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EFFECT OF FLOW RATES AND THE SLOPE OF THE LINEAR CONCENTRATION GRADIENT ON PEAK AREAS IN HIGH PRESSURE LIQUID CHROMATOGRAPHY

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SUMMARY

The effect of flow rates and the slope of the linear concentration gradient on peak areas in high pressure liquid chromatography was investigated. It was found that within limits the slope of the gradient had no effect on the peak areas. However, the peak areas were inversely proportional to the flow rates. Therefore, for accurate quantitative analyses by high pressure liquid chromatography using a linear gradient elution mode, flow rates must be accurately determined and controlled. Then calibrations obtained at one set of flow rates can be applied to other flow rates with the appropriate proportionality factor.

INTRODUCTION

In the analysis of nucleotide concentrations in cell extracts by high pressure liquid chromatography, it is often desirable to decrease the time necessary for each analysis; hence, the retention times of the nucleotides must be reduced. This is especially true in carrying out time studies on the effect of drugs on nucleotide pools since one study alone may involve 30–40 cell extracts. At other times, it is impossible to achieve a difficult separation and it is necessary to reduce flow rates and/or the slope of the linear concentration gradient in order to provide better resolution.

HORVATH and coworkers thoroughly investigated the operating parameters in high pressure liquid chromatography and their effect on the separation of nucleotides[1] and nucleosides[2], and KIRKLAND[3] studied the performance of the UV detector with efficient liquid chromatographic columns. Although BURTIS AND GERE[4] did research on the relationship of buffer concentration with flow rates and starting volumes, the effect of these parameters on peak areas was not studied. Theoretically, when using a UV detector with a fixed speed recorder, the peak area should be inversely proportional to the flow rate[5]. However, whether this proportionality holds true using the gradient elution mode must be determined experimentally. Since it was found that changing the flow rates and the slope of the linear concentration gradient were two of the better ways to reduce the retention times[6], the effects of these variables on peak areas were investigated. "Starting volume" (V_0) refers to the volume of low concen-

tration eluent at t_0 when gradient elution is used. It has been found[7] that V_0 is inversely proportional to the slope of the linear concentration gradient and that in this particular system[5] either the salt concentration of the strong eluent or V_0 can be changed in order to vary the slope without changing the flow rate. Although it is more precise to refer to the effect of the slope of the linear concentration gradient rather than to starting volume, the term starting volume will be used to denote the actual changes in the starting volume of the low concentration eluent.

Three separate studies of the effect of flow rates on peak areas were carried out; two using standard solutions of the mono-, di- and triphosphate mononucleotides and one using extracts of human erythrocytes. One study was made on the effect of slope of the linear concentration gradient on peak areas using a standard solution of the adenine and guanine nucleotides*.

EXPERIMENTAL

Apparatus

In the Varian LCS-1000 Nucleic Acid Analyzer used, the cylindrical flow cell has a volume of 8 μl, a diameter of 1 mm and a pathlength of 10 mm. The double beam UV detector operates at 254 μm. A 10 mV Texas Instrument Recorder was used. The column, purchased from Varian Aerograph Corp., is 1.0 mm I.D., 3 m in length and is packed with a pellicular anion-exchange resin (labeled LFS).

Mode of operation

For all samples, the gradient elution method was used. The low concentration eluent was 0.015 M KH_2PO_4 and the high concentration eluent was 0.25 M KH_2PO_4 in 2.2 M KCl. In the study of the effect of flow rates on peak areas of standard solution, the mixing chamber was filled with low concentration eluent to the 50 ml mark (V_0) and five 5 μl aliquots of the same sample were run at five different flow rates. The rates were checked before starting each run, in the middle of it and after it was finished. The same operator injected the samples to minimize sampling errors. In order to maintain the linearity of the gradient, the ratio of column flow rate (flow rate of eluent into the column) to the gradient flow rate (flow rate of high concentration eluent into the low) remained constant**. In the study of the effect of the slope of the linear concentration gradient on peak areas, the starting volumes were varied and the flow rates were kept constant at 12 ml/h for the column flow rate and 6 ml/h for the gradient flow rate.

Chemicals

Reagent grade KH_2PO_4 and KCl were purchased from Mallinkrodt. Standard solutions of the adenine and guanine nucleotides were prepared from commercially available nucleotides purchased from P-L Biochemicals, Inc.

Procedure

In the first study 5 μl aliquots of a solution of 0.25 mM adenine and guanine

* The following abbreviations are used: AMP, ADP, ATP = adenosine 5'-mono, di- and triphosphate. GMP, GDP, GTP = guanosine 5'-mono, di- and triphosphate.

** Hereafter, in referring to flow rates, since the gradients are linear, and the gradient flow rate is always one half the column flow rate, only the column flow rate will be noted.

mono-, di- and triphosphate mononucleotides were run at flow rates of 24, 12 and 6 ml/h. The starting volume was 50 ml and the UV output was 0.08 absorbancy units (a.u.). It was noted that the ratio of flow rate to area appeared to be the same for all six nucleotides, so another study was carried out using 2 μl aliquots of a solution of only the monophosphate nucleotides (AMP and GMP) (0.5 m*M*) but at five different flow rates instead of three: 24, 20, 15, 12 and 8 ml/h. In a third study, the nucleotides extracted from human erythrocytes were run on the analyzer at 24 and 12 ml/h to compare the validity of the results obtained on the standard solutions with those obtained on cell extracts. The extracts of human erythrocytes were prepared by precipitating the protein with cold 12 % TCA and then removing the TCA from the supernatant with water saturated diethyl ether. 10 μl aliquots were injected, the starting volume was 50 ml and the UV output was 0.04 a.u.

In order to find out if the slope of the linear concentration gradient affected the peak areas, a study was carried out using the solution of 0.25 m*M* adenine and guanine nucleotides, keeping the flow rates constant but varying the starting volume (50, 35, 20 and 10 ml).

RESULTS

There was no significant difference in the peak areas when the slope of the linear concentration gradient was varied by changing the starting volume of the low concentration buffer from 50 to 20 ml (Table I). When the starting volume was decreased to 10 ml, however, the peak areas of the triphosphates and GDP increased; however only the GTP increased in are when the starting volume was 20 ml. With a linear gradient on the Varian LCS 1000, retention times can be shortened (Fig. 1) by decreasing the starting volume (down to 20 ml) without significantly affecting peak areas. Thus, the quantitative calibrations as determined on this instrument using a flow rate of 12 ml/h and a starting volume of 50 ml can be applied to samples run with varying starting volumes as small as 20 ml.

TABLE I

EFFECT OF STARTING VOLUME ON PEAK AREAS (in cm^2) OF STANDARD SOLUTION OF ADENINE AND GUANINE NUCLEOTIDES

Flow rates: 12 ml/h (gradient into column); 6 ml/h (high concentration eluent into low). Eluents: 0.015 *M* KH_2PO_4 and 0.25 *M* KH_2PO_4 in 2.2 *M* KCl. Samples: 5 μl of solution of *ca.* 0.25 *M* adenine and guanine nucleotides. UV output: 0.08 a.u. The values given are the average of 3–5 samples run at each starting volume.

Starting volume	*Peak areas* (cm^2)					
	AMP	*ADP*	*ATP*	*GMP*	*GDP*	*GTP*
50	3.23	5.84	3.70	5.88[a]	2.82	3.59
35	3.13	5.29	3.39	5.42	2.66	3.30
20	3.07	5.77	3.91	5.49	3.03	4.00
10	3.13	5.94	4.38	5.51	4.85	4.51

[a] An example of the typical individual values of this average figure are: 5.81, 5.92, 5.82, 5.94 and 5.88. The standard deviation is 0.05 and the coefficient of variation is 0.89 %. However, with the small areas, the coefficient of variation is larger.

With the change in flow rates, it was found that peak area changed appreciably. The areas decreased as the flow rate increased (Tables II and III). The extracts of the erythrocytes were only run at two different flow rates. The ratio of peak areas obtained with flow rates of 12 ml/h to those obtained with flow rates of 24 ml/h for eight different samples are shown in Table IV. By plotting the area *vs.* flow rates in both studies, it can be seen that the plots for all six nucleotides are similar (Figs. 2 and 3). Since the peaks were not significantly higher at the lowest flow rate, there seems to be no drastic increase in column efficiency at the lower flow rates which might have been responsible for the larger increases in peak areas. It was noted that it is more difficult to control flow rates in the lower region and some differences in results may be due to fluctuations in the flow rates.

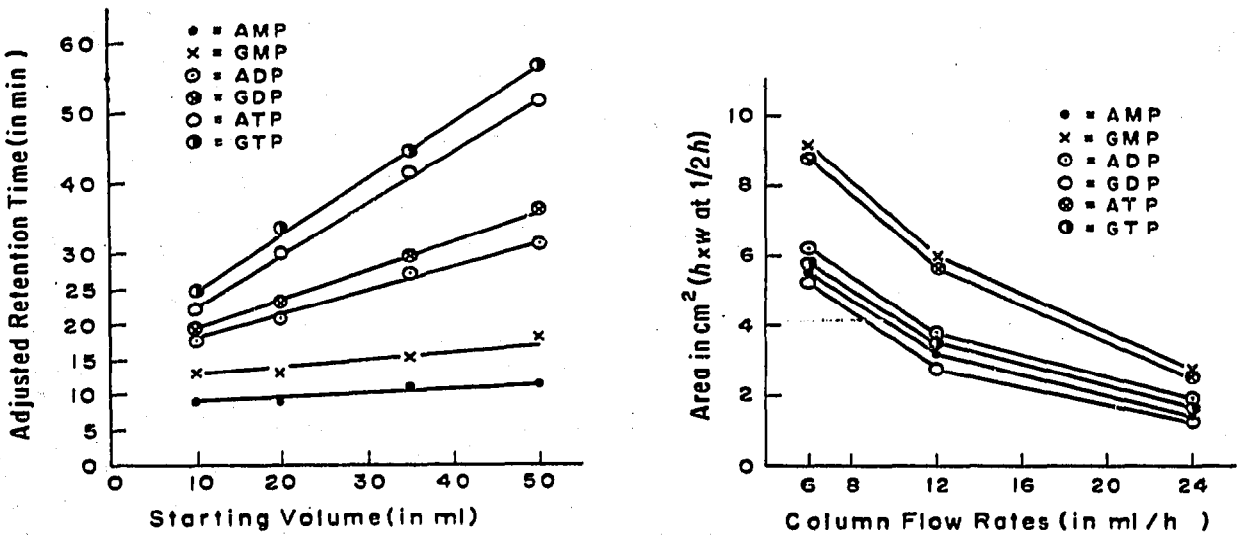


Fig. 1. Plot of adjusted retention time *vs.* the slope of the linear concentration gradient. Flow rates: 12 ml/h, 6 ml/h. Eluents: 0.015 *M* KH_2PO_4 and 0.25 *M* KH_2PO_4 in 2.2 *M* KCl. Samples: 5 μl of a solution of *ca.* 0.25 m*M* adenine and guanine nucleotides. UV output: 0.08 a.u. Adjusted retention time = retention time — hold-up time. Starting volume = volume of low concentration eluent in gradient chamber at t_0.

Fig. 2. Effect of flow rates on peak areas of adenine and guanine nucleotides. V_0 = 50 ml. Eluents: 0.015 *M* KH_2PO_4 and 0.25 *M* KH_2PO_4 in 2.2 *M* KCl. Samples: 5 μl of a solution of *ca.* 0.25 m*M* adenine and guanine nucleotides. UV output: 0.08 a.u.

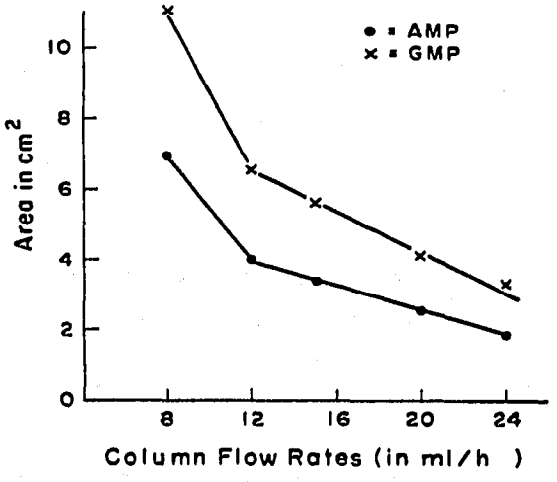
TABLE II

EFFECT OF FLOW RATES ON PEAK AREAS OF STANDARD SOLUTIONS OF ADENINE AND GUANINE NUCLEOTIDES

V_0: 50 ml. Eluents: 0.015 *M* KH_2PO_4 and 0.25 *M* KH_2PO_4 in 2.2 *M* KCl. Samples: 5 μl of a solution of *ca.* 0.25 m*M* adenine and guanine nucleotides. UV output: 0.08 a.u. The values given are an average of 3–5 samples run at each flow rate.

Column flow rate	*Gradient flow rate*	*Peak areas in nucleotides (in cm²)*					
		AMP	*ADP*	*ATP*	*GMP*	*GDP*	*GTP*
24	12	1.56	2.69	1.92	3.06	1.81	1.81
12	6	3.23	5.84	3.70	5.85	3.59	3.59
6	3	5.76	9.20	6.24	8.76	5.32	5.86

Since it was important to learn whether the same proportionality constant can be applied both to the adenine and guanine nucleotides, the area of AMP *vs.* GMP at different flow rates was plotted (Table III and Fig. 4). The relationship between the two nucleotides was linear and the plot went through the origin. Therefore the same



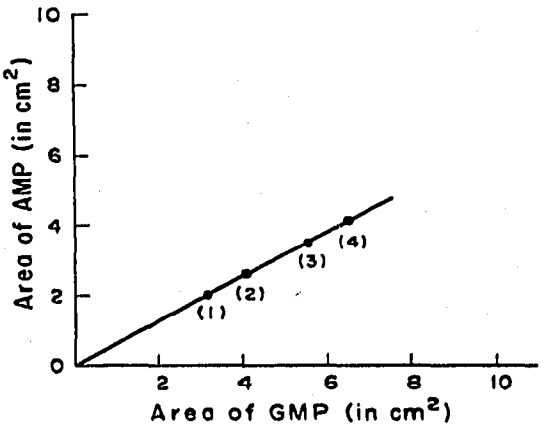


Fig. 3. Effect of flow rates on peak areas of AMP + GMP. V_0 = 50 ml. Eluents: 0.015 *M* KH_2PO_4 and 0.25 *M* KH_2PO_4 in 2.2 *M* KCl. Samples: 2 μl of a solution of *ca.* 0.5 m*M* AMP and GMP. UV output: 0.08 a.u.

Fig. 4. Plot of area of AMP *vs.* area of GMP at different flow rates. Flow rates: (1) = 24 ml/h; (2) = 12 ml/h; (3) = 15 ml/h; (4) = 12 ml/h. Eluents: 0.015 *M* KH_2PO_4 and 0.25 *M* KH_2PO_4 in 2.2 *M* KCl. UV output: 0.08 a.u. Samples: 2 μl of a solution of *ca.* 0.5 m*M* AMP and GMP. V_0 = 50 ml.

TABLE III

EFFECT OF FLOW RATES ON PEAK AREAS OF STANDARD SOLUTION OF AMP AND GMP

V_0: 50 ml. Eluents: 0.015 *M* KH_2PO_4 and 0.25 *M* KH_2PO_4 in 2.2 *M* KCl. Samples: 2 μl of a solution of *ca.* 0.5 m*M* AMP and GMP. UV output: 0.08 a.u. The values given are an average of 5 samples run at each flow rate.

Column flow rate	*Gradient flow rate*	*Peak areas (in cm²)*	
		AMP	*GMP*
24	12	1.98	3.29
20	10	2.56	4.12
15	7.5	3.60	5.61
12	6	4.05	6.64
8	4	6.96	10.63

correction factor can be applied to all adenine and guanine nucleotides when the flow rate is increased from 12 to 24 ml/h. Thus the retention times can be decreased (Fig. 5) but separate calibrations for quantitative determinations do not have to be carried out.

DISCUSSION

In gas chromatography chromatograms are generally described in terms of retention time, peak shape and sharpness. The number of theoretical plates is used as

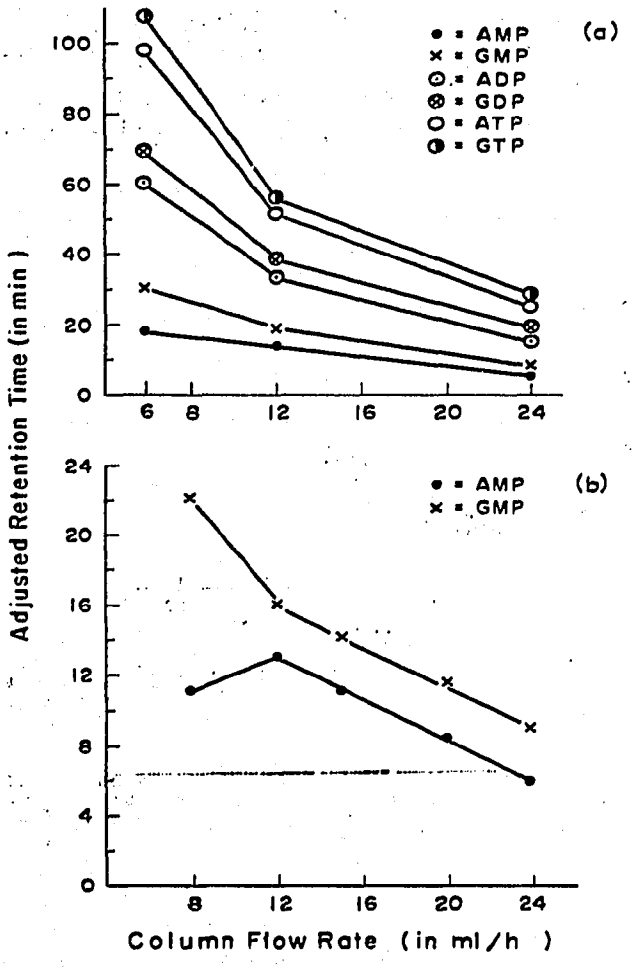


Fig. 5. Adjusted retention time *vs.* flow rates. V_0 = 50 ml. Eluents: 0.015 *M* KH_2PO_4 and 0.25 *M* KH_2PO_4 in 2.2 *M* KCl. (a) Samples: 5 μl of solution of *ca.* 0.25 m*M* adenine and guanine nucleotides. (b) Samples: 2 μl of solution of *ca.* 0.5 m*M* AMP and GMP. UV output: 0.08 a.u. Adjusted retention time = retention time − hold-up time.

TABLE IV

RATIO OF PEAK AREAS OF ADENINE NUCLEOTIDE EXTRACTS FROM HUMAN ERYTHROCYTES WHEN ANALYZED AT 12 ml AND 24 ml/h

Ratio = peak areas at 12 ml/h to peak areas at 24 ml/h. V_0 = 50 ml. Eluents: 0.015 *M* KH_2PO_4 and 0.25 *M* KH_2PO_4 in 2.2 *M* KCl. Samples: 10 μl of nucleotide extract from human erythrocytes. UV output: 0.04 a.u.

Sample No.	*Peak areas (in cm²)*		
	AMP	*ADP*	*ATP*
A-30	2.01	2.25	2.28
A-60	2.23	1.93	1.77
A-120	1.83	1.67	1.73
B-0	2.50	2.35	2.11
B-30	2.16	2.04	2.11
B-120	1.73	2.07	1.99
B-240	1.77	2.21	—
Average	2.03	2.07	2.00

a measure of peak sharpness and is an inverse function of the height equivalent to a theoretical plate (H). The VAN DEEMTER equation[8]

$$H = A + B/u + Cu$$

is useful in gas chromatography, but in liquid chromatography the B term does not apply since B accounts for the molecular diffusion of the solute in the gas phase. Therefore, the plate height is linear with flow velocity and is expressed by the equation

$$H = A + Cu$$

when C is related to kinetic and diffusional mass transfer resistance, u is the eluent velocity and A is a constant representing the eddy diffusion path. This has been demonstrated by HORVATH and coworkers[1,2]. However, in gas as well as in high pressure liquid chromatography, the type of detector is the determining factor in peak area[9]. Using linear gradient elution on the LCS 1000 which has a UV detector with a cylindrical flow cell and fixed speed recorder, our data indicate that the peak area is an inverse function of the flow rate. Theoretically, this is the result expected since it is known that the UV detector signal is proportional to the solute concentration but at fixed recorder speed, the peak area is inversely proportional to the flow rate[5]. Therefore, when using the linear gradient elution mode this system is analogous to a "differentiating" detector in gas chromatography such as a thermal conductivity detector in which the peak area is directly proportional to the total mass of the component but inversely proportional to the flow rate[10].

From our results, it can be seen that for quantitative analysis by high pressure liquid chromatography using a linear concentration gradient, it is imperative that the flow rate be accurately determined and kept constant. If these conditions are met, then quantitative calibrations run at one flow rate can be applied to chromatograms run at other flow rates by using the appropriate proportionality factor. In gradient elution in high pressure liquid chromatography, retention times may be varied within limits by changing the flow rates or the slope of the linear concentration gradient without affecting the accuracy of the quantitative analysis.

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