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EFFECT OF SAMPLE SIZES ON PEAK SHAPES IN PREPARATIVE LIQUID CHROMATOGRAPHY

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Abstract—Cyclobutane pyrimidine dimers and monomers of thymine were separated on a C_{18} reversed-phase high performance liquid chromatographic column. Using the two mathematical models, the effect of the sample sizes on peak shapes in preparative liquid chromatography was investigated. One of them is the linear kinetic model, and the other is based on the nonlinear adsorption isotherm. In case of small injections of sample, the good agreements between the calculated value by the linear kinetic model and experimental data were achieved. However, when sample size is increased, this model lacks in the prediction of large concentration profile of sample. However, the nonlinear model permits the accurate prediction of the location of the component band and the determination of the appropriate time to start and stop collection of the enriched fraction at the higher concentrations of monomer and the lower that of dimer. Therefore, extremely small amounts of dimer can be extracted from monomer solutions.

INTRODUCTION

The reported chemical lesions in the DNA material of some animal and plant systems include classes of pyrimidine cyclobutane dimers, pyrimidine hydrates, glycols, various addition products and some purine photoproducts [1]. The pyrimidine cyclobutane dimers(thymine thymine, thymine cytosine and cytosine cytosine) are the most abundant, representing as much as 90% of the UV damage. The concentration of these compounds is extremely low, of the order of 0.02% or less of the base concentration. Exposure of deoxyribonucleic acids in living cells to ultraviolet radiation results in damaging structural alterations, and they are thought to be the major DNA photoproduct lesion [2]. In the present work, the cyclobutane pyrimidine dimer and monomer of thymine are considered for reversed-phase high performance chromatographic (HPLC) separation. Because of the low concentration of the dimer and the small quantity of material that is usually available for analysis, sensitive methods are required for accurate determination.

A complete analytical procedure involves the quantitative extraction of the dimers from a mixture of regular bases, followed by derivatization of the dimers and their quantitative analysis by gas chromatography with electron-capture detection. This scheme makes possible the achievement of very low detection limits in spite of the small size of the samples available which do not exceed 500 μg.

To aid in the selection of optimum conditions for the isolation and determination of trace amounts of thymine thymine dimer changes in the elution profile of this dimer on a HPLC column overloaded with thymine have been examined both experimentally and theoretically using the semi-ideal model of non-linear chromatography described previously [3]. One is the model equations based on the linearity of the equilibrium isotherm [4]. The other is the theory of nonlinear liquid preparative chromatography, where the volume of sample or the concentration is increased to obtain large products [4, 5]. The purpose of this work is to estimate the kinetic parameters used in the linear chromatography, to compare the calculated values by the two models mentioned above to the experimental data when the concentration of monomer higher than that of dimer, and to observe the shape of concentration profiles with increase in sample sizes.

MATHEMATICAL MODELS

1. Linear Kinetic Model

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This model is used to predict the concentration profile of monomers and dimers and to assist in optimization of cut points for HPLC fraction collection. The equations in the kinetic model present the local average solute concentration in the column and in the intraparticle voids in terms of the various transport parameters such as axial dispersion coefficient, interparticle coefficient, intraparticle coefficient, and effective intraparticle diffusion coefficient [4, 5].

$$\frac{\partial c}{\partial t}+u\frac{\partial c}{\partial z}=\frac{E\partial^2 c}{\partial z^2}-\frac{3}{r_p}\frac{(1-\varepsilon)\varepsilon_p}{\varepsilon}D_e\frac{\partial q}{\partial r}\Big|_{r=r_p} \quad (1)$$

$$\frac{\partial q}{\partial t}=D_e\frac{1}{r^2}\frac{\partial}{\partial r}\left(r^2\frac{\partial q}{\partial r}\right)-\frac{\partial n}{\partial t} \quad (2)$$

$$\frac{\partial n}{\partial t}=k_a(q-n/K_e) \quad (3)$$

The initial and boundary conditions are:

$$c=q=n=0 \quad \text{(for t 0, z>0)} \quad (4)$$

$$c=\delta(t) \quad \text{(for t>0, z=0)} \quad (5)$$

$$c=\text{finite} \quad \text{(for t>0, z}\rightarrow\infty\text{)} \quad (6)$$

$$\frac{\partial q}{\partial r}=0 \quad \text{(for t>0, x=0)} \quad (7)$$

$$D_e\frac{\partial q}{\partial r}=k_f(c-q) \quad \text{(for t>0, r=r}_p\text{)} \quad (8)$$

It is assumed that the adsorption effect is dominant with a linear equilibrium isotherm. The solution of Eq. (1) to Eq. (8) in the Laplace domain is

$$\bar{C}(s, L)=\exp\left[\frac{Lu}{2E}-L\left\{\left(\frac{u}{2E}\right)^2+\frac{\gamma}{E}\right\}^{1/2}\right] \quad (9)$$

at the bed exit, z=L, where

$$\gamma=s+\frac{3k_f}{r_p}\gamma_3\left(1-\frac{1}{1-\gamma_1+\gamma_1\gamma_2\coth\gamma_2}\right) \quad (10)$$

$$\gamma_1=\frac{D_e}{r_p k_f} \quad (11)$$

$$\gamma_2=r_p\sqrt{\frac{s}{D_e}\left(1+\frac{K_e k_a}{K_e s+k_a}\right)} \quad (12)$$

$$\gamma_3=\frac{(1-\varepsilon)\varepsilon_p}{\varepsilon} \quad (13)$$

The first two moments can be obtained by applying the final value theorem of Laplace transform.

$$m_1'=\frac{L}{u}\{1+\gamma_3(1+K_e)\} \quad (14)$$

$$m_2=\frac{2EL}{u^3}\{1+\gamma_3(1+K_e)\}^2+\frac{2L\gamma_3}{u}\left\{\frac{K_e^2}{k_a}+\frac{r_p(1+K_e)^2}{3}\left(\frac{1}{k_f}+\frac{r_p}{5D_e}\right)\right\} \quad (15)$$

where m_1' indicates the first noncentral moment and m_2 is the second central moment, i.e., mean and variance.

To predict the concentration profiles, the kinetic parameters should be estimated. In this work, the moment method is used to evaluate rate and equilibrium constants and the first and second moments are sufficient to establish resolution criteria.

2. Nonlinear Semi-ideal Model

Neglecting axial dispersion and assuming fast kinetics of radial mass transfer, the material balance for each component can be written as follows [6, 7]:

$$(1+k_i')\frac{\partial C_i}{\partial t}+u\frac{\partial C_i}{\partial z}=0 \quad (16)$$

$$k_i'=\frac{\partial q_i}{\partial C_i} \quad (17)$$

where C_i is the concentration of each species in the mobile phase and q_i the concentration of each species in the stationary phase at equilibrium, and k_i' the stationary phase to mobile phase ratio. These equations were also employed to model the separation of the thymine thymine dimer from thymine monomer, and they were solved using numerical dispersion to account for the finite column efficiency [6, 7].

The competitive Langmuir adsorption isotherm was employed to relate the amount adsorbed in the stationary liquid phase at equilibrium to the concentration in the mobile phase. The relationship of a binary system is expressed,

$$q_i=a_iC_i/(1+b_1C_1+b_2C_2), \quad (18)$$

where a_i, b_i=Langmuir isotherm parameters

The concentration of thymine thymine dimer is very low, so the amount of sample injected is almost always insufficient for the dimer band to exhibit non-linear elution behavior. Therefore, we can neglect the corresponding terms($b_1=0$) and write as follows for the second component(thymine) after rearrangement:

$$C_2/q_2=1/a_2+(b_2/a_2)C_2 \quad (19)$$

Therefore, the parameters of a_2 and b_2 was calculated from the intercept and slope of the plot of c_2/q_2 to c_2. Using the above simplified isotherm model, Eq. (19), the values of the coefficients, a_2 and b_2 are determined experimentally [8].

EXPERIMENTS

The samples were analyzed on a Waters liquid chromatographic system equipped with a Model 600E multisolvent delivery system, a Model U6K injector, and a Model 490 programmable multiwavelength detector (Waters Chromatography Division, Millipore, Milford, MA, U.S.A.). A MAXIMA 820 chromatography workstation was used for data acquisition(Dynamic Solutions, Millipore, Ventura, CA, U.S.A.).

Thymine thymine dimer is monitored at 210 nm, because its response remains within the linear range of the detector, whereas thymine monomer is detected at 254 nm, where the absorbance is small enough to provide a linear response in the concentration range investigated. The LC effluents were simultaneously monitored at two different wavelengths corresponding to the absorption maxima, and two calibration graphs were constructed from the peak heights at different concentrations.

An Alltech C_{18} HS(7 μm) column(250 mm×4.6 mm I.D.) was used for the separations. High-purity water was purchased from Burdick & Jackson Labs.(Muskegon, MI. U.S.A.) for the mobile phase. Data were obtained at ambient temperature at a flow-rate of 1.0 ml/min.

Thymine and uracil was purchased from Sigma(SL Louis, MO. U.S.A.) and the cisyn-5.6-thymine-thymine dimer was prepared by Dr. Ho at the Oak Ridge National Laboratory. The dead time determined from the retention time of uracil is 195 sec. All standards were prepared in aqueous solution.

RESULTS AND DISCUSSION

In the linear kinetic model, the linear relationship of solute distribution between stationary phase and mobile phase is assumed. The relation is expressed as the equilibrium coefficient, and the higher value of it means a substance is more retained in the column. The effect of sample size on the retention time is shown in Fig. 1, which points that they were independent of the concentration in the injected pulse. The thymine dimer is eluted earlier than thymine. The simple linear regression method was adopted to calculate the partition coefficients of the two components from Eq. (14). From the equation, it follows that

$$\frac{m_1'}{L} = \frac{1}{u}\{1+\gamma_3(1+K_e)\} \tag{20}$$

A relationship of m_1'/L vs. $1/u$ should be linear through the origin, and the slope gives $\{1+\gamma_3(1+K_e)\}$. The order of the magnitude of the slopes is identical to the elution order of the components. The resulting adsorption rate constants are listed in Table 1. This linear condition is basic and important concept for developing the linear mathematical models within the concentration range(0.01-20 μg) in this experiments.

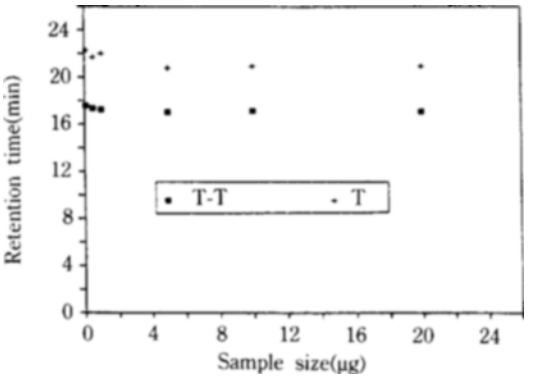

Fig. 1. Effect of sample size on retention time. (flow rate 1.0 ml/min)

Table 1. Equilibrium and rate constants of thymine-thymine dimer and thymine from the moments

	T-T	T
K_e	11.7	13.3
η	19.3	39.4
k_a(1/min)	1194.7	1965.5
D_e(cm²/min)	5.3	7.0
a_1(ml/ml)	14.03	
a_2(ml/ml)		15.908
b_1(ml/mg)	0.0	
b_2(ml/mg)		0.11774

Fig. 2 shows that the increase in sample size of thymine thymine dimer affects negligibly the peak width, but for thymine larger amount broads the peak width.

Each of the response curves was fit to the transient material balance with the adjustable parameters to find the variance. The void fraction of the bed(ε) and the internal porosity of the particle(ε_p) were assumed to be 0.4 and 0.5, respectively. The axial dispersion coefficient was obtained by the equation, $E = \eta r_p u$. The interparticle mass transfer coefficients, k_a, with the superficial flow rates were 19.85, 23.95, 27.40, 30.44, and 33.19 cm/min for 0.50, 0.75, 1.00, 1.25, and 1.50 ml/min, respectively from the correlation of Foo and Rice [9]. Therefore, Eq. (15) can be used to evaluate the rate constants, k_a, E, and D_e. They cannot be determined by the simple linear regression because of the

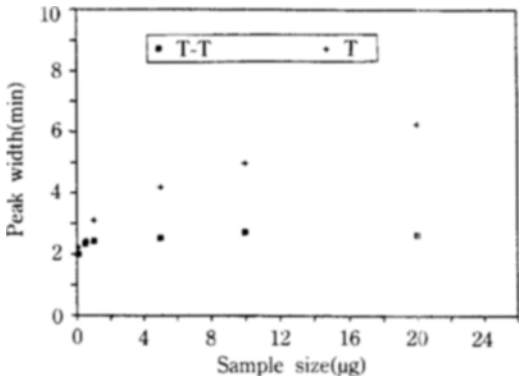

Fig. 2. Effect of sample size on peak width.
(flow rate 1.0 ml/min)

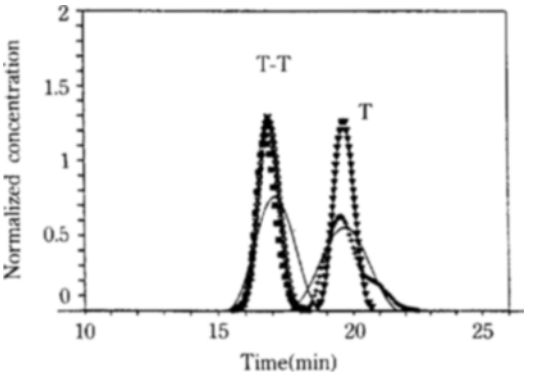

Fig. 3. Comparison of experimental data with the calculated values.
(The symbols: experimental data, the symbols with solid lines: the calculation from the semi-ideal model, and the solid lines: from the linear chromatography).

Table 2. Equilibrium constants and residence time of thymine-thymine dimer and thymine in case of very small amounts

	T-T	T
K_c	14.03	15.56
Retention time(min)	16.85	18.35

nonlinearity of the expression with respect to eluant velocity. Consequently, the nonlinear optimization method was used. Table 1 lists the resulting values.

In the linear chromatography, the adsorption rate and kinetic constants determined can be used to predict the elution curves by transforming Eqs. (9) to (13) into the real time domain [10]. Among many approximation techniques, the equations were inverted numerically from the curve fitting procedure by Dang and Gibilaro [11].

The equilibrium constant in Eq. (17), k_i', was rewritten as follows,

$$K_c = (V_R - V_0)/V_{sp}, \tag{21}$$

where V_R is retention volume of solute, V_0 retention volume of unretained solute, and V_{sp} volume of stationary phase. The results are listed in Table 2. Compared to the values in Table 1, the equilibrium constant in Eq. (17) is a little bit larger. Knowing the all parameters used both in the linear kinetic model and in the nonlinear semi-ideal model, it is possible to make accurate predictions of the band profiles of the thymine and the thymine thymine dimer at very low concentrations enough to assume the linearity of the adsorption isotherm. The agreements between the experimental data and the predicted elution curves are shown in Fig. 3. The points are represented by experimental data, the solid lines by the calculated values

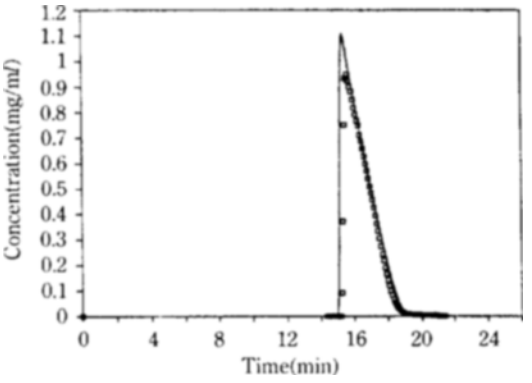

Fig. 4. Elution profiles of thymine in large concentration.
(The symbols: experimental data, the solid line: the calculation from the semi-ideal model, flow rate 1.0 ml/min, thymine concentration 2.0 mg/ml)

of the linear chromatography, and the solid lines with symbols those of the nonlinear semi-ideal chromatography. This is interesting results, and both model can not completely match the experimental data, but they can be used as rough approximations.

Referring to the paper [8], the Langmuir adsorption coefficients obtained by a least squares regression are $a_2 = 15.908$ ml/ml and $b_2 = 0.11774$ ml/mg. The coefficient, a_1, is determined from the retention time of the pure dimer and is equal to 14.03 ml/ml.

When the sample size of thymine is increased to mg order of magnitude, the peak shape is greatly deviated from a Gaussian curve, and shows sharp front

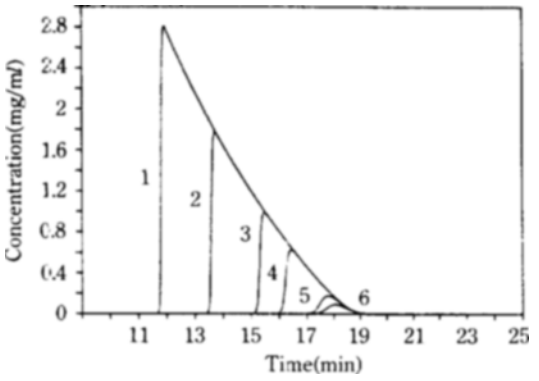

Fig. 5. Calculated elution profiles of thymine.
(amount of sample: 1=8.3 mg, 2=4.15 mg, 3=1.66 mg, 4=830.0 μg, 5=166.0 μg, 6=83.0 μg)

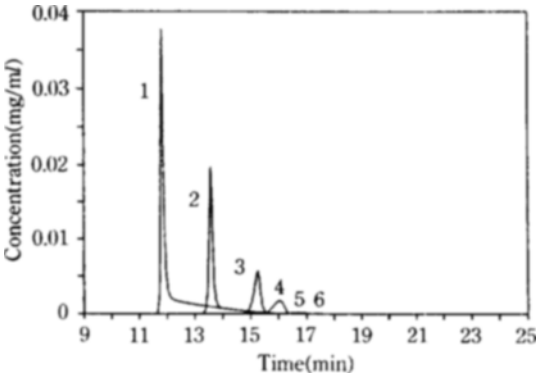

Fig. 6. Calculated elution profiles of thymine-thymine dimer.
(amount of sample: 1=8.3 μg, 2=4.15 μg, 3=1.66 μg, 4=830.0 μg, 5=166.0 μg, 6=83.0 μg)

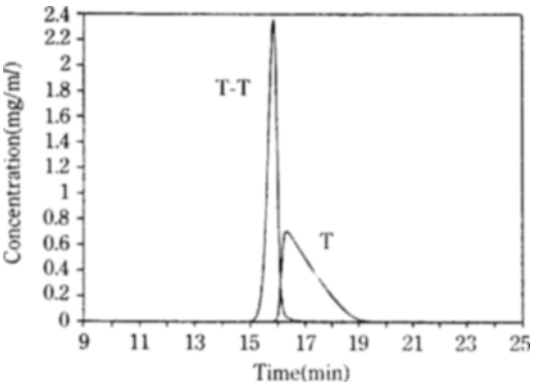

Fig. 7. Calculated elution profiles of thymine and thymine-thymine dimer.
(amount of thymine=1.0 mg, amount of thymine thymine dimer=1.0 mg)

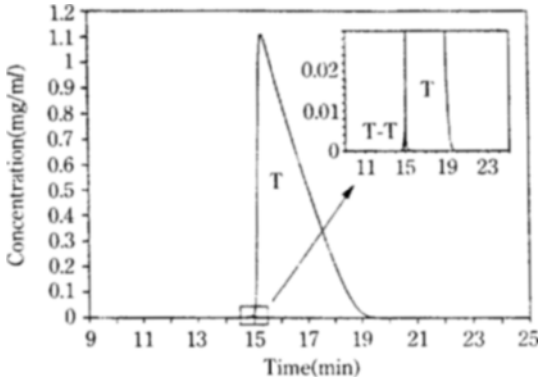

Fig. 8. Calculated elution profiles of thymine and thymine-thymine dimer.
(amount of thymine=2.0 mg, amount of thymine thymine dimer=2.0 μg)

edging. So it looks like a triangle. Consider the injection of a pure thymine solution at a concentration of 2 mg/ml. In Fig. 4, the solid-line curve is calculated from the nonlinear semi-ideal chromatography. They are in good agreement except for a slight tail at the end of the column band.

In the nonlinear semi-ideal model, the investigation of series of numerical solutions of the system of mass balance equations of the two components in the chromatographic column allows the determination of the conditions of optimum operation [12]. Fig. 5 shows the elution profiles of thymine in samples containing decreasing masses of thymine(8.3, 4.15, 1.66, 0.83, 0.166, 0.083 mg). The concentration profile of 6 in the Fig. forms nearly a Gaussian curve. With increase in sample size, the peaks are distorted. In a real sample, the concentration ratio of thymine monomer to thymine thymine dimer is believed to be in the range of 500:1 -2000:1, so the simulation is performed at the ratio of 1000:1. Fig. 6 shows the variation of the elution profiles of 8.3, 4.15, 1.66, 0.83, 0.166, 0.083 μg of dimer. The concentration profile of 6 in the Fig. is hardly seen, because the outlet concentration is extremely low. From Figs. 5 and 6, it can be clearly seen that, when the amount of thymine is increased, the retention time of both components decreases and that the maximum concentration of the dimer band increases(Fig. 6) And the resolution between the two components get worse as sample sizes are increased. In the same amount of 1 mg for monomer and dimer, the concentration profiles are shown in Fig. 7, which indicates that the concentration profile of dimer is symmetrical even though the injected amount is 1.0 mg. This is

attributed to the linear relationship assumed in the model equations.

At very large values of the monomer and at very low relative dimer concentrations(1000:1 ratio), the tiny band of the dimer is hidden by the huge monomer band(see Fig. 8). The inset is an expansion of the dimer peak, recorded at 210 nm(see Experiments). This simulation permits the determination of the optimum cutting time when the dimer is collected.

CONCLUSION

Using the linear kinetic model and the nonlinear semi-ideal model, the effect of the sample sizes on peak shapes in preparative liquid chromatographic separation of cyclobutane pyrimidine dimers and monomers of thymine on a C_{18} reversed-phase column was investigated.

It is very meaningful and interesting work to compare the calculated values from the two different models, because the allowable amount of sample that can be used for each model is respectively determined by the experimental data, and the limitation of the linear chromatography is revealed at the higher concentration.

In case of small injections of sample(μg amount), the linear kinetic model gives good predictive tool. However, at the higher concentrations of monomer and the lower that of dimer the nonlinear model permits the accurate prediction of the location of the component band and the determination of the appropriate time to start and stop collection of the enriched fraction. Therefore, due to the prediction by the nonlinear semi-ideal model, extremely small amounts of dimer can be extracted from monomer solutions.

ACKNOWLEDGEMENTS

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NOMENCLATURE

- c : concentration of solute in the mobile phase [mol/m^3]
- c_0 : inlet concentration of solute [mol/m^3]
- $C(s)$: Laplace transform of c(t)
- D_e : diffusion coefficient in the pore spacing [m^2/sec]
- E : axial dispersion coefficient [m^2/sec]
- k_a : adsorption rate constant [1/sec]
- k_f : interparticle mass transfer coefficient [m/sec]
- k_i' : stationary phase to mobile phase ratio in Eq. (17)
- K_r : adsorption rate constant
- L : column length in the partition section and desorption section [m]
- n : concentration of solute adsorbed on the pore surface [mol/m^3]
- q : concentration of solute in the pore spacing [mol/m^3]
- r : radial distance [m]
- r_p : radius of porous particle [m]
- s : variable of Laplace transform
- t : time [sec]
- u : interstitial velocity of carrier gas or desorbent [m/sec]
- V_0 : retention volume of unretained solute [m^3]
- V_R : retention volume of solute [m^3]
- V_{sp} : volume of stationary phase [m^3]
- x : distance perpendicular to surface of porous particle [m]
- z : axial distance [m]

Greek Letters

- $\gamma, \gamma_1, \gamma_2, \gamma_3$: values defined by Eqs. (10) to (13)
- ε : void fraction of chromatographic column
- ε_p : intraparticle porosity
- η : coefficient for axial dispersion

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