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COMPUTER-ASSISTED DEVELOPMENT OF A HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR FRACTIONATING SELECTED NITRO DERIVATIVES OF POLYAROMATIC HYDROCARBONS

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SUMMARY

The process of developing a high-performance liquid chromatographic (HPLC) method for fractionating nitro derivatives of polyaromatic hydrocarbons using the HPLC simulation program DryLab G is presented. A comparison of the computer-simulated results and experimental data revealed retention time prediction errors during preliminary stages of the method development. These errors resulted from the simulation of mobile phase conditions that required the program, based on the input data, to extrapolate beyond the point at which accurate retention times could be reasonably predicted. We discuss the cause and solution of these retention time prediction errors. After entering new input data, the final "optimum" HPLC method developed using DryLab G simulations correlated well with the experimentally observed chromatogram.

INTRODUCTION

Nitro derivatives of polyaromatic hydrocarbons (nitro-PAHs) are direct-acting mutagens that are formed by the reaction of PAHs with oxides of nitrogen and nitric acid[1–5]. Nitro-PAHs can form during primary source emissions or from post-emission atmospheric reactions. For example, 2-nitropyrene and 2-nitrofluoranthene are believed to be formed by atmospheric reactions, whereas 1-nitropyrene and 3-nitrofluoranthene are believed to be formed during primary source emissions[6,7]. The relative concentrations of these selected nitro-PAHs in ambient air particulate extracts can be used as a guide to assess the importance of primary emissions *vs.* post-emission atmospheric reactions in the production of nitro-PAHs. To measure the relative concentrations of the selected nitro-PAHs, a reversed-phase high-performance liquid chromatographic (HPLC) method was developed with the assistance of the DryLab G HPLC simulation program.

The development of HPLC methods traditionally has been a time-consuming process of trial and error. Several HPLC test runs are usually required to optimize the capacity factors, the plate number and the band spacing for any given separation problem. Recently, computer software based on well documented equations and as-

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sumptions has been developed to enable quick, easy and reasonably accurate simulations of experimental chromatographic test runs to be made[8–15]. In general, the computer simulations require only two test gradients to simulate multiple chromatograms.

Snyder *et al.*[9] have reported the use of DryLab to develop an HPLC method of separating nitro derivatives of benzene. In this work, the DryLab program was used to develop an HPLC method for the analysis of selected nitro-PAHs. In particular, the program DryLab G was used to develop a segmented isocratic HPLC method for analysing 1-nitropyrene, 2-nitropyrene, 2-nitrofluoranthene, 3-nitrofluoranthene and 6-nitrobenz[*a*]pyrene in a standard mixture. The process of using the DryLab G program to develop an isocratic HPLC method for fractionating nitro-PAHs is presented together with comparable experimental data.

EXPERIMENTAL

Reagents

All solvents were obtained from Baxter Burdick and Jackson (Muskegon, MI, U.S.A.). A nitro-PAH mixture was prepared with NBS Standard Reference Material SRM 1587 (National Bureau of Standards, Gaithersburg, MD, U.S.A.), 2-nitrofluoranthene, 2-nitropyrene and 1-nitro[$^2H_9$]pyrene (Chemsyn Science Labs., Lenexa, KA, U.S.A.). The concentrations of the targeted nitro-PAHs in the final mixture were 1-nitro[$^2H_9$]pyrene 0.62, 1-nitropyrene 0.46, 3-nitrofluoranthene 0.47, 2-nitrofluoranthene 0.41, 2-nitropyrene 0.84 and 6-nitrobenzo[*a*]pyrene 0.31 ng/μl.

HPLC

The HPLC experiments were performed with a Varian Model 5000 instrument in conjunction with a Valco Instruments six-port HPLC valve and a Perkin-Elmer (Maywood, IL, U.S.A.) LS-4 fluorescence spectrophotometer equipped with a 3.0-μl flow cell. A DuPont (Wilmington, DE, U.S.A.) Zorbax ODS reversed-phase HPLC column was used. The mobile phase was methanol–water at a flow-rate of 1.0 ml/min. A 10-μl volume of the nitro-PAH mixture was injected for each HPLC run. Fluorescence detection of amino-PAHs was accomplished by in-line catalytic reductions of the nitro-PAHs to aromatic amines[16]. The HPLC computer simulations were carried out with DryLab G software from LC Resources (Lafayette, CA, U.S.A.) using an IBM AT personal computer. The dwell volume for the HPLC system was determined using a linear gradient profile method[13,17].

RESULTS AND DISCUSSION

To use the DryLab program, two HPLC test gradients are required to obtain data from which chromatographic simulations can be calculated. Therefore, two HPLC test gradients of 75–100% methanol–water with gradient times of 30 and 90 min were performed. The retention times and peak heights, together with other system variables listed in Table I, were entered into the DryLab G program.

Based on the k' (capacity factor) ratio of the first and last peaks, the DryLab program automatically determined that an isocratic method would be best for sample fractionation. Although the DryLab G program is designed to assist in the devel-

TABLE I

SYSTEM VARIABLES AND RETENTION TIME (t_R) DATA FROM THE 75–100% TEST GRADIENTS THAT WERE ENTERED INTO THE DRYLAB PROGRAM

The analyte name was not entered.

System variables

Variable	*Value*
Dwell volume (ml)	3.32
Column length (cm)	25.00
Column diameter (cm)	0.46
Flow-rate (ml/min)	1.00
Starting %B	75.00
Final %B	100.00
Gradient time, 1st run (min)	30.00
Gradient time, 2nd run (min)	90.00

Retention entries (No. of bands = 8)

Peak No.	*Analyte*	*Run 1*		*Run 2*
		t_R *(min)*	*Peak height (mm)*	t_R *(min)*
1	1-Nitro[$^2H_9$]pyrene	20.30	170	26.89
2	1-Nitropyrene	20.81	218	27.87
3	3-Nitrofluoranthene	21.65	56	29.79
4	2-Nitrofluoranthene	22.00	136	30.57
5	2-Nitropyrene	23.04	38	32.65
6	Unknown nitro-PAH	23.04	5	33.88
7	Unknown nitro-PAH	24.96	77	37.77
8	6-Nitrobenzo[*a*]pyrene	28.83	14	47.80

opment of gradient methods, it also can aid in the development of isocratic methods. This is accomplished by representing isocratic methods as extremely shallow gradients with a change in solvent of 0.001% over the course of the isocratic simulation. Therefore, we continued to use the DryLab G program to develop an isocratic HPLC method.

A series of HPLC methods, both isocratic and segmented, was tested using DryLab simulations to determine the optimum HPLC conditions for fractionation of the selected nitro-PAHs. The chromatographic parameters used in the computer simulations were the same as those which would have been used in actual HPLC runs. It is important to note that each simulation required only aproximately 1–5 min, whereas an actual HPLC run would require about 1 h. The optimum method that balanced resolution with peak width and gradient elution time was an isocratic segmented gradient program with an isocratic step at 75% methanol–water for 40.0 min, followed by a 2.0-min step to 100% methanol that was held until the last peak eluted. The retention times and resolution values predicted by DryLab are given in Table II, A.

TABLE II

RETENTION TIME (t_R) AND RESOLUTION (R_s) VALUES FROM THE LISTED METHOD FOR (A) THE DRYLAB PREDICTIONS BASED ON THE 75–100% METHANOL–WATER TEST GRADIENTS, (B) THE ACTUAL HPLC RUN AND (C) THE 75–85% METHANOL–WATER TEST GRADIENT DRYLAB PREDICTIONS

	Gradient	
	Time (min)	*Methanol (%)*
	0	75
	40	75
	42	100

Peak	*(A) Predicted from 75-100% test*		*(B) Observed*		*(C) Predicted from 75-85% test*	
	t_R *(min)*	R_s	t_R *(min)*	R_s	t_R *(min)*	R_s
1	34.96	1.28	29.88	1.35	29.83	1.35
2	36.79	2.71	31.46	2.67	31.49	2.57
3	41.01	1.04	34.63	1.55	34.90	1.31
4	42.76	3.03	36.55	2.46	36.78	1.87
5	46.61	1.53	39.76	3.65	39.65	3.12
6	47.48	2.31	45.20	2.83	44.92	2.50
7	48.09	6.38	48.03	6.29	47.57	5.25
8	49.70		50.27		49.18	

An actual HPLC run was made to test the proposed HPLC method; the observed retention time and resolution values are listed in Table II, B. A comparison of the retention times from the predicted and observed chromatogramphic runs showed poor correlation. The retention times of peaks 1–6, which eluted during the isocratic 75% methanol portion of the actual HPLC run, were as much as 2.3–6.9 min shorter than those predicted by DryLab G. Peaks 7 and 8, which eluted during the 100% methanol step of the gradient, had retention times that were within 0.06 and 0.57 min, respectively, of the corresponding predicted values. Although the predicted retention times did not correlate well with those observed experimentally, reasonable estimates of the relative resolution between peaks were achieved. For example, the smallest difference between the predicted and experimental resolution values was 0.04, and although for one peak pair (peaks 5 and 6) the difference was 2.02, the average difference between the predicted and observed resolutions of the remaining peaks was only 0.30.

Two possible causes for the retention-time prediction errors are a malfunctioning solvent delivery system and inadequate input data. An analysis of the solvent delivery system using the mobile phase partitioning test[18] indicated that the solvent partitioning error for our HPLC system was less than 1% in the working range of 75–100% methanol. Therefore, the discrepancy between the predicted and the observed retention times was presumed to have been due to errors based on the initial input data.

To understand how errors in predicting retention times may result from inadequate input data, we must first understand how DryLab G uses the input data from the two initial test gradients. The retention-time prediction is based on the following relationship:

$$\log k' = \log k_w - S\varphi \tag{1}$$

where k' is the gradient capacity factor of the solute, k_w is the value of k' for water as the mobile phase and φ is the volume percentage of organic component in the mobile phase. S is a constant for a given solute and solvent that corresponds to the slope of plot of log k' *vs.* φ. Using the above relationship with the data from the two test gradients, DryLab G calculates k_w and S for each solute (peak); this permits the calculation of solute retention as a function of mobile-phase organic concentration (φ). (For more detailed descriptions on how DryLab G predicts retention times, see refs. 8–15.)

The accuracy of retention-time predictions based on the input data is dependent on three basic factors related to the two test gradients and to the sample[12,13,15]. First, variations in solute retention times during the test gradients can result in deviations in the calculated S value. Thus, in some instances, the predictions are most accurate only within a small range of k' values; in a practical sense this means that the predictions are accurate only within a small range of chromatographic conditions. This effect can be amplified if the ratio of the k' values from the two test gradients is less than 3 (ref. 13). Second, when band overlapping occurs in the test chromatograms, the retention time for the resulting single peak usually is entered into the program for both of the coeluting peaks. However, this observed retention time is really the "average" retention time of the two peaks[14]; the actual retention time for each peak is unknown, and therefore the actual calculated S value for each peak is unknown. Finally, some samples exhibit deviations from linearity in the relationship $\log k' = \log k_w - S\varphi$. When this occurs, the predictions again would be accurate only within a limited range of k' values for which the actual curved log k' *vs.* φ plot correlates with the linear relationship. The primary cause of error in these examples is extrapolation beyond the point at which the input data can be used reasonably to predict retention times. [When fractionating compounds that are chemically similar, errors of this type are generally uniform among the compounds and often result in correct predictions of separations, but for an actual isocratic mobile phase of slightly different composition than predicted by computer simulation. For example, in the present instance, the use of 76.5% B instead of 75% B in the simulation (Table II, A) reduces the retention time error from ±12% to only ±1.5%. This suggests that predictions based on a "corrected" mobile phase composition (+1.5% B) will yield adequately reliable results. Alternatively, if the analytes are not similar, new test gradients could be run that reduce the amount of extrapolation required for accurates simulations.]

For this example, two new test gradients were chosen using the DryLab G program. The chosen test gradients were predicted by the program to have k' values closer to the value of the 75% methanol isocratic gradient already developed using DryLab. The new test gradients of 75–85% methanol in 60 and 180 min were run, and the retention times and peak heights were entered into the DryLab G program together with the other system variables listed in Table III. With these test gradients, band

TABLE III

SYSTEM VARIABLES AND RETENTION TIME (t_R) DATA FROM THE 75–85% METHANOL TEST GRADIENTS THAT WERE ENTERED INTO THE DRYLAB PROGRAM

System variables

Variable	*Value*
Dwell volume (ml)	3.32
Column length (cm)	25.00
Column diameter (cm)	0.46
Flow-rate (ml/min)	1.00
Starting %B	75.00
Final %B	85.00
Gradient time, 1st run (min)	60.00
Gradient time, 2nd run (min)	180.00

Retention entries (No. of bands = 8)

Peak	*Run 1*		*Run 2*
	t_R *(min)*	*Peak height (mm)*	t_R *(min)*
1	26.52	114	28.56
2	27.62	148	29.99
3	29.63	29	32.80
4	30.77	80	34.36
5	32.95	25	36.94
6	35.20	4	40.80
7	39.66	39	46.93
8	52.06	8	66.54

overlapping was not a problem, and less extrapolation by DryLab was required to predict retention times in the 75–85% methanol range.

The simulated retention times and resolution values of the 75% isocratic method as predicted using the data generated from the new 75–85% test gradients are presented in Tabel II, C. As shown, the correlation between the predicted and observed retention time values was greatly improved. The predicted retention times of peaks 1–6, which eluted in the isocratic 75% methanol portion of the actual HPLC run, were within 0.05–0.28 min of the actual values. Peaks 7 and 8, which eluted during the 100% methanol step of the gradient, were within 0.46 and 1.09 min, respectively, of the predicted values. We could now proceed to develop an improved HPLC method using DryLab G simulations with reasonable accuracy.

A second series of HPLC methods was tested using DryLab simulations based on data from the 75–85% test gradients. A second segmented isocratic gradient method was developed for optimum separation. The method involved an isocratic step at 78% methanol for 25 min, followed by a 2.0-min step to 100% methanol that was held until the last peak eluted at 34.31 min. Although other methods yielding better peak resolution were developed using DryLab G simulation, the chosen method provided the best balance among gradient elution time, resolution and peak width.

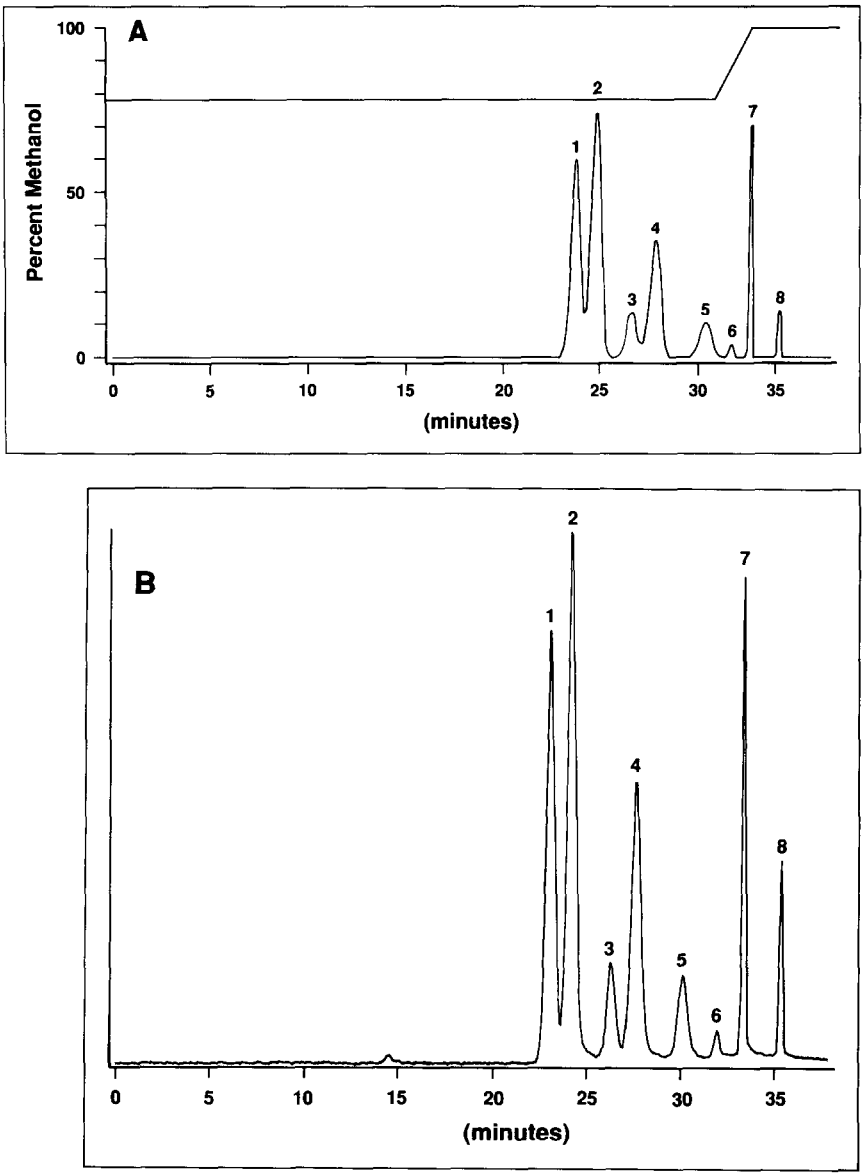

Fig. 1. Optimum isocratic segmented gradient: (A) DryLab G simulation and (B) actual separation using a Zorbax ODS (25 × 0.46 cm I.D.) column with an isocratic gradient of 78% methanol in water for 25 min followed by a 2-min step to 100% methanol held until the last peak eluted; flow-rate, 1.0 ml/min.

An actual HPLC run was made to test the DryLab G-simulated method for retention time and resolution accuracy. As shown in Fig. 1, the predicted and observed chromatograms are remarkably similar. The slight differences between the predicted and observed retention times and resolution values can be more clearly

TABLE IV

RETENTION TIME (t_R) AND RESOLUTION (R_s) VALUES FROM THE LISTED METHOD FOR (A) THE DRYLAB PREDICTIONS BASED ON THE 75–85% METHANOL–WATER TEST GRADIENTS AND (B) THE ACTUAL HPLC RUN WITH THE LISTED GRADIENT

	Gradient	
	Time (min)	*Methanol (%)*
	0	78
	25	78
	27	100

Peak	*(A) Predicted*		*(B) Observed*	
	t_R *(min)*	R_s	t_R *(min)*	R_s
1	23.77	1.07	22.96	1.40
2	24.82	1.83	24.12	2.56
3	26.70	1.09	26.29	1.38
4	27.90	2.25	27.53	2.67
5	30.53	1.58	30.11	2.37
6	31.89	2.45	31.96	3.14
7	32.83	5.41	33.29	6.59
8	34.31		35.30	

examined in Table IV. The retention times of peaks 1–6 in the actual chromatogram eluted within 0.07–0.81 min of the corresponding predicted values. The retention times of peaks 7 and 8 were within 0.46 to 1.07 min, respectively, of their predicted values. The relative resolution values of the predicted and observed chromatograms are reasonably similar.

CONCLUSIONS

The HPLC simulation program DryLab G was used to develop a segmented isocratic HPLC method for fractionating nitro-PAHs. Difficulties in the prediction of retention times were encountered when attempting to simulate mobile phase conditions that required DryLab to extrapolate beyond reasonable input data limits. These difficulties were overcome by using DryLab G to establish new test gradients from which accurate retention-time predictions could be made in the optimum range of mobile phase conditions. The difficulties encountered with the prediction of retention times demonstrated the importance of the two test gradients and the usefulness of the DryLab simulations in choosing new test gradients when necessary. Even with these minor difficulties, developing an HPLC method with the aid of the DryLab program required only a fraction of the time a classical approach to methods development would have taken. This allowed the examination of many more possible method variations for fractionating selected nitro-PAHs than would have been practical using a classical approach.

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