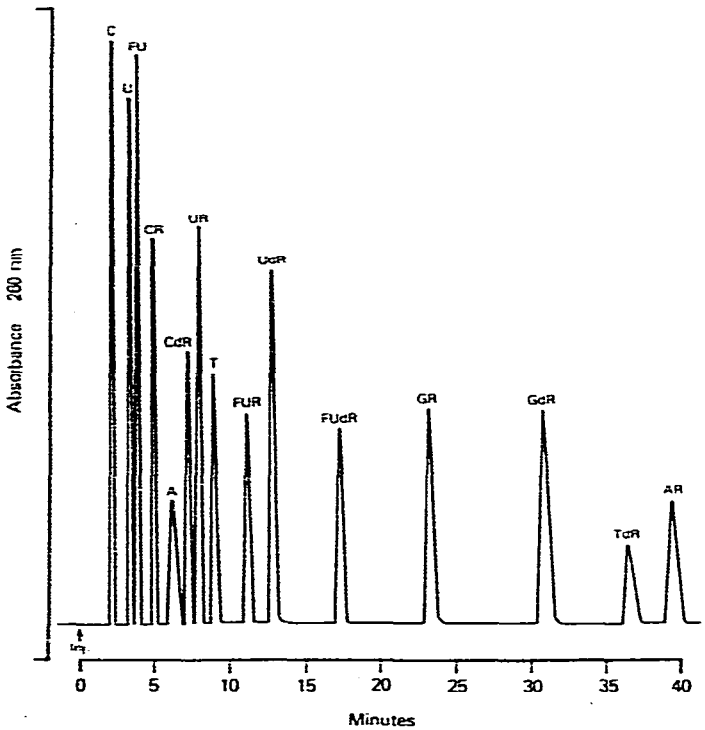

Fig. 2. Reversed-phase HPLC of nucleic acid bases and nucleosides (standards, 3 nmol each) on a Spherisorb ODS-2 (5 μm particle size) column with 0.05 M monobasic ammonium phosphate (pH 3.5) as solvent (isocratic conditions, flow-rate 1.5 ml/min, detector set at 0.1 a.u.f.s.).

Table III summarizes the capacity factors of this column with two solvents. The reproducibility of capacity factors and peak areas was evaluated by five analyses on this column with ammonium phosphate of pH 3.5 as mobile phase, injecting 3 nmol of each standard in a volume of 50 μl (as in Fig. 2). The coefficients of variation are shown in Table IV.

TABLE IV

COEFFICIENTS OF VARIATION OF CAPACITY FACTORS AND PEAK AREAS

Five analyses on a 5-μm Spherisorb ODS-2 column with 0.05 *M* ammonium phosphate (pH 3.5) as solvent, 3 nmol of each compound in 50-μl injection volume, detector set at 0.1 a.u.f.s.

Compound	Coefficient of variation (%)	
	Capacity factor	Peak area
C	0.8	2.5
U	0.9	1.5
FU	0.8	2.1
CR	0.9	1.0
CdR	1.0	2.9
UR	1.1	2.7
T	0.8	2.8
FUR	1.2	0.5
UdR	1.3	0.5
FUdR	1.4	0.9
TdR	1.3	2.1

To demonstrate the potential applications of this method for biological samples, deproteinized plasma of a patient treated with PALA, TdR, and 5-FU was analysed. The chromatogram (Fig. 3) showed that the fluoropyrimidines and pyrimidines of interest could be separated and no interfering peaks occurred.

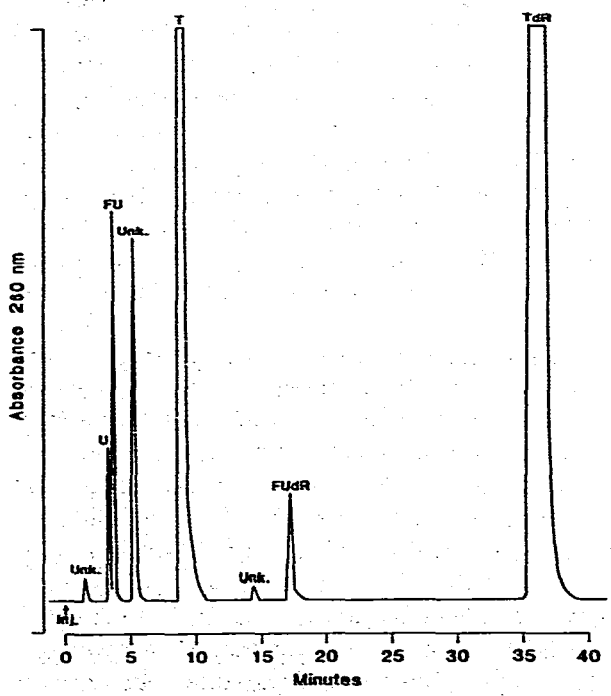

Fig. 3. Reversed-phase HPLC of ultrafiltered plasma from a patient after treatment with PALA, TdR, and 5-FU (identical conditions as in Fig. 2; Unk. = unknown compounds).

Other columns

The μBondapak Phenyl column (10 μm) was not useful with any of the described mobile phases, mainly because the capacity factors for pyrimidines were too low. The chromatograms obtained with the Radial-Pak C_{18} cartridge (10 μm) showed broad peaks and insufficient separation. With the Spherisorb Hexyl (5 μm) column, chromatograms revealed short retention times and poor separation for all pyrimidines and fluoropyrimidines.

DISCUSSION

The objective of this study was to compare and optimize the resolution (Tables I and II) of fluoropyrimidines and pyrimidines within separation times of 30—60 min employing isocratic, reversed-phase techniques at ambient temperature. Resolution is achieved by differential migration of compounds as determined by the composition of the mobile phase (Table I), the nature of the stationary phase, and the separation temperature, and is shown by the sharpness of the peaks and the distance between peak maxima on chromatograms (Figs. 1—3). Three variables define resolution [23], namely, efficiency, selectivity, and capacity factor.

The efficiency of a column determines the peak widths. Band spreading is caused by eddy diffusion or multiple flow paths, mass transfer, and molecular diffusion. These phenomena are influenced by particle characteristics, column dimensions, and the flow-rate. A variety of packings and columns was investigated in this study. Spherical particles, like in the Radial-Pak and Spherisorb columns, are better permeable and more stable. Irregular particles, like in μBondapak, cause more flow inequalities. Decreasing the particle size from 10 to 3 μm yields higher efficiency, better resolution, and shorter separation times. The particle size distribution affects the uniformity of the packing and interparticle void spaces. Advanced control of the particle size improved the separation with Spherisorb packings: in ODS-1 70% of the spheres have a size distribution of ± 1 μm compared to 95% in ODS-2. These small (3—5 μm), thinly coated particles homogeneously packed gave the best resolution (Table II). In columns with larger diameters, like Radial-Pak cartridges (8 mm I.D.), more flow inequalities and different microscopic flowstreams between particles tend to occur, which leads to broader peaks.

The selectivity or nature of the stationary and mobile phases determines the peak separation. A number of bonded and mobile phases was investigated (Table I). Monobasic ammonium phosphate as solvent allowed the best separations. Changing the pH influenced the retention times and was used to improve the separation, especially of purines (Figs. 1 and 2, Table III). Although the mechanism of retention has not been established, the organic bonded phase is considered to play the most important role. Fully hydrolyzed silica contains about 8 μmol of silanol groups per m^2 of surface [23]. Under optimum conditions about 4.7 $\mu mol/m^2$ of these silanol groups can be reacted with trimethylsilyl groups, the smallest modifier available; the remaining silanol groups are shielded by reacted groups. This dense monolayer of functional groups or monomeric coverage, like in Spherisorb ODS-2, appears to improve selectivity. The presence of bulky groups on the silane, like in μBondapak Phenyl, reduces

the possible surface coverage. Residual acidic silanol groups, like in the uncapped Radial-Pak C_{18} cartridges, impair separation when compared with capped μBondapak C_{18}.

The capacity factor determines the retention time of a peak, and depends on the relative amount of stationary to mobile phase and the effective utilization of the stationary phase. Increasing the column length results in longer retention times. However, a shorter column packed with 3-μm particles can have similar capacity factors as a longer column with 5 μm particles (Table III). The length of the alkyl chains on the silica particles also affects the capacity factor. Increasing the chain length from C_6 in Spherisorb Hexyl to C_{18} in Spherisorb ODS increased the capacity factors markedly. However, when residual silanol groups are present, like in Radial-Pak cartridges, decreasing the chain length from C_{18} (10 μm) to C_8 (10 μm) improved the separation.

By observing the three variables of resolution, the separation of fluoropyrimidines and pyrimidines can be optimized. If especially reproducible separations of fluoropyrimidines and all major nucleic acid bases and nucleosides are required, the system with Spherisorb ODS-2 as stationary phase and ammonium phosphate as mobile phase is recommended. For less complex applications, other columns have been proved to be satisfactory. U and FU were resolved by several systems (Table I). The separation of FU nucleosides was achieved on μBondapak C_{18}, Radial-Pak C_{18} (5 μm) or C_8 (10 μm), and on Spherisorb ODS-2 with ammonium phosphate or sodium acetate buffer of pH 3.5 as mobile phase. The resolution of naturally occurring pyrimidines was possible on μBondapak C_{18}, Radial-Pak C_8, and on Spherisorb ODS with ammonium phosphate (preferably pH 3.5) as solvent.

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REFERENCES

1 D.S. Martin, R. Nayak, R.C. Sawyer, R.L. Stolfi, C.W. Young, T. Woodcock and S. Spiegelman, Cancer Bull., 30 (1978) 219.
2 A. Floridi, C.A. Palmerini and C. Fini, J. Chromatogr., 138 (1977) 203.
3 H.G. Schneider and A.J. Glazko, J. Chromatogr., 139 (1977) 370.
4 H.J. Breter, G. Seibert and R.K. Zahn, J. Chromatogr., 140 (1977) 251.
5 B.C. Pal, J. Chromatogr., 148 (1978) 545.
6 B. Bakay, E. Nissinen and L. Sweetman, Anal. Biochem., 86 (1978) 65.
7 A.M. Krstulovic, P.R. Brown and D.M. Rosie, Anal. Chem., 49 (1977) 2237.
8 C.W. Gehrke, K.C. Kuo, G.E. Davis, R.D. Suits, T.P. Waalkes and E. Borek, J. Chromatogr., 150 (1978) 455.
9 P.R. Brown and A.M. Krstulovic, Anal. Biochem., 99 (1979) 1.
10 A. Wakizaka, K. Kurosaka and E. Okuhara, J. Chromatogr., 162 (1979) 319.
11 M. Ehrlich and K. Ehrlich, J. Chromatogr. Sci., 17 (1979) 531.
12 R.A. Hartwick, S.P. Assenza and P.R. Brown, J. Chromatogr., 186 (1979) 647.
13 G.A. Taylor, P.J. Dady and K.R. Harrap, J. Chromatogr., 183 (1980) 421.

14 K.C. Kuo, R.A. McCune, C.W. Gehrke, R. Midgett and M. Ehrlich, Nucleic Acids Res., 8 (1980) 4763.
15 C.W. Gehrke, K.C. Kuo and R.W. Zumwalt, J. Chromatogr., 188 (1980) 129.
16 H.K. Webster and J.M. Whaun, J. Chromatogr., 209 (1981) 283.
17 B. Matz, J. Chromatogr., 187 (1980) 453.
18 C.F. Gelijkens and A.P. De Leenheer, J. Chromatogr., 194 (1980) 305.
19 J.L. Cohen and R.E. Brown, J. Chromatogr., 151 (1978) 237.
20 W.E. Wung and S.B. Howell, Clin. Chem., 26 (1980) 1704.
21 A.R. Buckpitt and M.R. Boyd, Anal. Biochem., 106 (1980) 432.
22 L.S. Ettre, J. Chromatogr., 220 (1981) 29.
23 L.R. Snyder and J.J. Kirkland, Introduction to Modern Liquid Chromatography, 2nd ed., Wiley, New York, Chichester, Brisbane, Toronto, 1979, Ch. 2, p. 34.