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INFLUENCE OF THE STATIONARY PHASE ON THE RETENTION CHARACTERISTICS OF PURINE AND PYRIMIDINE BASES, RIBONUCLEOSIDES AND DEOXYRIBONUCLEOSIDES IN REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The influence of the chain length of alkylsilica stationary phases on the selectivity of the separation of purine and pyrimidine bases and nucleosides is investigated, and the reproducibility of the retention when applying a constant gradient program with different packing materials is discussed. The use of different alkylsilica stationary phases leads to appreciable changes in the retention, but peak identification on the basis of retention data remains possible for most of the compounds when an appropriate internal standardization method is employed. The investigations were performed by applying a gradient program which allows the separation of up to 20 physiologically important purine and pyrimidine bases, ribonucleosides and 2'-deoxyribonucleosides on alkylsilica adsorbents.

INTRODUCTION

The qualitative and quantitative determination of purine and pyrimidine bases and nucleosides in body fluids and cellular extracts is a field of extensive investigations. High-performance liquid chromatography (HPLC) offers the possibility to determine these compounds simultaneously and is therefore very well suited for this analytical problem. The majority of the chromatographic separations of these compounds described in the literature used reversed-phase chromatography, where the mobile phase consisted of an aqueous buffer solution with a constant or increasing content of an organic solvent component[1].

Whereas the influence of the mobile phase composition on the retention of nucleic acid components in reversed-phase chromatography has been investigated by many authors, *e.g.*, refs. 2–5, limited attention has been given to studying the influence of the support material[7]. This paper deals with the influence of the stationary phase material on the separation of nucleobases and nucleosides in reversed-phase

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chromatography. Of main interest are selectivity effects which can be obtained in practice by choosing different alkyl-modified surfaces when applying a given gradient elution program.

Gradient elution programs are very often used in reversed-phase chromatography of nucleosides and bases, especially when a great number of compounds has to be determined which have very different retention behaviours[8–14]. The gradient program used in this investigation allows the separation of the most important physiologically occurring purine and pyrimidine bases, nucleosides and 2'-deoxynucleosides including 3',5'-cyclic AMP. It involves increasing the concentration of an organic component (methanol) in an aqueous buffer solution and is similar to the elution program presented by De Abreu *et al.*[9].

Concerning the influence of the stationary phase material, the different length of the alkyl chains and the influence of pore diameter and specific surface area are investigated as parameters for adjusting the selectivity of separation. The influence of chain length was investigated previously in a somewhat different way, together with the bonded-phase polymericity, by Zakaria and Brown[7], where the accessibility of residual silanols under various isocratic conditions and the implications for retention mechanisms were of main interest. The selectivity effects are discussed here also from the viewpoint of the reproducibility of the retention when applying a constant gradient program with similar packing materials, first because an optimized gradient program should be applicable to similar packing materials without the need for reoptimization, and secondly because a certain degree of reproducibility in the retention data is necessary for peak identification, especially when automatic, *i.e.*, computer-assisted, methods are used. Usually the reproducibility of data is enhanced by internal standardization. The limitations inherent to an internal standardization when applying a gradient program are investigated for the mentioned compounds.

EXPERIMENTAL

Instrumentation

The separations were performed on a Varian 5060 chromatograph equipped with a Vista data system CDS 401 (Varian), a Valco injector using 10-μl or 50-μl loops and a Varian Series 50 detector, monitoring at 280 or 254 nm. The data processing including peak-area integration and peak-height evaluation was done by the Vista data system.

Columns

Prepacked stainless-steel columns (250 × 4.0 mm I.D.) with chemically bonded alkylsilica packings (LiChrosorb® RP-2, RP-8 and RP-18: E. Merck, Darmstadt, F.R.G.) were used. The specification of the packing materials are given in Table I.

Reagents and solutions

Solute standards were obtained from Sigma (Taufkirchen, F.R.G.). Methanol and acetonitrile chromatography grade solvents were obtained from E. Merck (LiChrosolv®) as were analytical-reagent grade potassium dihydrogen phosphate and phosphoric acid. Water was doubly distilled.

The eluent mixture was prepared by the chromatographic system using three

TABLE I

SPECIFICATIONS OF THE PACKING MATERIALS USED

DMS = Dimethylsilica; OS = octylsilica; ODS = octadecylsilica.

Column number	*Packing material*	*Alkyl chain length*	*Particle size (μm)*	*Pore diameter (Å)*	*Specific surface area of the silica support*[17] *(m^2/g)*
1	LiChrosorb RP-2	C_2 (DMS)	5	60	480
2	LiChrosorb RP-8	C_8 (OS)	5	60	480
3	LiChrosorb RP-8	C_8 (OS)	10	100	310
4.1	LiChrosorb RP-18	C_{18} (ODS)	5	100	310
4.2	LiChrosorb RP-18	C_{18} (ODS)	7	100	310
4.3	LiChrosorb RP-18	C_{18} (ODS)	10	100	310
4.4	LiChrosorb RP-18	C_{18} (ODS)	10	100	310
5	MicroPak MCH10	C_{18} (ODS)	10	100	–

stock solutions: A, pure methanol; B, 0.15 *M* potassium dihydrogen phosphate solution, pH 4.5 (the pH was adjusted with phosphoric acid); C, water containing 2% of acetonitrile.

Procedure

The chromatographic separation was performed by use of the gradient elution program in Table II. After each experiment the column was reconditioned for 20 min. After five experiments, at least each evening, the column was rigorously washed by use of a methanol–acetonitrile–water gradient program.

The time specifications of the gradient program shown in Table II are given for a flow-rate of 0.7 ml/min. At higher flow-rates the gradient can be adjusted by using correspondingly shorter times.

All measurements were done at 40°C. Values of t_{R0} for the calculation of capacity factors were determined by the injection of phloroglucinol.

TABLE II

GRADIENT ELUTION PROGRAM USED FOR SEPARATION OF THE MAIN PURINE AND PYRIMIDINE BASES AND NUCLEOSIDES

It has been optimized for the ODS column 4.2, described in Table I. The time specification refers to a flow-rate of 0.7 ml/min. Volume between mixing of solvents and the top of the column was 2 ml. A = Pure methanol; B = 0.15 *M* aqueous potassium dihydrogenphosphate solution, pH 4.5; C = water containing 2% acetonitrile.

Time (min)	*Solvents (%)*			*Comments*
	A	*B*	*C*	
0 to 9.5	1	99	–	Isocratic elution
9.5 to 37.0	15	70	15	Linear gradient
37.0 to 52.0	26	48	26	Linear gradient

RESULTS AND DISCUSSION

Description of the gradient

In order to investigate the influence of the stationary phase material on the retention behaviour of solutes, the mobile phase program must not be changed. Therefore the elution program has been established using one particular support material: the octadecylsilica (ODS) material LiChrosorb RP-18,7 μm, 100 Å, referred to in Table I by the number 4.2. This was quite an arbitrary choice within the materials investigated. The gradient program described allows the separation of the most important physiologically occurring purine and pyrimidine bases, ribonucleosides and 2'-deoxyribonucleosides on this column. It is similar to that presented by De Abreu *et al.*[9] but has a higher ionic strength, a lower pH and a slightly different profile (Table II).

The program starts with an isocratic part using an aqueous 0.15 *M* potassium dihydrogenphosphate buffer solution (pH 4.5) with low modifier concentration (1% methanol to allow wetting of the stationary phase and to facilitate the reconditioning of the column) for 9.5 min which leads to a good separation of uracil from uric acid and of hypoxanthine from xanthine. Increasing the methanol concentration and reducing the buffer concentration in the way described enables sufficient separation of all investigated compounds. The retention patterns are similar to those obtained by De Abreu *et al.*[9], except for 3',5'-cyclic AMP. Compared with other similar elution programs, an improvement in the resolution between certain pairs of compounds is observed: hypoxanthine–xanthine[9], adenine–guanosine[10], adenine–inosine[8], 3',5'-

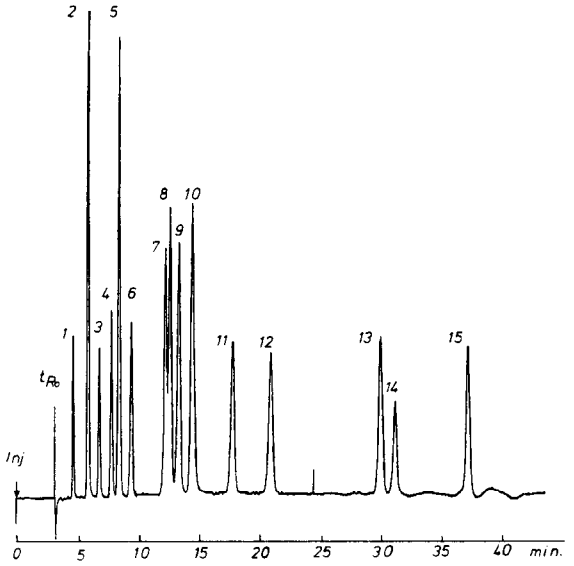

Fig. 1. Chromatogram of purine and pyrimidine bases, nucleosides and 2'-deoxynucleosides on a RP-2 column (particle diameter 5 μm). The gradient program applied is given in Table II. The flow-rate was 0.7 ml/min. Temperature: 40°C. Peaks: 1 = orotic acid; 2 = uracil and cytidine; 3 = uric acid; 4 = uridine; 5 = hypoxanthine; 6 = xanthine; 7 = inosine; 8 = allopurinol; 9 = guanosine; 10 = adenine; 11 = 2'-deoxyinosine; 12 = 2'-deoxyguanosine; 13 = adenosine; 14 = 3',5'-cyclic AMP; 15 = 2'--deoxyadenosine.

cyclic AMP–2′-deoxyadenosine[8] and 2′-deoxyinosine–guanosine[11]. However, with this method, hypoxanthine and guanine are not separated. Fig. 1 shows the chromatographic separation of fifteen of the most important bases and nucleosides in about 35 min. Complete baseline separation is obtained in all cases except for allopurinol, which does not occur physiologically. The retention data for the investigated compounds on the stationary phase for which the gradient was optimized are summarized in Table III.

If one investigates the metabolism in cells, cellular extracts are found to contain a great number of nucleotides. With our gradient program, all nucleotides except AMP and TMP and the cyclic monophosphates appear in the first region of the chromatogram before the peaks of hypoxanthine and xanthine, interfering mainly with pyrimidine bases and nucleosides. The retention data of some of the important nucleotides are also shown in Table III. The relatively narrow region of about 2.0 ml, where most of the nucleotides are eluted, offers good possibilities for coupled column chromatography, combining the reversed-phase column with an anion-exchange column which allows a subsequent separation of the nucleotides.

TABLE III

RETENTION DATA AND REPRODUCIBILITY ON ODS COLUMN 4.2

Retention times, t_{Ri}, and capacity factors, κ_i (within the isocratic region), for purine and pyrimidine bases, nucleosides and 2′-deoxynucleosides (A) and nucleotides (B). The data were obtained by use of the elution program described in Table II and the packing material LiChrosorb RP-18, 7 μm (pore diameter 100 Å), at 40°C. The reproducibility is given by the standard deviation of the retention time, $s(t_{Ri})$; $N = 6$. The nucleotides investigated are 5′-mono-, di- or -triphosphates. t_{R0} (3.10 min) was determined by use of phloroglucinol.

Compounds A	t_{Ri} *(min)*	κ_i	$s(t_R)$ *(%)*	*Compounds B*	t_{Ri} *(min)*	κ_i
Orotic acid (OA)	4.05	0.31	1.0	CTP	3.28	0.06
Cytosine (Cys)	4.39	0.42		UTP	3.29	0.06
Uracil (Ura)	5.65	0.82	1.1	XTP	3.68	0.19
Uric acid (UA)	6.25	1.02	1.5	GTP	3.68	0.19
Cytidine (Cyt)	6.93	1.24	2.3	ITP	3.74	0.21
Hypoxanthine (Hyp)	7.90	1.55	1.6	ATP	4.72	0.52
Guanine (Gua)	7.92	1.55	3.3	TTP	4.74	0.53
Xanthine (Xan)	8.90	1.87	2.0	CDP	3.38	0.09
Uridine (Urd)	9.60	2.10	1.4	XDP	3.93	0.27
Allopurinol (Allo)	12.2	2.94	1.5	GDP	4.00	0.29
Adenine (Ade)	17.6		1.4	IDP	4.09	0.32
7-Methylguanine	18.5			ADP	5.65	0.82
Inosine (Ino)	18.9		2.5	TDP	5.66	0.83
Guanosine (Guo)	20.2		3.0	CMP	3.95	0.27
2′-Deoxyinosine (2dIno)	23.6		0.6	GMP	4.56	0.47
2′-Deoxyguanosine (2dGuo)	25.0		2.9	IMP	5.93	0.91
Thymidine (Thy)	28.2			AMP	10.98	2.54
Adenosine (Ado)	33.3		1.0	TMP	10.89	2.51
3′,5′-cyclic AMP (cAMP)	33.9		2.4	3′,5′-cyclic CMP	10.83	2.49
2′-Deoxyadenosine (2dAdo)	36.3		1.7	3′,5′-cyclic XMP	11.72	2.78
				3′,5′-cyclic AMP	33.90	
				3′,5′-cyclic TMP	25.87	

Reproducibility of retention data on individual columns

The reproducibility of the retention data on a single column is specified by the standard deviation, $s(t_R)$, shown also in Table III. The data are obtained from measurements performed during a period of a few days. Statistical outliers could always be associated with improper washing or conditioning of the columns. The standard deviations of t_{Ri} values for the compounds eluted in the isocratic part of the gradient program are generally about 1.5–2.5%. This scatter probably results from an imperfect equilibration after the washing procedure. For the compounds eluted in the steeper part of the gradient the standard deviations are found to be not significantly different from the values quoted above.

Influence of alkyl chain length of the stationary phase

The main topic of interest in this work is the influence of the alkylsilica stationary phases on the separation of bases and nucleosides and concerns the following two questions:

(i) Is the length of the alkyl chains of alkyl-modified silica a useful parameter for rational adjustment of selectivity?

(ii) Is the reproducibility of the retention data, on packing materials of equal alkyl chain length, high enough to allow the application of an optimized mobile phase gradient program to all stationary phases without significant loss in resolution? Are the variations obtained in the retention data small enough to allow an unique identification of a peak?

In our investigation we used monomeric alkyl-modified silica materials with three different chain lengths, namely RP-2 material (dimethylsilica), RP-8 (octylsilica) and RP-18 (octadecylsilica). The effect of the alkyl chain length has to be discussed in relation to the variation of the retention behaviour on different packing materials with the same chain length. Eight different columns packed with five different adsorbents have been investigated. Table I gives the chain lengths, pore diameters and specific surface areas of the silica supports. Four columns (Nos. 4.1 to 4.4) contain packing materials having the same specifications. An identical gradient program, which has been described before, has been applied to all columns. It has been optimized for one particular column and is therefore not necessarily optimal for the other columns. However, in comparing selectivity effects of stationary phases or for the determination of reproducibility within similar materials it was necessary to keep constant the influence of the mobile phase.

The retention time data measured for the most important bases and nucleosides on these eight columns are reported in Table IV and are illustrated (for clearity) in Fig. 2. Some significant and sometimes important changes in the retention behaviour are caused by the stationary phase material, even when comparing stationary phases having the same specification. The following general trends can be discerned.

(i) Generally, the retention time is longer on C_{18} than on C_8. This is seen particularly clearly by comparing materials of equal specific surface areas. The retention times of bases are affected much less than those of nucleosides and deoxynucleosides. This effect leads to significant selectivity changes for base–nucleoside pairs, as illustrated in Table V, and can be used for a distinct improvement of separation. No significant differences in the retention time data or in the selectivity of separation could be found on comparing (Table VI) the C_2 and C_8 materials (Nos.

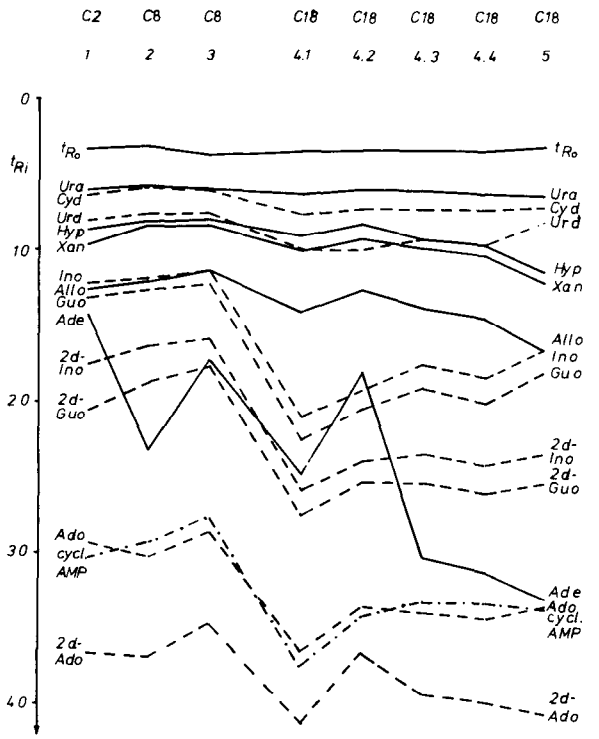

Fig. 2. Retention time data, t_{Ri}, of the investigated compounds on eight different columns. The elution program is given in Table II. Flow-rate: 0.7 ml/min. Column: 250 × 4 mm I.D.

1 and 2) of equal pore diameters. This finding is in agreement with a minimum chain length needed for a stronger adsorption of the nucleosides due to a better and more complete enclosure of the nucleoside molecules by the alkyl chains.

(ii) Fig. 2 shows that the variation in the retention data due to different surface areas (and pore diameters) of the two C_8 materials is about as high as the differences obtained on the C_2 and the C_8 materials of equal surface areas. For the compounds eluted in the isocratic part of the elution program, the ratio of the capacity factors measured on the two C_8 materials is approximately equal to the ratio of their specific surface areas. Table VI therefore shows that the variation of the chain length from dimethyl- to octylsilica does not result in significantly different selectivity coefficients for most pairs of compounds, nor does a reduction in specific surface area or an increase in pore diameter.

(iii) Within the group of bases, most compounds undergo a change in retention in the same direction when changing the stationary phase. The same is true with the group of nucleosides and 2'-deoxynucleosides. In some cases the changes for these two groups are found to occur in opposite directions. This suggests a certain difference in the geometric arrangement in the adsorbed state for compounds belonging to these two groups.

(iv) Irregular patterns are observed for adenine. This may be due partially to the fact that the pH of the mobile phase, 4.5, is near to the pK value of adenine. The retention in this region is very sensitive to small changes in pH (ref. 2). In addition,

TABLE IV

UNIFORMITY OF RETENTION DATA ON DIFFERENT ALKYLSILICA COLUMNS

Retention times, t_{Ri}, are given for bases, nucleosides and 2'-deoxynucleosides on eight different columns specified as in Table I. The applied gradient is given in Table II.

	Column							
	1	*2*	*3*	*4.1*	*4.2*	*4.3*	*4.4*	*5*
	Chain length							
	C_2	C_8	C_8	C_{18}	C_{18}	C_{18}	C_{18}	C_{18}
	Pore diameter (Å)							
	60	*60*	*100*	*100*	*100*	*100*	*100*	*100*
t_{R0}	3.00	2.80	3.40	3.10	3.10	3.05	3.10	2.90
OA	4.50	3.90	4.22	4.20	4.05	3.95	4.02	4.02
Ura	5.75	5.40	5.63	5.95	5.65	5.72	5.90	6.05
UA	5.65	5.35	5.60	6.70	6.25	6.52	6.70	6.80
Cyd	5.80	5.45	5.70	7.35	6.93	6.95	7.45	6.85
Hyp	8.20	7.75	7.60	8.70	7.90	8.90	9.25	11.1
Xan	9.20	8.05	8.00	10.2	8.90	9.50	9.90	11.8
Urd	7.60	7.20	7.20	9.70	9.60	8.90	9.25	7.80
Allo	12.2	11.8	11.0	13.7	12.2	13.5	14.1	16.2
Ade	14.0	22.9	16.9	24.5	17.6	30.0	30.9	32.7
Ino	11.8	11.5	11.0	20.6	18.9	17.2	18.1	16.2
Guo	12.8	12.3	11.9	22.2	20.2	18.8	19.8	17.7
2dIno	17.2	16.0	15.5	25.5	23.6	23.1	23.9	23.1
2dGuo	20.2	18.4	17.4	27.2	25.0	25.0	25.7	25.0
Ado	29.0	29.9	28.2	36.2	33.3	33.6	34.0	33.3
cAMP	30.0	29.1	27.3	37.2	33.9	32.9	33.0	33.4
2dAdo	36.3	36.6	34.4	40.9	36.3	39.0	39.5	40.3

the retention of this compound also seems to depend on the method of preparation of the packing material, *i.e.*, on the selection of the silica base material and on the percentage coating of its surface by the alkyl groups. This is supported by the strong tailing of the adenine peak observed in some cases. Fig. 2 shows that the irregular retention behaviour of adenine is also exhibited to a certain extent by adenine-con-

TABLE V

SELECTIVITY COEFFICIENTS OF NUCLEOSIDE-BASE PAIRS, MEASURED ON DIMETHYL-, OCTYL- AND OCTADECYLSILICA MATERIALS

Pair	*Column*							
	1 C_2	*2* C_8	*3* C_8	*4.1* C_{18}	*4.2* C_{18}	*4.3* C_{18}	*4.4* C_{18}	*5* C_{18}
Cyd–Ura	1.01	1.02	1.03	1.49	1.51	1.46	1.54	1.25
Cyd–UA	1.06	1.04	1.05	1.18	1.22	1.12	1.20	1.02
Ino–Allo	0.95	0.97	1.00	1.65	1.74	1.35	1.36	1.00

TABLE VI

SELECTIVITY COEFFICIENTS ON DIMETHYL- AND OCTYLSILICA MATERIALS OF DIFFERENT SPECIFIC SURFACE AREAS

Pair	*Column*		
	1	*2*	*3*
	Chain length		
	C_2	C_8	C_8
	Pore diameter (Å)		
	60	*60*	*100*
	Specific surface area (m^2/g)		
	480	*480*	*310*
Cyd–Ura	1.01	1.02	1.03
Cyd–UA	1.06	1.04	1.05
Xan–Hyp	1.20	1.06	1.09
Hyp–Urd	1.13	1.13	1.11
Allo–Ino	1.05	1.03	1.00
Guo–Ino	1.12	1.09	1.12

taining nucleosides. The behaviour of adenosine, 2′-deoxyadenosine and 3′,5′-cyclic AMP appears to be a superposition of the "normal" behaviour of the nucleosides and that of adenine.

Summarizing the possibilities of selectivity adjustment by appropriate selection of the stationary phase, the use of long alkyl chains gives the best results for pairs comprising a base and a nucleoside, *e.g.*, uracil–cytidine, uric acid–cytidine and allopurinol–inosine in Table V. In these cases the use of C_{18} material is advantageous. Our investigations have shown that the different pore diameters and specific surface areas of different adsorbents cannot be utilized in a predictable way to achieve significant changes in selectivity coefficients. As pointed out before, the dependence of the retention behaviour of adenine on the choice of stationary phase cannot be discussed purely in terms of the chain length or of the specific surface area.

Constant pattern in the retention characteristics of different packings

Beside the selectivity effects discussed so far, Fig. 2 gives the impression that similar trends in the retention behaviour are dominant. These regularities within the retention of the investigated compounds can be used to establish algorithms for peak identification when a new packing material is used. Fig. 2 shows that the changes in retention time, accompanying the changes in stationary phase, are very similar within the group of bases and very similar within the group of nucleosides. These similarities within the two groups can be quantified by means of two reference compounds, one belonging to the group of bases, *e.g.*, allopurinol, and one belonging to the group of nucleosides, *e.g.*, guanosine. These two reference compounds can serve as internal standards (ISs) for the peak identification. However, one has to choose different

TABLE VII

REPRODUCIBILITY OF THE RETENTION TIMES, t_{Ri}, ON DIFFERENT ALKYLSILICA COLUMNS WITHOUT AND WITH APPLICATION OF AN INTERNAL STANDARD

All eight columns were investigated, five of which have different specifications. Internal standardization for compounds eluted in the isocratic part of the elution program is done by use of the capacity factor ratios, $\kappa_i\kappa_{(IS)}$; for nucleosides, which are eluted in the gradient part, differences in the retention times, $t_{Ri} - t_{R(IS)}$, are used.

	Mean t_{Ri} *(min)*	$s(t_{Ri})$		*IS*	$s(\kappa_i/\kappa_{(IS)})$ *(%)*	$s(t_{Ri} - t_{R(IS)})$ *(%)*
		min	%			
Isocratic region						
Bases						
Uracil	5.76	0.21	3.6	Allo	3.8	
Uric acid	6.20	0.58	9.4	Allo	4.3	
Hypoxanthine	8.68	1.14	13.1	Allo	3.2	
Xanthine	9.44	1.24	13.1	Allo	3.6	
Gradient region						
Nucleosides						
Inosine	15.7	3.73	23.8	Guo		2.3
2'-Deoxyinosine	21.0	4.03	19.2	Guo		3.3
2'-Deoxyguanosine	23.0	3.73	16.2	Guo		4.2
Adenosine	32.2	2.81	8.7	Guo		4.6
3',5'-cyclic AMP	32.1	3.14	9.8	Guo		4.5
2'-Deoxyadenosine	37.9	2.32	6.1	Guo		7.3

approaches for the internal standardization for isocratic and gradient elution. In the case of isocratic elution, applied at the beginning of our elution program, the ratio of the capacity factors, $\kappa_i/\kappa_{(IS)}$, or of the net retention times, $t'_{Ri}/t'_{R(IS)}$, can be used for the internal standardization because this ratio is constant if no changes in selectivity are induced by the stationary phase. In the case of gradient elution, the equations derived by Schoenmakers *et al.*[15] for linear gradients and a fairly linear dependence of ln κ on the volume fraction of the stronger solvent component suggest that the differences in the retention time values should be approximately constant. This is discussed briefly in Appendix I, and is valid for the nucleosides and deoxynucleosides eluted in the later part of the gradient elution program. Fig. 2 shows in a qualitative way that for many of these compounds the differences in the retention times, $t_{Ri} - t_{R(IS)}$, are approximately constant when applying this gradient.

The column-to-column reproducibility of data is shown in Tables VII and VIII. Table VII gives by the standard deviation of the retention data measured on the eight different columns, including packing materials with different alkyl chain lengths. The standard deviation of the retention time, $s(t_{Ri})$, and the standard deviation after internal standardization will now be discussed, first by use of the selectivity coefficients with respect to the internal standard compound, $\kappa_i/\kappa_{(IS)}$, for the bases eluted in the initial, isocratic part of the elution program, and secondly using the differences in the retention times, $t_{Ri} - t_{R(IS)}$, for the compounds eluted in the later part of the gradient program. Allopurinol is used as the internal standard for the bases and guanosine for the nucleosides.

TABLE VIII

REPRODUCIBILITY OF THE RETENTION TIMES ON COLUMNS 4.1 TO 4.4 HAVING THE SAME SPECIFICATIONS

	Mean t_{Ri} *(min)*	$s(t_{Ri})$		*IS*	$s(\kappa_i/\kappa_{(IS)})$ *(%)*	$s(t_{Ri} - t_{R(IS)})$ *(%)*
		min	*%*			
Isocratic region						
Bases						
Uracil	5.81	0.09	1.6	Allo	1.4	
Uric acid	6.54	0.14	2.1	Allo	0.9	
Hypoxanthine	8.69	0.38	4.3	Allo	1.4	
Xanthine	9.63	0.37	3.8	Allo	1.7	
Gradient region						
Nucleosides						
Inosine	18.7	0.95	5.1	Guo		0.6
2'-Deoxyinosine	24.0	0.68	2.8	Guo		1.4
2'-Deoxyguanosine	25.7	0.68	2.6	Guo		1.7
Adenosine	34.3	0.86	2.5	Guo		1.3
3',5'-cyclic AMP	34.3	1.32	3.9	Guo		1.5
2'-Deoxyadenosine	38.9	1.26	3.2	Guo		3.1

The use of allopurinol as internal standard for bases results in a reduction in the standard deviation for most of the investigated bases to about 3.5–5.0%. For nucleosides eluted in the gradient part of the program there is a remarkable reduction in the standard deviation in most cases, when using $s(t_{Ri} - t_{R(IS)})$. Guanosine served as internal standard. The standard deviations are less than 5%, except for 2'-deoxyadenosine due to the reasons already mentioned. Table VIII shows the corresponding standard deviations on the four packing materials with the same specification (columns 4.1 to 4.4).

With regard to the question of reoptimization of the elution program when using different support materials, Fig. 2 reveals that the variations in the retention behaviour induced by the stationary phases are too large to obtain a satisfactory

TABLE IX

SELECTIVITY COEFFICIENTS, r_{ji}, OF PAIRS OF COMPOUNDS WHERE A REOPTIMIZATION OF THE GRADIENT PROGRAM IS NEEDED FOR SOME COLUMNS

Compounds	*Column*							
	1	*2*	*3*	*4.1*	*4.2*	*4.3*	*4.4*	*5*
Cyd–UA	1.06	1.04	1.05	1.18	1.22	1.12	1.20	1.02
Hyp–Urd	1.13	1.13	1.11	0.83	0.74	1.00	1.00	1.67
Ino–Allo	0.95	0.97	1.00	1.65	1.74	1.35	1.36	1.00
Ado–cAMP*	0.96	1.03	1.04	0.97	0.98	1.02	1.03	0.995

* These compounds are eluted in the gradient part of the elution program. Selectivity coefficients are given in formal analogy to the isocratic case only for the purpose of illustrating the need for reoptimization.

separation of all compounds with the same gradient program. This effect is illustrated in Table IX by the selectivity coefficients for the pairs cytidine–uric acid (column 5), hypoxanthine–uridine (columns 4.3 and 4.4), inosine–allopurinol (column 5) and adenosine–3′,5′-cyclic AMP (column 5). Unfortunately, a loss in resolution is found even for packing materials having the same specification. For these pairs and for the columns mentioned, the gradient program has to be reoptimized.

CONCLUSIONS

The influence of alkyl-modified surfaces on the retention characteristics and the separation of purines and pyrimidines in reversed-phase chromatography has been investigated. A gradient elution program has been used which allows the separation of at least fifteen of the purine and pyrimidine bases, ribonucleosides and 2′-deoxyribonucleosides and 3′,5′-cyclic AMP.

The use of long alkyl chain lengths (octadecylsilica) results in significantly enhanced retention times for nucleosides. This allows an improved separation in those cases where advantage can be taken of the increased retention of nucleosides relative to the bases which show significantly smaller changes in retention time. The pore diameter has a definite but small influence: it cannot be used in a rational way for the adjustment of selectivities.

When using different but similar stationary phases, a variation of the retention data is generally observed, even on materials having the same specifications. However, similar compounds were found to undergo similar trends in retention. This facilitates the peak identification by use of internal standards. Wheras the ratios of capacity factors are suitable in the isocratic range at the beginning of the elution program, retention time differences should be used in the region where the gradient is applied. However, a change of packing material in most cases requires a reoptimization of the elution program, even for materials having the same specifications. This unsatisfactory batch-to-batch reproducibility with respect to the investigated compounds is a serious limitation upon the application of an optimized elution program, especially when there is a high density of peaks on the chromatogram.

APPENDIX I

Starting from the equations derived by Schoenmakers *et al.*[15], one obtains for the net retention time, t'_{Ri}, of a component i

$$t'_{Ri} = (1/S_i b) \ln [1 + S_i b t_0 \kappa_i(a)] \tag{A1}$$

where t_0 is the hold-up time, κ is the capacity factor, S is the slope of the relationship between ln κ and the volume fraction of the organic modifier in the solution, φ, according to

$$\ln \kappa(\varphi) = \ln \kappa_0 - S\varphi \tag{A2}$$

and b is the slope of the gradient with respect to time:

$$\varphi = a + bt \tag{A3}$$

Calculating the net retention time for a compound 2 with a capacity factor, κ_2, at the concentration a according to

$$\kappa_2(a) = \kappa_1(a) r_{21}(a) \tag{A4}$$

where $r_{21}(a)$ is the selectivity coefficient at the modifier concentration, a, one obtains:

$$t'_{R2} = (1/S_2 b) \ln [1 + S_2 b t_0 \kappa_1(a) r_{21}(a)] \tag{A5}$$

Assuming the S values for components 1 and 2 to be approximately equal in the region investigated (a reasonable assumption for similar nucleic acid components[15,16]) and for $S_i b t_0 \kappa_i(a) \gg 1$, eqn. A5 can be rewritten:

$$t'_{R2} = t'_{R1} + (1/S_2 b) \ln r_{21}(a) \tag{A6}$$

Eqn. A6 shows that, under the condition of constant selectivity, the differences in retention times are constant with a linear gradient.

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