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Correlative retention time peak identification method for glycated haemoglobin in high-performance liquid chromatography

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ABSTRACT

A convenient peak identification method in stepwise elution was investigated and correlation among the retention times of peaks in ion-exchange chromatography of glycated haemoglobin was assessed. By using a correlation method, accuracy of peak identification among columns with degradation and product deviations can be maintained. The correlative retention time identification procedure is treated theoretically.

INTRODUCTION

In chromatographic separations, peaks are usually identified by measuring the retention times. Data processors are usually equipped with a function to assign and print the component names for the peaks appearing in the time windows set up in advance. However, retention time variations often occur in HPLC [1], which could disturb peak identification. To minimize the variations, the column temperature, flow-rate and quality control of the stationary and mobile phases are important considerations for chromatographers. However, small variation cannot be avoided because of degradation of the stationary phase and also reproducibility. Even when retention times vary, it is known that correlation among the retention times of peaks

seems to be conserved in the ion-exchange chromatography of glycated haemoglobin [2,3].

Glycated haemoglobins are classified as HbA_{1a} (A_{1a}), HbA_{1b} (A_{1b}), labile HbA_{1c}(l-A_{1c}), HbA_{1c} (A_{1c}), etc. The concentration of A_{1c} is one of the indices of diabetes. Human haemoglobins consist not only of glycated haemoglobin but also of non-glycated haemoglobins such as HbF and HbA_0 [4,5].

In this paper, focusing discussion on the analysis of glycated haemoglobin, correlation among the retention times of peaks is considered. A method of peak identification based on the correlation is presented. This method is similar to a peak identification method based on the relative retention times with internal standards [6,7]. The relative retention method is based on the assumption that each retention time varies is proportion to the retention time of the internal standard. With stepwise elution, some peaks will be identified incorrectly, because this assumption

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is not always valid. The relative retention time is a kind of linear transformation on a common time axis for each peak. On the other hand, the present method, based on the correlation among the retention times of peaks, can treat each peak individually. In other words, this method has linear transformations of retention times, the number of which is the same as that of the peaks to be identified.

A general equation for the retention mechanism can be applied theoretically [8,9]. This supports the correlation among the retention times of peaks in glycated haemoglobin analysis.

THEORY

Retention model of ion-exchange chromatography

In the ion-exchange chromatography of haemoglobin, as the valence of potassium ion in the mobile phase is 1, the capacity factor of the ith peak (k_i') can be expressed as

$$k_i' \sim \frac{V_s}{V_m} \cdot K_i Q^{n_i} X^{-n_i} \tag{1}$$

where V_s and V_m are the total volumes of the stationary (s) and mobile (m) phases in the column, K_i is the conventional selectivity constant, Q is the ion-exchange capacity of the stationary phase, X is the concentration of potassium ion in the mobile phase and n_i is the effective valence of ith haemoglobin molecule, Hb_i. The conventional selectivity constant is defined as $K_i = X^{n_i}(Hb_i)/\{(K)^{n_i}[Hb_i]\}$, where (K) is the concentration of potassium ion in the stationary phase, (Hb_i) is that of Hb_i in the stationary phase and $[Hb_i]$ is that of Hb_i in the mobile phase [8].

The selectivity between the ith and jth peaks, a_{ij}, is defined as

$$\alpha_{ij} = k_j'/k_i' \tag{2}$$

By combining eqns. 1 and 2, the following equation is obtained:

$$\alpha_{ij} \sim \frac{K_j}{K_i} \cdot Q^{n_j - n_i} X^{-(n_j - n_i)} \tag{3}$$

If the n_j value is very close to n_i, the α_{ij} values

will not be influenced by the ion-exchange capacity of the stationary phase (Q) and the concentration of potassium ion in the mobile phase (X), and then α_{ij} is almost constant.

HbF and A_{1c} with isocratic elution

In isocratic elution, the retention time of the ith peak, t_{R_i}, can be expressed as

$$t_{R_i} = t_0 k_i' + t_0 \tag{4}$$

where t_0 is mobile phase hold-up time.

The retention time of the jth peak (t_{R_j}) can be expressed as a function of the retention time of the ith peak (t_{R_i}) using α_{ij}:

$$t_{R_j} = f(t_{R_i}) = \alpha_{ij} t_{R_i} + (1 - \alpha_{ij})t_0 \tag{5}$$

When α_{ij} = constant is assumed, t_{R_j} can be represented by a linear form of t_{R_i}. In addition, the mobile phase hold-up time (t_0) is a function of flow-rate, column length and available column cross-section; t_0 can be regarded as a constant when degradation of the stationary phase or reproducibility occurs, because these three variables are regarded as constant.

For example, the jth and ith peaks can be considered as HbF and A_{1c}, respectively. If $\alpha_{A_{1c}F}$ is constant, the retention time of HbF (t_{RF}) can have a correlation with the retention time of $A_{1c}(t_{RA_{1c}})$ as follows;

$$t_{RF} = \alpha_{A_{1c}F} t_{RA_{1c}} + (1 - \alpha_{A_{1c}F})t_0 \tag{6}$$

The value of $\alpha_{A_{1c}F}$ is the inverse of $\alpha_{FA_{1c}}$. Although the selectivity α is generally defined as $\alpha_{FA_{1c}}$, $\alpha_{A_{1c}F}$ is used in this paper. Thus, $\alpha_{A_{1c}F}$ varies between 0 and 1.

HbF and A_{1c} with stepwise elution

This method can be evolved with stepwise elution of glycated haemoglobin. The programme for stepwise elution is shown in Fig. 1. The first mobile phase A carries a solute to a migration distance q_A in a total elution period τ_A; τ_A is the sum of the time corresponding to the volume of the instrumentation causing a stepwise delay (τ_Z) [8], the programme period of the
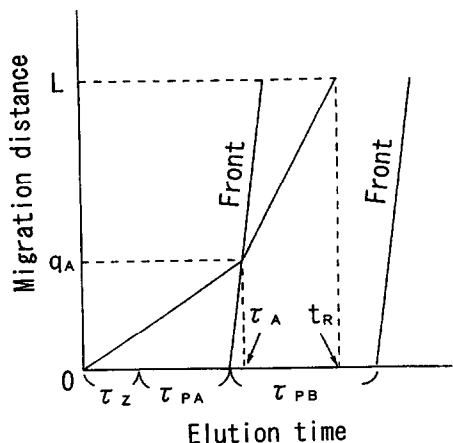
Fig. 1. Migration distance *versus* elution time with stepwise elution. L = column length; q_A = migration distance by mobile phase A; t_R = retention time; τ_A = total elution period of mobile phase A; τ_Z = stepwise delay time; τ_{PA} and τ_{PB} = programme period with mobile phases A and B.

mobile phase A (τ_{PA}) and the time corresponding to the migration distance of the ith solute with mobile phase A $[(\tau_Z + \tau_{PA})/k_i'(X_A)]$, where $k_i'(X_A)$ is the capacity factor of the ith solute [9]. The third term is equal to the elution period of mobile phase B from the column inlet to the ith solute. When the value of $k_i'(X_A)$ is sufficiently larger than 1 $(k_i' > 10)$, τ_A can be regarded as constant approximately; $\tau_A = \tau_Z + \tau_{PA}$.

Using $k_i'(X_A)$, τ_A can be expressed as

$$\tau_A = [1 + k_i'(X_A)]q_A/v \qquad (7)$$

where X_A is the concentration of the gradient-forming ion in the first mobile phase A and v is the velocity of the mobile phase. This equation is similar to eqn. 4. Then the second mobile phase B carries the analyte through the remaining column length $(L - q_A)$ for the remaining retention time $(t_{R_i} - \tau_A)$. In the same way, using the capacity factor for the mobile phase B $[k_i'(X_B)]$, $t_{R_i} - \tau_A$ can be expressed as

$$t_{R_i} - \tau_A = [1 + k_i'(X_B)](L - q_A)/v \qquad (8)$$

The retention time of the ith solute in stepwise elution (t_{R_i}) can be calculated from eqns. 7 and 8 as

$$t_{R_i} = \frac{k_i'(X_A) - k_i'(X_B)}{1 + k_i'(X_A)}\tau_A + [1k_i'(X_B)]t_0$$

$$= \frac{1 + k_i'(X_B)}{1 + k_i'(X_A)} \cdot t_0 k_i'(X_A)$$

$$+ \left[1 - \frac{1 + k_i'(X_B)}{1 + k_i'(X_A)}\right]\tau_A + \frac{1 + k_i'(X_B)}{1 + k_i'(X_A)} \cdot t_0$$

$$= C_1(X_A, X_B)k_i'(X_A) + C_0(X_A, X_B) \qquad (9)$$

where $C_1(X_A, X_B)$ and $C_0(X_A, X_B)$ are substituted coefficients which are introduced for a convenient expression and $t_0 = L/v$. Considering eqn. 4, the retention time ratio for different concentrations of mobile phase $[\beta_i(X_A, X_B)]$ in the isocratic elution mode is defined as

$$\beta_i(X_A, X_B) = [1 + k_i'(X_B)]/[1 + k_i'(X_A)] \qquad (10)$$

When the value of k_i' is sufficiently larger than 1 $(k_i' > 10)$, substitution of k_i' using eqn. 1 in eqn. 10 approximately gives the equation

$$\beta_i(X_A, X_B) \sim k_i'(X_B)/k_i'(X_A) = (X_A/X_B)^{n_i} \qquad (11)$$

Generally, the retention time ratio $[\beta_i(X_A, X_B)]$ is nearly constant with variation of the ion-exchange capacity in the stationary phase. Then $C_1(X_A, X_B)$ and $C_0(X_A, X_B)$ are also constant, and t_{R_i} can be represented by the linear form of $k_i'(X_A)$, when eqn. 9 is considered.

In our analysis of glycated haemoglobin, A_{1c} is eluted with both the mobile phases A and B using stepwise elution. The retention time of $A_{1c}(t_{RA_{1c}})$ can be described as

$$t_{RA_{1c}} = C_1(X_A, X_B)k_{A_{1c}}'(X_A) + C_0(X_A, X_B) \qquad (12)$$

where

$$C_1(X_A, X_B) = \beta_{A_{1c}}(X_A, X_B)t_0$$

and

$$C_0(X_A, X_B) = [1 - \beta_{A_{1c}}(X_A, X_B)]\tau_A$$

$$+ \beta_{A_{1c}}(X_A, X_B)t_0$$

Both $C_1(X_A, X_B)$ and $C_0(X_A, X_B)$ can be regarded as the functions of $\beta_{A_{1c}}(X_A, X_B)$. $\beta_{A_{1c}}(X_A, X_B)$ is the retention time ratio of A_{1c} in the isocratic elution mode.

On the other hand, HbF is eluted only with

the mobile phase A. From eqn. 4, the retention time of HbF (t_{RF}) can be expressed as

$$t_{RF} = t_0 k'_F(X_A) + t_0 \qquad (13)$$

Combination of eqns. 12 and 13 gives

$$t_{RF} = \frac{t_0}{C_1(X_A, X_B)} \cdot \alpha_{A_{1c}F}(X_A) t_{RA_{1c}}$$

$$+ \left[1 - \frac{C_0(X_A, X_B)}{C_1(X_A, X_B)} \cdot \alpha_{A_{1c}F}(X_A) \right] t_0$$

$$= \frac{\alpha_{A_{1c}F}(X_A)}{\beta_{A_{1c}}(X_A, X_B)} \cdot t_{RA_{1c}} + \left[1 - \alpha_{A_{1c}F}(X_A) \right] t_0$$

$$- \frac{1 - \beta_{A_{1c}}(X_A, X_B)}{\beta_{A_{1c}}(X_A, X_B)} \cdot \alpha_{A_{1c}F}(X_A) \tau_A \qquad (14)$$

where $\alpha_{A_{1c}F}(X_A)$ is the selectivity between HbF and A_{1c} in mobile phase A.

If both $\alpha_{A_{1c}F}(X_A)$ and $\beta_{A_{1c}}(X_A, X_B)$ are constant with variation in the ion-exchange capacity among the stationary phases, t_{RF} correlates with $t_{RA_{1c}}$ even in the stepwise elution mode.

In the case of $\beta_{A_{1c}}(X_A, X_B) \sim 1$, t_{RF} can be approximated as follows:

$$t_{RF} \sim \alpha_{A_{1c}F}(X_A) t_{RA_{1c}} + [1 - \alpha_{A_{1c}F}(X_A)] t_0$$

$$+ [1 - \beta_{A_{1c}}(X_A, X_B)] \alpha_{A_{1c}F}(X_A)(t_{RA_{1c}} - \tau_A) \qquad (15)$$

Compared with eqn. 6, the effect of the second mobile phase B appears in the third term of eqn. 15.

l-A_{1c} and A_{1c} with stepwise elution

Both l-A_{1c} and A_{1c} are eluted with mobile phases A and B using stepwise elution. By using eqn. 9, the retention times of l-A_{1c}($t_{Rl\text{-}A_{1c}}$) and A_{1c}($t_{RA_{1c}}$) are expressed as follows:

$$t_{Rl\text{-}A_{1c}} = C_{1l\text{-}A_{1c}}(X_A, X_B) k'_{l\text{-}A_{1c}}(X_A)$$

$$+ C_{0l\text{-}A_{1c}}(X_A, X_B) \qquad (16)$$

$$t_{RA_{1c}} = C_{1A_{1c}}(X_A, X_B) k'_{A_{1c}}(X_A) + C_{0A_{1c}}(X_A, X_B) \qquad (17)$$

where $C_{1l\text{-}A_{1c}}(X_A, X_B)$, $C_{0l\text{-}A_{1c}}(X_A, X_B)$,

$C_{1A_{1c}}(X_A, X_B)$ and $C_{0A_{1c}}(X_A, X_B)$ are constants. By using $\alpha_{A_{1c}l\text{-}A_{1c}}(X_A)$, the selectivity between l-A_{1c} and A_{1c} in mobile phase A, combination of eqns. 16 and 17 give

$$t_{Rl\text{-}A_{1c}} = \frac{C_{1l\text{-}A_{1c}}(X_A, X_B)}{C_{1A_{1c}}(X_A, A_B)} \cdot \alpha_{A_{1c}l\text{-}A_{1c}}(X_A) t_{RA_{1c}}$$

$$- \frac{C_{1l\text{-}A_{1c}}(X_A, X_B) C_{0A_{1c}}(X_A, X_B)}{C_{1A_{1c}}(X_A, X_B)}$$

$$\cdot \alpha_{A_{1c}l\text{-}A_{1c}}(X_A) + C_{0l\text{-}A_{1c}}(X_A, X_B) \qquad (18)$$

Both $t_{Rl\text{-}A_{1c}}$ and t_{RF} can be represented with a linear form of $t_{RA_{1c}}$ in the stepwise elution mode. Then t_{RF} or $t_{Rl\text{-}A_{1c}}$ can be estimated by using $t_{RA_{1c}}$, employing the capacity factors as more important retention values than retention times. In other words, retention times can be regarded as secondary retention values.

In the isocratic elution mode, by employing eqns. 2 and 4, t_{RF} is related to $t_{RA_{1c}}$ (eqn. 6). In a similar way, in the stepwise elution mode of HbF and A_{1c}, the algorithm can be explained as follows: (1) $t_{RA_{1c}}$ is transformed into $k'_{A_{1c}}(X_A)$ by using t_0, τ_A and $\beta_{A_{1c}}(X_A, X_B)$; (2) $k'_{A_{1c}}(X_A)$ is changed into $k'_F(X_A)$ by using $\alpha_{A_{1c}F}(X_A)$; and (3) t_{RF} is obtained from $k'_F(X_A)$ by using t_0. The retention time correlation of l-A_{1c} and A_{1c} can be explained by a similar algorithm.

EXPERIMENTAL

Materials

Apparatus

The HPLC system was a Hitachi Model L-9100 glycated haemoglobin analyser. It had two analytical modes: high-speed mode and high-resolution mode. The two modes used the same mobile phases as follows: mobile phase A = 64 mM potassium phosphate buffer (pH 6.2); mo-

bile phase B = 75 mM potassium phosphate buffer (pH 6.2); and mobile phase C = 207 mM sodium phosphate buffer (pH 6.1).

In the high-speed mode, a 35 mm × 4.6 mm I.D. column packed with weak cation-exchange polymethacrylate resin was used. The mobile phase hold-up time (t_0) was about 0.2 min, the diameter of the resin particles was 3.5 μm and the ion-exchange capacity was about 0.3 mequiv./g. The time course of the stepwise elution was as follows: 0.0–0.6 min with A, 0.6–1.6 min with B, 1.6–2.0 min with C and 2.0–3.3 min with A. The flow-rate was adjusted to 1.4 ml/min. Samples were injected at 3.3-min intervals.

In the high-resolution mode, an 80 mm × 4.6 mm I.D. column packed with the same resin in the high-speed mode was used. The mobile phase hold-up time (t_0) was about 0.6 min. The time course of the elution was follows: 0.0–3.0 min with B, 3.0–3.9 min with C, 3.9–6.5 min with A and 6.5–7.0 min with B. The flow-rate was set to 1.0 ml/min. Samples were injected at 7.0-min intervals.

Other conditions for the two modes were identical. The column temperature was 40°C. The absorbance was measured at 415 nm, using the absorbance at 530 nm as a reference. Samples were whole blood diluted 160-fold with water. The injection volume was 10 μl by the complete-fill mode.

Methods

In order to simulate the retention time variations, the concentration of potassium ion in mobile phases A and B was varied, maintaining a constant ratio of potassium ion concentrations in mobile phases A and B. In the high-speed mode, the concentration of potassium ion in mobile phases A and B was varied from 94% to 106% and in the high-resolution mode from 94% to 111% of the standard concentration. These concentration ranges represent almost the limits to obtain satisfactory separations.

The integrator was set to have a delayed start time of 0.2 min after the injection time in the high-speed mode and 0.5 min in the high-resolution mode.

RESULTS AND DISCUSSION

High-speed mode

A chromatogram obtained using the high-speed mode is shown in Fig. 2a. It has six assigned peaks: A_{1a}, A_{1b}, HbF, $1\text{-}A_{1c}$, A_{1c} and HbA_0. There is a small valley between $1\text{-}A_{1c}$ and
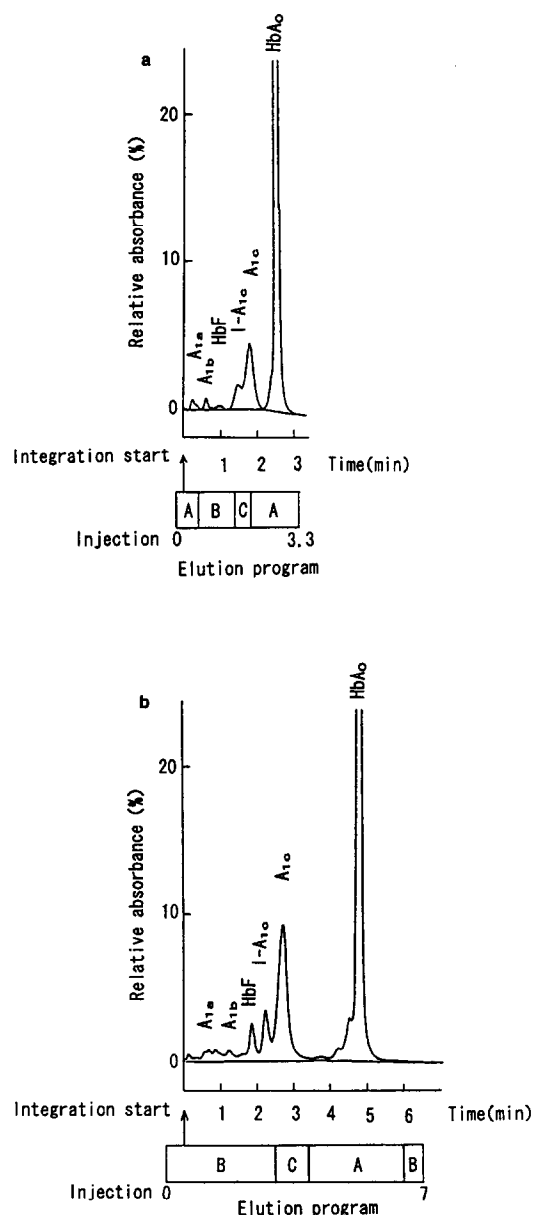

Fig. 2. Representative chromatograms of glycated haemoglobins obtained with stepwise elution. (a) High-speed mode; (b) high-resolution mode.

A_{1c}. Mobile phase A elutes mainly A_{1a}, A_{1b} and HbF. Mobile phase B elutes predominantly l-A_{1c} and A_{1c}. Mobile phase C elutes mainly HbA_0 drastically. The difference in time between a mobile phase change being detected at the detector and the switching of the mobile phases at the pump was about 0.5 min. The ordinate axis (relative absorbance) is normalized as the peak height of A_{1c} corresponding to the percentage of A_{1c} in total haemoglobin.

Simulation of stationary phase variation

In the high-speed mode, retention time variation was simulated by using three pairs of mobile phases A and B with different concentrations. The retention time of A_{1c} ($t_{RA_{1c}}$) was changed on purpose between 1.2 and 1.8 min for the simulation. The retention times of A_{1b} and HbF are correlated with $t_{RA_{1c}}$ in Fig. 3a, although mobile phase B influenced only A_{1c}. The retention time of HbF (t_{RF}) can be fitted to the linear form of the retention time of A_{1c}($t_{RA_{1c}}$):

$$t_{RF} = 0.65t_{RA_{1c}} - 0.11 \tag{19}$$

This corresponds to eqn. 14. The coefficients for the other peaks are given in Table I.

High-resolution mode

A chromatogram obtained using the high-resolution mode is shown in Fig. 2b. The sample was obtained for a diabetic patient. The separation between l-A_{1c} and A_{1c} in this mode was much better than that in the high-speed mode and the tail of the HbF peak reached the baseline.

In the high-resolution mode, mobile phase B elutes the solutes between A_{1a} and A_{1c}. In other words, the influence of mobile phase A on the elution of these solutes will be negligible. The difference in time between a mobile phase change detected at the detector and the switching of the mobile phases at the pump was about 1.0 min. The high-resolution mode showed good correlations between the retention times (Fig. 3b). The coefficients for the peaks are given in Table I.
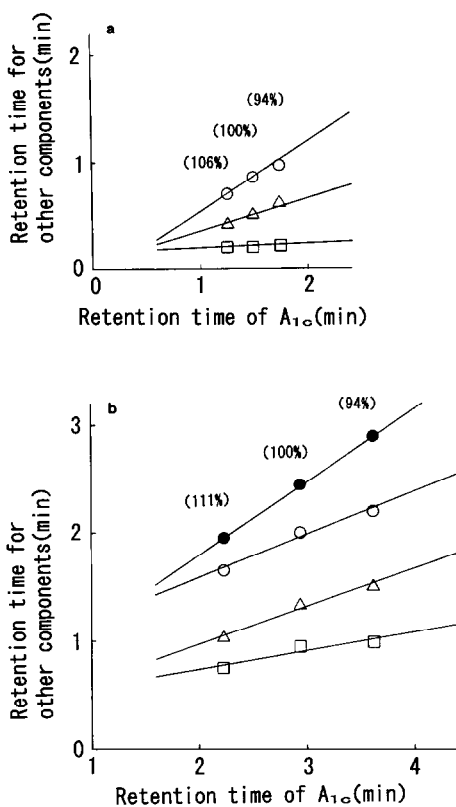

Fig. 3. Correlation of retention times between A_{1c} and the others with stepwise elution. The values in parentheses are the concentrations of mobile phases A and B as a percentage of that of the standard. $\square = A_{1a}$; $\triangle = A_{1b}$; $\bigcirc = HbF$; $\bullet = l$-A_{1c}. (a) High-speed mode; (b) high-resolution mode.

TABLE I

FITTED LINEAR EQUATION FOR RETENTION TIME CORRELATION

High-speed mode

$t_{RA_{1a}} = 0.04t_{RA_{1c}} + 0.16$
$t_{RA_{1b}} = 0.30t_{RA_{1c}} + 0.06$
$t_{RF} = 0.65t_{RA_{1c}} - 0.11$
$t_{Rl-A_{1c}} = 1.03t_{RA_{1c}} - 0.39^a$

High-resolution mode

$t_{RA_{1a}} = 0.17t_{RA_{1c}} + 0.40$
$t_{RA_{1b}} = 0.35t_{RA_{1c}} + 0.27$
$t_{RF} = 0.40t_{RA_{1c}} + 0.79$
$t_{Rl-A_{1c}} = 0.68t_{RA_{1c}} + 0.43$

^{a} The coefficients were obtained using several columns with retention time $t_{RA_{1c}}$ between 1.40 and 2.01 min.
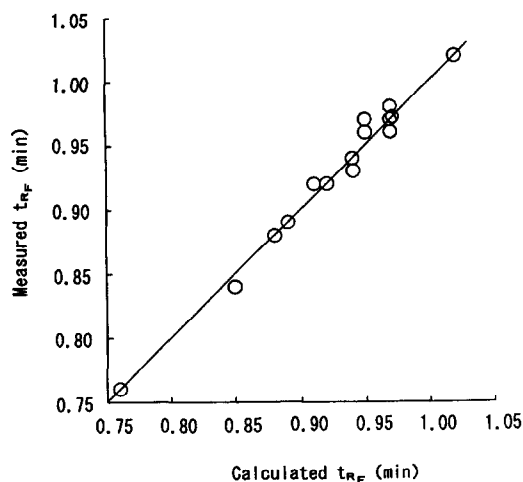
Fig. 4. Relationship between measured retention time of HbF and the calculated value in the high-speed mode. The retention times were calculated by using eqn. 19.

Correlation in the different stationary phases

In the high-speed mode, t_F measured on several different columns correlated with the calculated retention time from eqn. 19 as shown in Fig. 4. The correlation coefficient was found to be as 0.99 for fifteen columns. The difference between the measured and the calculated retention times was 0.03 min at most. This shows that the simulation for changes in mobile phases is almost equivalent to that for variations in the

ion-exchange capacity in the stationary phase. Provided that the composition of the mobile phase is well controlled, even if the stationary phase has a slightly different nature, the retention times will correlate well with each other.

Some chromatographic values can be examined in detail. Both the selectivity of two solutes in the mobile phase A $[\alpha_{A_{1c}F}(X_A)]$ and the retention time ratio of A_{1c} between two mobile phases $[\beta_{A_{1c}}(X_A, X_B)]$ are considered to be almost constant owing to the small relative standard deviations (R.S.D.) among stationary phases (Table II). This constancy is the essential reason for the retention time correlation as estimated in eqn. 14.

Table III shows the variation of the capacity factors. The values in Table II were calculated from these factors. From eqn. 11, by using measured capacity factors, $k'_{A_{1c}}(X_A)$, $k'_{A_{1c}}(X_B)$, $k'_F(X_A)$ and $k'_F(X_B)$, and the concentration ratio (X_A/X_B), the effective valences of HbF (n_F) and A_{1c} $(n_{A_{1c}})$ can be estimated. Both n_F and $n_{A_{1c}}$ were ca. 4 (Table III). This is the reason why $\alpha_{A_{1c}F}(X_A)$ is almost constant despite variations in the stationary phase (eqn. 3). On the other hand, it is the constancy of $\beta_{A_{1c}}(X_A, X_B)$ that makes both $k'_{A_{1c}}(X_A)$ and $k'_{A_{1c}}(X_B)$ sufficiently higher than 1 (eqn. 11).

The R.S.D. of $k'_{A_{1c}}(X_B)$ was 15.4% (Table III). Hence the R.S.D. of the ion-exchange

TABLE II

RETENTION TIME, SELECTIVITY AND RETENTION TIME RATIO BETWEEN MOBILE PHASES

No.	Column[a]	$t_{RA_{1c}}$(min)	$\alpha_{FA_{1c}}(X_A)$	$\alpha_{FA_{1c}}(X_B)$	$\beta_{A_{1c}}(X_A,X_B)$	$\beta_F(X_A,X_B)$
1	Used column G[b]	1.40	2.60	2.12	0.51	0.65
2	Used column K[b]	1.44	2.61	2.11	0.53	0.68
3	New column K	1.62	2.65	2.20	0.52	0.66
4	New column G	1.77	2.60	2.16	0.49	0.62
5	New column K	1.85	2.64	2.21	0.50	0.62
6	New column B	1.90	2.56	2.22	0.53	0.64
7	New column B	2.01	2.66	2.25	0.48	0.60
Mean		1.713	2.617[c]	2.181[d]	0.509	0.639
S.D.		0.233	0.035	0.053	0.020	0.027
R.S.D. (%)		13.62	1.34	2.42	3.84	4.28

[a] G, K and B represent the resin lots. Columns with the same name, such as 6 and 7, have different packings.
[b] The stationary phase had experienced more than 3000 injections.
[c] $\alpha_{A_{1c}F}(X_A) = 1/\alpha_{FA_{1c}}(X_A) = 0.382$.
[d] $\alpha_{A_{1c}F}(X_B) = 1/\alpha_{FA_{1c}}(X_B) = 0.459$.

TABLE III

CAPACITY FACTOR AND EFFECTIVE VALENCE OF HEMOGLOBIN

The results were obtained using isocratic elution. The value of t_0 was measured as 0.2 min by using a non-retention peak from blood.

No.	Column	$k'_{A_{1c}}(X_A)$	$k'_{A_{1c}}(X_B)$	$n_{A_{1c}}$	$k'_F(X_A)$	$k'_F(X_B)$	n_F
1	Used column G[a]	11.20	5.20	4.8	4.30	2.45	3.5
2	Used column K[a]	11.50	5.60	4.5	4.40	2.65	3.2
3	New column K	13.50	6.60	4.5	5.10	3.00	3.3
4	New column G	12.67	5.75	5.0	4.88	2.67	3.8
5	New column K	16.65	7.75	4.8	6.30	3.50	3.7
6	New column B	15.00	7.46	4.4	5.86	3.36	3.5
7	New column B	15.96	7.21	5.0	6.00	3.21	3.9
Mean		13.78	6.51	4.71	5.26	2.98	3.56
S.D.		2.15	1.00	0.25	0.80	0.40	0.26
R.S.D. (%)		15.6	15.4	5.3	15.2	13.4	7.2

[a] The stationary phase had experienced more than 3000 injections.

capacity (Q) in the stationary phases can be estimated to be as small as *ca.* 4%, because $k'_{A_{1c}}(X_B)$ is proportional to the fourth power of Q (eqn. 1).

Further, the fitted parameters can be examined. The fitted first differential coefficient for HbF is 0.65 in eqn. 19, while the estimated value, which is calculated from eqn. 14 using the values in Table II, is 0.75. This difference can be explained as the difference between $\beta_{A_{1c}}(X_A, X_B)$ in the isocratic elution and stepwise elution modes. Actually, the value of $\beta_{A_{1c}}(X_A, X_B)$ with stepwise elution was to be about 15% larger than that with isocratic elution, because the influence of the third mobile phase C from the preceding sample analysis still remains in the succeeding analysis (eqn. 10).

The high-resolution mode can almost be regarded as isocratic elution with mobile phase B. The fitted first differential coefficient was 0.40 (Table I). This can be estimated as $\alpha_{A_{1c}F}(X_B)$ from eqn. 6 theoretically. From Table II, the selectivity in mobile phase A [$\alpha_{A_{1c}F}(X_A)$] is 0.38, whereas that in mobile phase B [$\alpha_{A_{1c}F}(X_B)$] is 0.46. In the high-resolution mode, the first differential coefficient will be an intermediate value between $\alpha_{A_{1c}F}(X_A)$ and $\alpha_{A_{1c}F}(X_B)$, since elution with mobile phase B should become slightly weaker owing to the remaining influence of the preceding mobile phase A.

Peak identification

The above-discussed correlation can be utilized in peak identification in HPLC. An example of the application of this method is shown in the chromatogram in Fig. 5. The first step of the method is a search for the largest peak as A_{1c} in the region with retention times shorter than 2.0
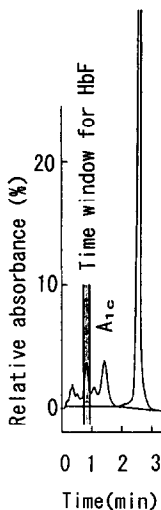

Fig. 5. Time window for peak identification with calculated retention time of HbF in the high-speed mode. The retention time of A_{1c} ($t_{RA_{1c}}$) was 1.37 min. By using the time window (0.77 ± 0.10 min) calculated from eqn. 19, the peak with a retention time of 0.78 min was identified as HbF. The shaded zone is the time window for HbF.

min. In Fig. 5, the retention time of A_{1c} $(t_{RA_{1c}})$ is regarded as 1.37 min. Next, the retention time of HbF (t_{RF}) can be calculated and is estimated as 0.77 min from eqn. 19. Then the time window for the peak identification can be adjusted to 0.77 ± 0.10 min. This width of the window (± 0.10 min) is allowed for compensation for the variation in the mobile phase. There is also the retention time variation caused by peak overlap. This can be estimated to be 0.02 min at most by simulating overlapped Gaussian peaks. The time window could identify the peak at a retention time of 0.78 min with HbF. This method is useful for the identification of other important components such as A_{1a}, A_{1b} and l-A_{1c}, and is also effective in the high-resolution mode.

CONCLUSION

Retention time correlation is preserved in stepwise elution in glycated haemoglobin analysis. This is based on the conservation of the selectivity and the retention time ratio in the two mobile phases in the event of stationary phase variations. The accuracy of peak identification can be improved by calculating the retention time relationship for each chromatogram instead of using time windows fixed in advance.

REFERENCES

1. E. Grushka and I. Zamir, *High Performance Liquid Chromatography*, Wiley, New York, 1989, pp. 529–561.
2. M. Mizuno, K. Tochigi, M. Ito and J. Miura, *Bunseki Kagaku*, 40 (1991) 395.
3. M. Mizuno, K. Tochigi, M. Ito and J. Miura, *Bunseki Kagaku*, 40 (1991) 907.
4. R. Fluckiger and H.B. Mortensen, *J. Chromatogr.*, 429 (1988) 279.
5. E. Bisse and H. Wieland, *J. Chromatogr.*, 434 (1988) 95.
6. S.H. Ashoor and M.A. Osman, *J. Chromatogr.*, 393 (1987) 329.
7. R. Gill, A.C. Moffat, R.M. Smith and T.G. Hurdley, *J. Chromatogr. Sci.*, 24 (1986) 153.
8. P. Jandera and J. Churacek, *J. Chromatogr.*, 91 (1974) 207.
9. W. Markowski and W. Golkiewicz, *Chromatographia*, 25 (1988) 339.