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Computer Optimization for RP-HPLC Separation of Some Nucleosides

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The DrylabG software (LC Resources, Lafayette, CA, USA) was applied for optimization of HPLC resolution of some nucleosides in the reversed-phase systems. Two preliminary runs based on linear gradient range of acetonitrile (ACN) from 0% (pure buffer) to 20% and of methanol (MeOH) from 0% to 50% are shown to be satisfactory for optimization of the resolution. A good agreement between simulated and experimental chromatograms was observed.

Zastosowano oprogramowanie DrylabG (LC Resources, Lafayette, CA, USA) do optymalizacji rozdzielenia wybranych nukleozydów w układach HPLC z odwróconymi fazami. Optymalizację oparto na danych uzykanych z dwóch wstępnych chromatogramów wykonanych przy zastosowaniu gradientu acetonitrylu od 0 % (bufor) do 20 % oraz metanolu od 0 % do 50 %. Otrzymano dobrą zgodność danych symulowanych i eksperymentalnych w obu badanych układach chromatograficznych.

Chromatographic analysis of nucleosides is of importance especially in the biomedical area, *e.g.* in methabolic profiling and the studies of disease processes. Nucleosides are present in human serum and urine as, among others, the products of RNA and particulary tRNA degradation. An elevated level of modified nucleosides in serum and urine was found to be a possible tumor marker [1–4].

Effective chromatographic analysis of these substances was reported for the reversed-phase, ion pair, and ion exchange liquid chromatography [4–6]. In the reversed-phase systems a gradient elution is preferred for the separation of variety of analytes. For this purpose even 50 cm long columns were applied [3]. It seems that

an improvement of the chromatographic separation of these solutes is still a challange for the chromatographers.

In our investigation we have applied the DrylabG software to this problem. The program enables to perform a simulation of a chromatographic process basing on the following relationships: 1) dependence of the retention on the mobile phase composition, 2) dependence of the column plate number on the experimental conditions, 3) interrelationships of the isocratic and gradient retention, and 4) predictability of the gradient retention as a function of gradient conditions [7,8]. The typical procedure applied for the chromatogram simulations in RP-HPLC systems, by means of this software, is based on the system variables and the retention data of the solutes obtained in two preliminary gradient runs, from 5% to 100% of organic modifier in water with different steepness. Usually the second run is three times longer than the first one. For some nucleosides the starting eluent concentration (5% org. modifier) is too high because these compounds are eluted in a very small retention time. Then the calculation based on this data is not precise. One way to solve this problem is to decrease the starting eluent concentration in two preliminary runs even to 0% of the modifier [9]. This leads to the stronger retention of the solutes which then can be determined more precisely and the data can be applied for the simulation of the chromatographic process by means of the DrylabG software.

EXPERIMENTAL

HPLC experiments were carried out using a HP-1050 liquid chromatograph (Hewlett–Packard, Palo Alto, CA, USA) equiped with a 20 µl sample injector Model 7125 (Rheodyne, Cotati, CA, USA) and a programable spectrophotometric detector operated at 230 and 254 nm. The chromatograms were recorded with a Hewlett–Packard Model 3396A reporting integrator. A stainless-steel columns, 100×4.6 mm and 250×4.6 mm I.D. were packed with 7 µm, LiChrosorb Si 100 (Merck, Darmstadt, Germany) after silanization with octadecyldimethylchlorosilane (O. Ch., Lublin, Poland). The first column had an efficiency of 3600 and the second of 8200 theoretical plates, as was determined using toluene as the test solute eluted with methanol + water (60 + 40) at a flow rate of 1 ml min^{-1}. The dwel volume of the equipment was determined by runing a blank gradient without the column. In the experiments, the eluent composed of acetonitrile or methanol gradient grade (Merck, Darmstadt, Germany) and buffer of pH 5.1 (0.01 mol l^{-1} ammonium dihydrogen phosphate, (Merck, Darmstadt, Germany) in doubly distilled water were used. The sample was a mixture of nine components (Sigma Chemical Company, St. Louis, Mo., USA), see Table 1.

RESULTS AND DISCUSSION

The preliminary data (Table 1) for the computer optomization by means of DrylabG software were obtained using a short column (10 cm) and the acetonitrile gradient from 0% to 20% in buffer (pH 5.1) from two runs in 15 and 45 min, respectively. The larger starting acetonitrile concentrations in preliminary runs were too high because the retention times of 5,6-dihydrouridine and pseudouridine were too short, near the dead time of the column. Figure 1 demonstrates the relative

resolution map, RRM, *i.e.* resolution, R_S, *vs.* gradient time, t_g. It can be seen that maximum resolution, 0.6, can be achieved with a gradient time about 50 min. A beter chromatographic efficiency was obtained applying a segmented gradient elution and a longer 25 cm column. In Table 2 the simulated and the real retention data are compared for this conditions, and in Fig. 2 the simulated and the real chromatograms are given. The data show a good agreement of the simulated and the experimental retention especially for the strongly retained solutes with exception of 1-methyladenosine.

Table 1. Input values for retention optimization by DrylagG software in acetonitrile system

System variables			
Dwell volume, ml		0.70	
Column length, cm		10.00	
Column diameter, cm		0.46	
Fow rate, ml min^{-1}		1.00	
Starting % B (ACN)		0.0	
Final % B (ACN)		20.0	
Gradient time, run 1, min		15.0	
Gradient time, run 2, min		45.0	
Retention Entries			
band	band name	run 1 t_R, min	run 2 t_R, min
1	5,6-dihydrouridine	2.26	2.29
2	pseudouridine	2.54	2.58
3	1-methyladenosine	3.97	4.92
4	7-methylguanosine	5.43	7.72
5	1-methylinosine	6.55	10.71
6	1-methylguanosine	6.75	11.26
7	*N*4-acetylcytidine	7.01	11.60
8	*N*2-methylguanosine	7.05	11.89
9	8-bromoguanosine	8.89	16.64

*The value was decreased by 0.2 ml to accept the retention time of 5,6-dihydrouridine by software.

Table 2. Simulated and experimental retention data for acetonitrile plus buffer system in a 0.0 to 25.0% segmented gradient run over 50.0 min, 25 cm column

Segment	% B	Time, min	
0	0.0	0.00	
1	4.00	25.00	
2	4.00	28.75	
3	25.00	50.00	
Band	Band name	Retention time, min (simulated)	Retention time, min (experimental)
1	5,6-dihydrouridine	4.72	5.96
2	pseudouridine	5.48	6.75

Table 2 (continuation)			
3	1-methyladenosine	11.44	14.08
4	7-methylguanosine	18.79	19.66
5	1-methylinosine	26.89	27.50
6	1-methylguanosine	28.40	28.81
7	*N*4-acetylcytidine	29.29	30.36
8	*N*2-methylguanosine	30.21	31.23
9	8-bromoguanosine	37.73	38.46

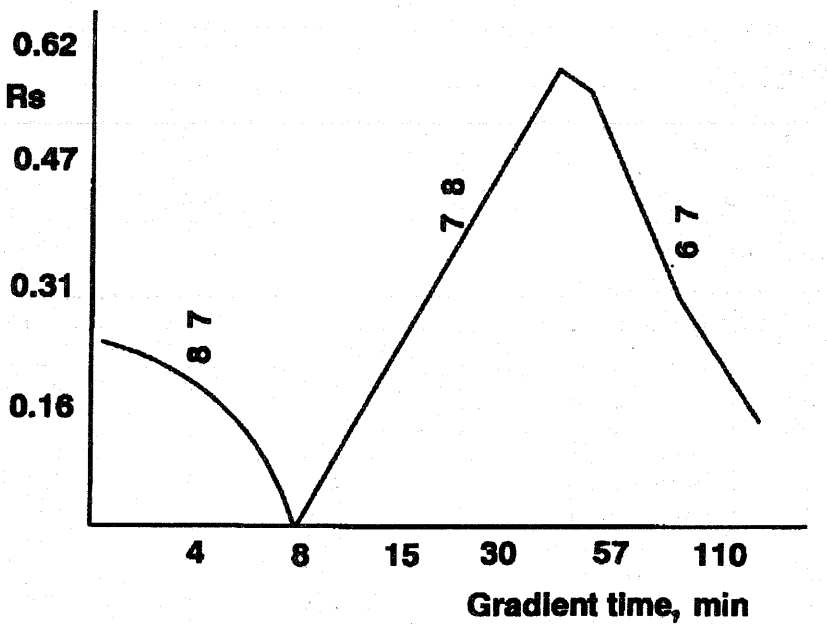

Figure 1. Relative resolution map for nucleosides. 0–20% acetonitrile–buffer gradient; compounds as in Table 1

A similar procedure was applied for the methanol system. The relative resolution map shows that the maximum resolution (about 0.7) can be achieved with the linear methanol gradient in 100 min and a 10 cm column (Fig. 3). After the computer optimization based on the retention data presented in Table 3, the final conditions with three segment gradient and a 25 cm long column were chosen. In Table 4 and in Fig. 4 the simulated and the real retention times and chromatograms are compared. The data show also an acceptable agreement between the simulated and the experimental chromatograms.

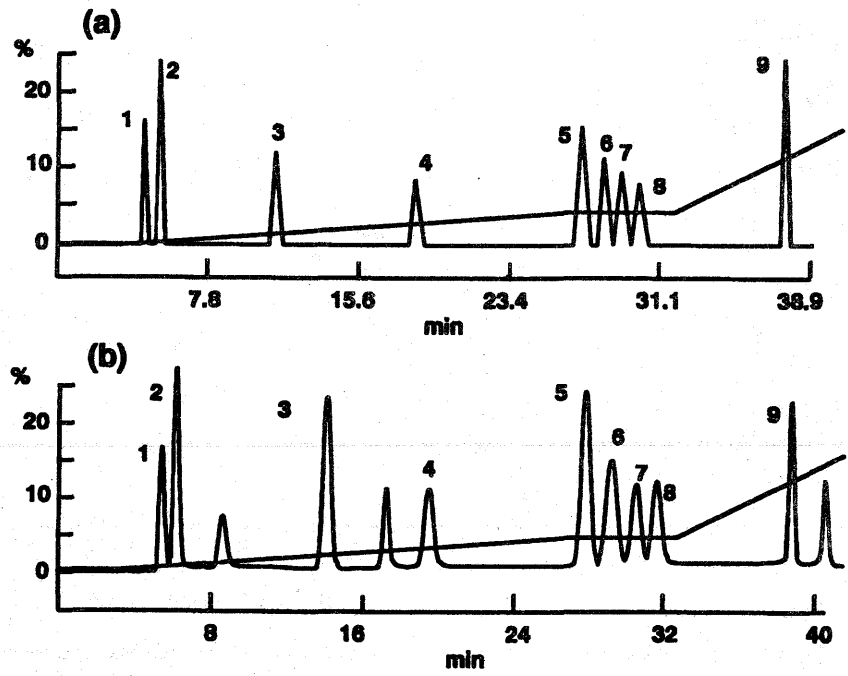

Figure 2. Simulated (a) and experimental (b) chromatograms of nucleosides; conditions as in Table 2

Table 3. Input values for retention optimization by DrylabG software in methanol system

System variables			
Dwell volume, ml		0.70*	
Column length, cm		10.00	
Column diameter, cm		0.46	
Flow rate, ml min^{-1}		1.00	
Starting % B (MeOH)		0.0	
Final % B (MeOH)		50.0	
Gradient time, run 1, min		15.0	
Gradient time, run 2, min		45.0	
Retention Entries			
band	band name	run 1 t_R, min	run 2 t_R, min
1	5,6-dihydrouridine	2.22	2.26
2	pseudouridine	2.45	2.53
3	1-methyladenosine	3.61	4.43
4	7-methylguanosine	5.35	7.43
5	1-methylinosine	6.18	9.99
6	1-methylguanosine	6.52	10.71
7	*N*4-acetylcytidine	6.71	11.09
8	*N*2-methylguanosine	6.74	11.32
9	8-bromoguanosine	8.71	16.09

*The value was decreased by 0.2 ml to accept the retention time of 5,6-dihydrouridine by software.

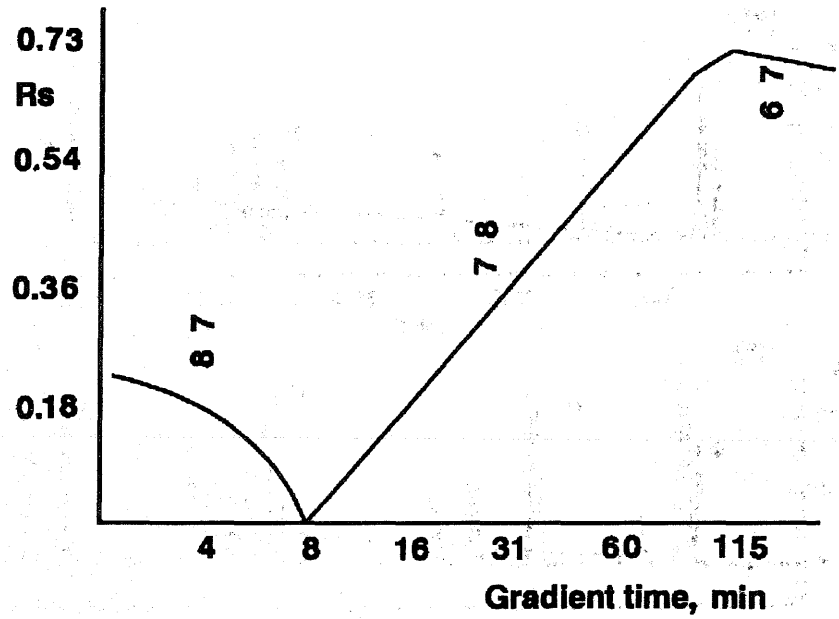

Figure 3. Relative resolution map for nucleosides. 0–50% methanol–buffer gradient; compounds as in Table 3

Table 4. Simulated and experimental retention data for methanol plus buffer system in a 0.0 to 34.0 % segmented gradient run over 52.5 min, 25 cm column

Segment	% B	Time, min	
0	0.0	0.00	
1	4.00	2.50	
2	4.00	35.00	
3	34.00	52.50	
Band	Band name	Retention time, min (simulated)	Retention time, min (experimental)
1	5,6-dihydrouridine	4.47	5.11
2	pseudouridine	5.08	5.83
3	1-methyladenosine	8.40	9.27
4	7-methylguanosine	17.12	18.51
5	1-methylinosine	28.98	30.01
6	1-methylguanosine	32.90	34.16
7	*N*4-acetylcytidine	34.97	36.28
8	*N*2-methylguanosine	37.07	38.47
9	8-bromoguanosine	48.12	49.84

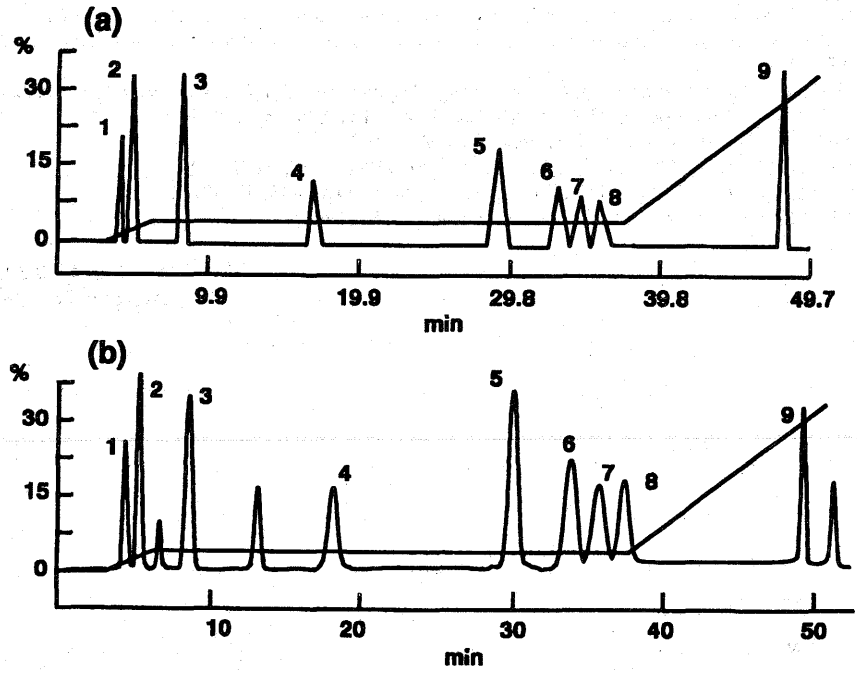

Figure 4. Simulated (a) and experimental (b) chromatograms of nucleosides; conditions as in Table 4

The optimization procedure in the RP-HPLC systems with the DrylabG software for the hydrophilic substances such as nucleosides can be applied if the preliminary data are based on the gradient range of the modifier started from pure buffer. It seems that the procedure can lead ocasionally to the retention discriepancy between the simulated and the experimental chromatograms, especially for the solutes with lower retention. However, the resolution should be similar, what can be useful in the optimization of such systems.

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