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Chromatographic characterization of some dideoxyribonucleosides

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ABSTRACT

Selectivity and retention surfaces were developed for the rapid high-performance liquid chromatographic separation of dideoxyribonucleosides which are promising antiretroviral agents. In this study, the dideoxyribonucleosides 2′,3′-dideoxyadenosine, 2′,3′-dideoxyinosine, 2′,3′-dideoxycytidine and 3′-deoxythymidine were used as model compounds and 2′-azido-3′-deoxythymidine (AZT) and AZT-5′-phosphate (AZT-5′-P) as experimental test compounds. The interdependence of the experimental variables, column temperature, mobile phase pH and percentage of methanol, and the effects of these variables on the reversed-phase liquid chromatographic (RPLC) behavior of the dideoxy compounds were investigated. The conditions for optimal resolution of these compounds, as determined from the surfaces, were mobile phases of pH 5.0 and either 14% methanol at 40°C or 15% methanol at 30°C. These surfaces served as guidelines in determining conditions for preparative and analytical scale RPLC separations of AZT from AZT-5′-P.

INTRODUCTION

Dideoxyribonucleosides (ddNs) have been shown to be inhibitors of the human immunodeficiency virus (HIV) *in vitro* [1–7]. At present, 3′-azido-3′-deoxythymidine (AZT), a ddN, is approved for use in the treatment of the acquired immunodeficiency syndrome (AIDS). Other ddNs, which are promising antiretroviral drugs, include 2′,3′-dideoxycytidine (ddCyd), 2′,3′-dideoxyinosine (ddIno), 3′-deoxythymidine (dThd) and 2′,3′-dideoxyadenosine (ddAdo). However, because these ddNs as well as AZT have serious toxic side-effects [8,9], new analogues, which have the potential for the same or greater efficacy but lower toxicity, are being synthesized.

Since high-performance liquid chromatography (HPLC) has been succesfully used for the past two decades for the separation of nucleotides and nucleosides [10,11], it is the method of choice for the separations of ddNs. In the past, we have investigated structure–retention relationships in reversed-phase liquid chromatography (RPLC) of nucleotides, nucleosides and their bases [12,13], and we have developed retention and selectivity surfaces for deoxyribonucleotides [14] and deoxyribonucleosides [15], which

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can be used to determine the conditions for various HPLC separations of these compounds. With these surfaces, optimal resolution and retention times can be obtained by two or more sets of experimental conditions; thus the best conditions for difference scale separations (*e.g.*, analytical, preparative) can be readily chosen.

In this study, we applied the results of our previous work [12–15] to the separation of the ddNs. First, we used structure–retention relations to determine the initial experimental conditions, retention and selectivity surfaces of the deoxyribonucleosides to find the approximate conditions for the separations of ddNs and the surfaces of the ddNs to fine-tune these separations. Finally, we used AZT and AZT-5′-phosphate (AZT-5′-P) as model compounds to test experimentally our predictions for the conditions needed both on the analytical and preparative scale to separate AZT-5′-P from its precursor AZT in the synthesis of the monophosphate [16]. Large amounts of the AZT-5′-P are needed to synthesize new analogues of AZT for animal studies and clinical trials.

EXPERIMENTAL

Instrumentation

The chromatographic system was equipped with a Waters 6000A pump (Waters Division, Millipore, Milford, MA, U.S.A.), a Rheodyne 7125 injector (Rheodyne, Berkeley, CA, U.S.A.) and a Waters M440 absorbance detector at 254 nm. Retention times were recorded on an HP 3390A integrator (Hewlett-Packard, Avondale, PA, U.S.A.) and an Omniscribe recorder (Houston, TX, U.S.A.). The column was a 110 mm × 4.70 mm I.D. cartridge packed with 5-μm C_{18} packing material (Whatman, Clifton, NJ, U.S.A.). A guard column (30 mm × 2.0 mm) packed with Whatman pellicular C_{18} glass beads was installed between the injector and the column.

Materials

The dideoxyribonucleosides ddAdo, ddCyd, ddIno and dThd, which were of the highest purity, were purchased from ICN Biochemicals (Cleveland, OH, U.S.A.). The dideoxyribonucleoside AZT (Sigma, St. Louis, MO, U.S.A.) and AZT-5′-P (synthesized in the Medicinal Chemistry Laboratory at URI) were used to test the optimization schemes. Stock solutions (20 m*M*) were prepared and stored at −20°C. Working standards, which were diluted to $2 \cdot 20^{-5}$ *M*) with doubly distilled, deionized water before injection into the chromatograph, were kept at 4°C. The pH of each solution was adjusted to 5.6 with potassium hydroxide (KOH). For the mobile phase, HPLC-grade potassium dihydrogenphosphate (KH_2PO_4) and methanol were obtained from Fisher Scientific (Fairlawn, NJ, U.S.A.).

Procedures

The mobile phase consisted of a solution of 10 m*M* KH_2PO_4 and methanol. The pH of the mobile phase was adjusted with phosphoric acid or KOH before the methanol had been added to the buffer. All experiments were carried out by changing one condition systematically while holding constant the two remaining conditions. The temperatures were 30, 35, 40, 45 and 50°C. The pH values were 3.5, 4.5, 5.0, 5.5 and 6.0. The methanol content (v/v) was 13.0, 13.5, 14.0, 14.5 or 15.0%. The flow-rate was 1 ml/min and the chart speed was 0.5 cm/min. The absorbance units of the detector were 0.02 absorbance units full scale.

Calculations

The void volume was determined using sodium nitrate as a void volume marker. The capacity factors (k') were the average of three measurements. The selectivity factors (α) were determined for adjacent peaks: ddAdo/dThd, ddIno/ddCyd, dThd/ddIno. The retention and selectivity surfaces were plotted by using Turbo Basic (Borland, CA, U.S.A.).

RESULTS AND DISCUSSION

Temperature effects

Capacity factors are related inversely to the temperature. The relationship is obtained from the following equation:

$$\ln k' = -\Delta H/RT + \Delta S/R + \ln \varphi \quad (1)$$

where ΔH and ΔS are the enthalpy and entropy of transfer of the solute between the phases, respectively, T is the absolute temperature, φ is the column phase ratio and R is the gas constant. By using the linear regression of the equation, the values of the slope ($-\Delta H/R$) and the intercept ($\Delta S/R + \ln \varphi$) were obtained. The slopes of all the ddNs in the plots of $\ln k'$ *versus* $1/T$ were positive, thus indicating that the capacity factors decrease with increasing column temperature (Fig. 1). Although these slopes were the same for the pyrimidine ddNs, they were different from those of the purine ddNs. Moreover, the capacity factors of ddAdo and ddIno decreased at different rates. Therefore, temperature can be utilized to manipulate retention time and to optimize the separation of ddAdo from its nearest neighbor dThd, ddIno from dThd as well as ddAdo from ddIno.

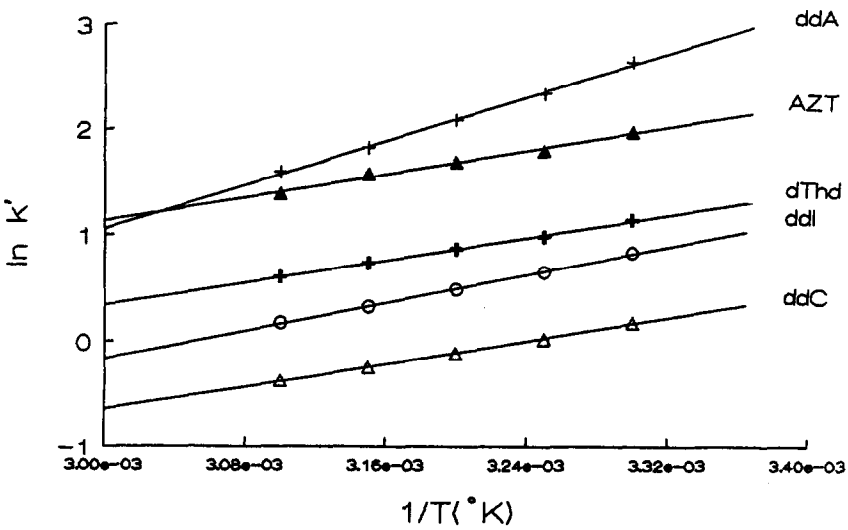

Fig. 1. Plots of $\ln k'$ values of ddAdo, ddCyd, ddIno, dThd and AZT as a function of the reciprocal of temperature at pH 5.0 and 14.0% methanol.

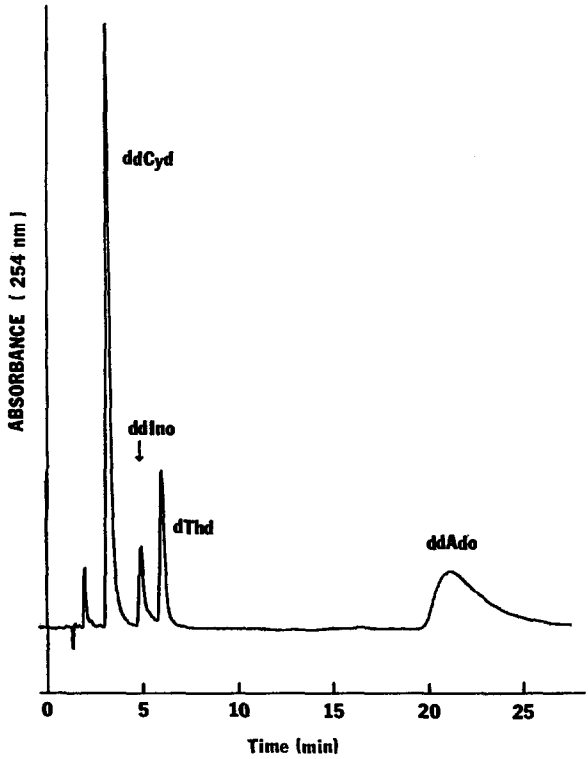

Fig. 2. Chromatogram of the four dideoxyribonucleosides in order of retention times: ddCyd, ddIno, dThd, ddAdo. Conditions: temperature, 30°C; pH, 5.0; methanol concentration, 14%; flow-rate, 1 ml/min.

With ddAdo, elevation of temperature not only decreased the capacity factor but also improved the peak shape as is shown in chromatograms obtained at 30°C (Fig. 2) and at 40°C (Fig. 3). With ddCyd, however, higher temperatures have an adverse effect on retention behavior because this compound had such a small capacity factor ($k' < 1$) at the lowest temperature (30°C) that it was eluted in the void volume at temperatures higher than 40°C.

The selectivity of two neighboring solutes is important in the determination of resolution. The selectivity (α) for any two solutes can be expressed as a function of changes in temperature:

$$\ln \alpha = (\Delta H_1 - \Delta H_2)/RT + (\Delta S_2 - \Delta S_1)/R \quad (2)$$

where subscript 2 indicates the more retained compound and subscript 1 is the less retained compound. The values of the slope, $(\Delta H_1 - \Delta H_2)/R$, and the intercept, $(\Delta S_2 - \Delta S_1)/R$, for all three pairs of ddNs (ddAdo/dThd, ddIno/ddCyd, and dThd/ddIno) are obtained by the linear regression of ln α *versus* $1/T$. The selectivities of both ddAdo/dThd and ddIno/ddCyd decreased with increases in the temperature; however, the selectivity of dThd/ddIno increased as the temperature increased (Fig. 4).

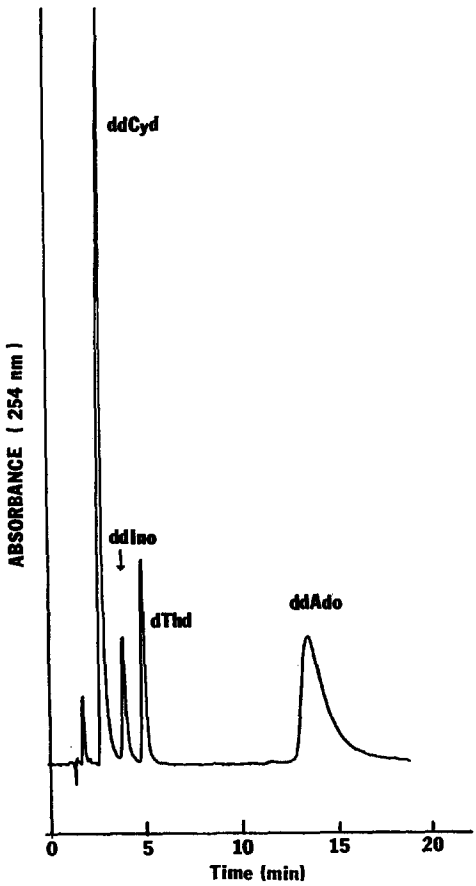

Fig. 3. Chromatogram of the four dideoxyribonucleosides in order of retention times (see Fig. 2). Conditions: temperature, 40°C; pH, 5.0; methanol concentration, 14%; flow-rate, 1 ml/min.

pH effects

The N-1 site in the purine ring of ddAdo has a pK value of 3.8 and the N-3 site in the pyrimidine ring of ddCyd has a value of 4.3. When the pH is 3.5, these two solutes are charged, thus it was predicted that their retention times would be lower than those at pH values of 4.5 and higher [12]. Experimentally it was found that at all temperatures and methanol contents, the capacity factors of ddAdo were at a maximum at pH 5.0 (Fig. 5). The capacity factors of ddCyd decreased slightly with a change of pH from 4.5 to 3.5 and the retention behavior of ddIno and dThd was not influenced by a change of pH in the range studied since the pK of ddIno is 8.9 and of dThd is 9.8.

Effects of methanol content

Based on structure–retention relationships [12], the elution order of a mixture of deoxyribonucleotides, deoxyribonucleosides and ddNs on a reversed-phase column can be predicted. Since deoxyribonucleotides contain phosphate groups which are ionic at all the pH values studied, they will be eluted first on reversed-phase columns;

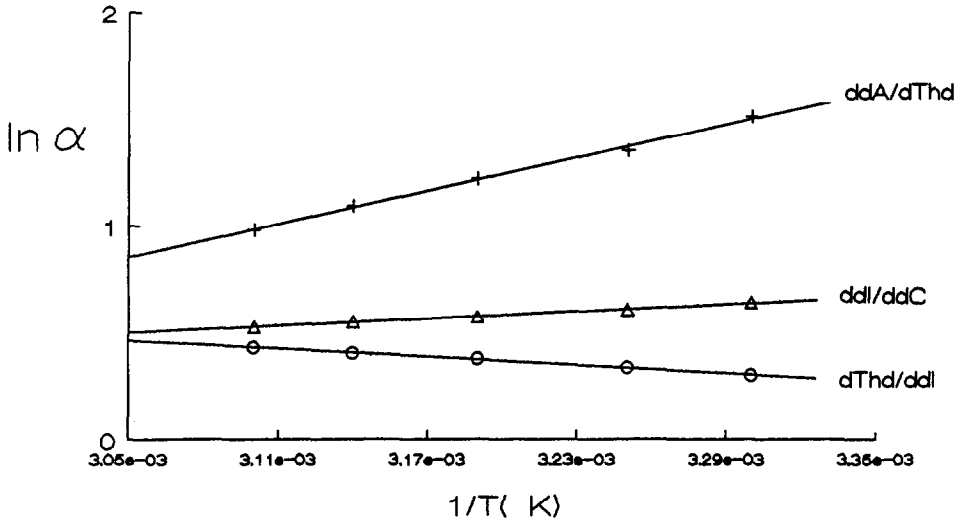

Fig. 4. Plots of ln α values of ddAdo/dThd, ddIno/ddCyc, dThd/ddIno as a function of the reciprocal of temperature at pH 5.0 and 14.0% methanol.

ddNs, which are less polar than deoxyribonucleosides, have the longest retention times. Therefore, it was predicted that a mobile phase containing more than 9% methanol, the maximum percentage of methanol used for the deoxyribonucleosides studies, would be needed in the separation of ddNs. It was also predicted from the work on deoxyribonucleosides [15] that the higher methanol concentrations will cause decreased retention of the ddNs without causing significant changes in their selectivities.

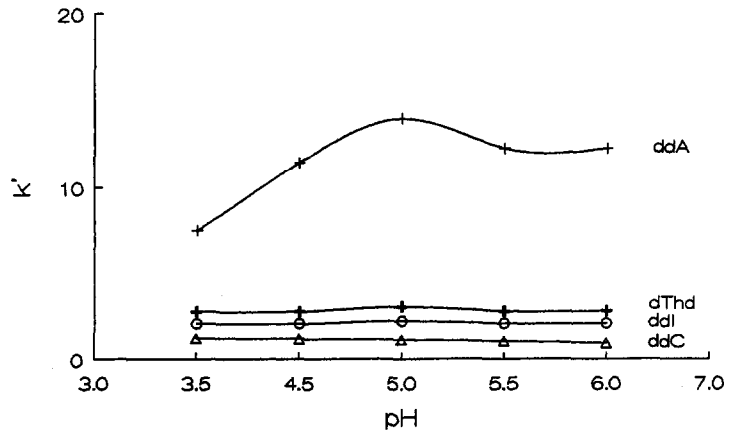

Fig. 5. Plot of k' values of ddAdo, ddCyd, ddIno and dThd as a function of the pH of the mobile phase at 30°C and 14% methanol.

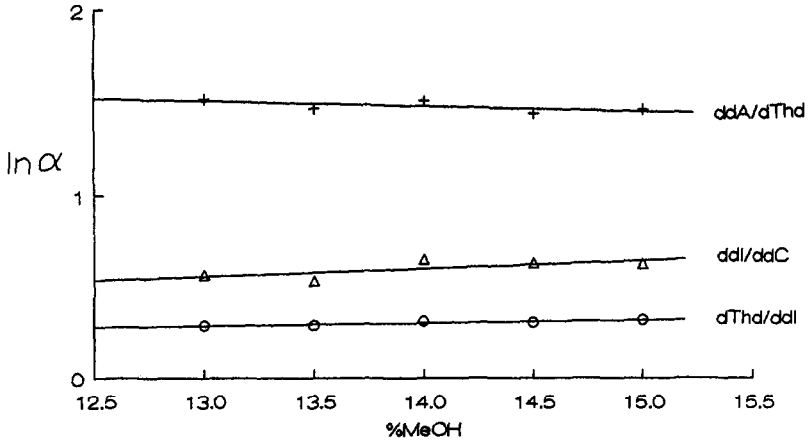

Fig. 6. Plot of ln α values of ddAdo/dThd, ddIno/ddCyd, dThd/ddIno as a function of the methanol content in the mobile phase at pH 5.0 and 30°C.

The dependence of selectivity on the percentage of organic modifier in the mobile phase (X) can be written as follows:

$$\ln \alpha = (a_1 - a_2)X + (b_2 - b_1) \tag{3}$$

where a and b are constants and the subscripts identify the solutes. The values of $(a_1 - a_2)$ and $(b_2 - b_1)$ were calculated by linear regression. The negative values of $(a_1 - a_2)$ for ddAdo and dThd indicated that the selectivity of ddAdo/dThd would

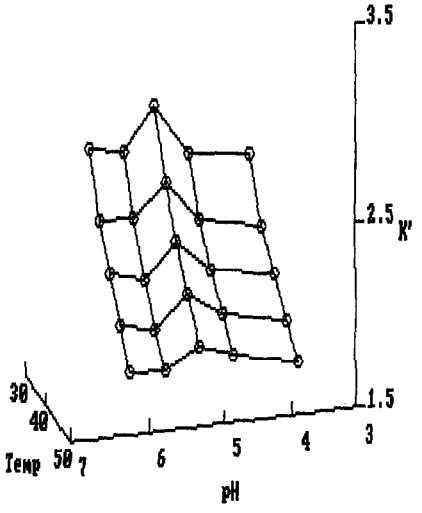

Fig. 7. Retention surface generated by plotting k' values of dThd as a function of the temperature (°C) and pH of the mobile phase that contains 14% methanol. Each circle on an intersection of two or more lines represents an experimentally determined k' value (average of triplicate determinations).

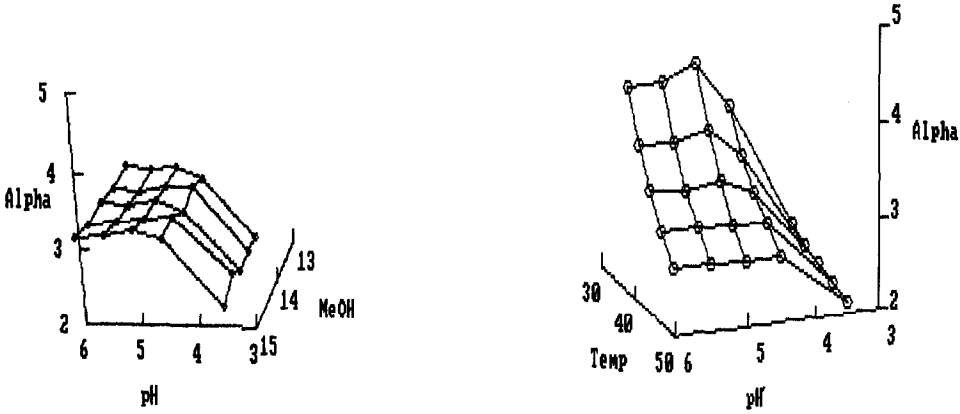

Fig. 8. Selectivity surface generated by plotting α values of ddAdo/dThd as a function of the methanol content and pH in the mobile phase at 40°C. Each circle on an intersection of two or more lines represents an experimentally determined α value (average of triplicate determinations).

Fig. 9. Selectivity surface generated by plotting α values of ddAdo/dThd as a function of temperature and pH of the mobile phase that contains 14.0% methanol. Each circle on an intersection of two or more lines represents an experimentally determined α value (average of triplicate determinations).

decrease with increasing methanol concentration (Fig. 6). However, with dThd/ddIno, since the values of $(a_1 - a_2)$ did not change, the selectivity for this pair of compounds would remain constant even with increases in the methanol content.

Selectivity and retention surfaces

The pH–temperature retention surfaces of ddAdo, ddIno and dThd have a maximum at pH 5.0 at all methanol concentrations. An example of this type of

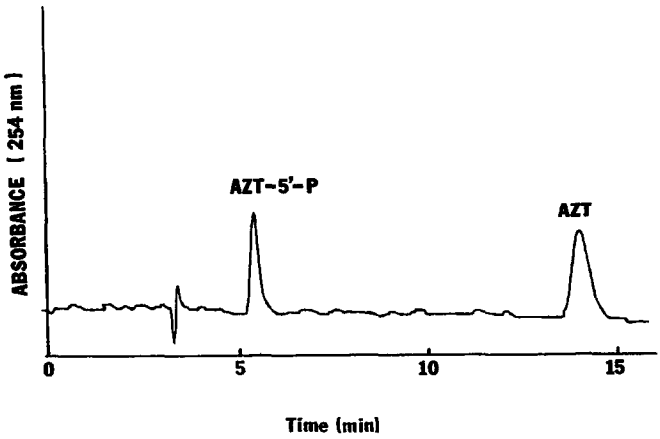

Fig. 10. Chromatogram of analytical separation of AZT and AZT-5'-P. Conditions: Waters μBondapak C_{18} column (30 cm × 4.6 mm I.D., 10 μm particle size); temperature, 26°C; pH, 5.0; methanol concentration, 15%; flow-rate, 1 ml/min.

surface is the pH–temperature retention surface (at 14% methanol) of dThd as shown in Fig. 7.

In the pH–methanol selectivity surface for ddAdo/dThd, the lowest α values are at the edge of pH 3.5 and there is a flat surface when the pH is higher than 4.5 (Fig. 8). In the pH–temperature selectivity surface plot of ddAdo/dThd, there is a maximum between pH 4.5 and 5.0 (Fig. 9), and the selectivity improves with increasing temperature.

The selectivity surface of dThd/ddIno is flat. However, the surface of ddIno/ddCyd at 13 and 13.5% methanol has a minimum at pH 4.5 and as the pH increases from 4.5 to 6.0, the selectivity increases.

These retention and selectivity surfaces show no interdependence of the experimental variables (*e.g.* pH, temperature and methanol content) in the ranges studied. However, if a certain analysis time or selectivity is desired, it can be achieved by selecting two or more sets of conditions from the surfaces. Using the surfaces, it was predicted that the best conditions for analyzing ddNs are a mobile phase of pH 5.0 and either 14% methanol and 40°C or 15% methanol and 30°C. A chromatogram of a separation of the ddNs using a mobile phase of pH 5.0 with 14% methanol at 40°C is shown in Fig. 3.

Discussion

From the guidelines we developed for the RPLC structure–retention relationships of purines, pyrimidines, their nucleosides and nucleotides [12], we predicted that the order of retention by groups would be deoxyribonucleotides prior to deoxyribonucleosides, and the ddNs would have the longest retention times. This order of retention was verified experimentally. Therefore, 13–15% methanol rather than the 7–9% used for the deoxyribonucleosides was needed to obtain k' values of 1–10 for the ddNs.

The experimental conditions necessary for good resolution of the ddNs differed from those of their deoxy analogues in several ways. With the deoxyribonucleosides the slopes in plots of ln k' *versus* percentage methanol (Fig. 2 in ref. 15) were steeper than the slopes of the dideoxy compounds; thus the percentage methanol had a larger effect on the separation factors of the deoxyribonucleosides than those of the ddNs. Within each group of compounds, the slopes of the four solutes were approximately the same; thus methanol concentration could be used to decrease the retention times without affecting the selectivity. Therefore, for the separation of a ddN from its parent deoxy compound, methanol can be a very effective parameter to manipulate the selectivity as the slopes of the methanol–capacity factor plots for the two groups of compounds differed.

In optimizing a separation, temperature can be used effectively to optimize selectivity, especially in the separation of purines frm pyrimidines (Fig. 1 in ref. 15) [17–19]. Although the slopes of all the pyrimidines are the same, these slopes are different from the slopes of the purines (ddIno and ddAdo) which also differ from each other. Therefore, temperature can be used to improve the separation of purines from pyrimidines as well as of ddIno from ddAdo. Since ddIno is a metabolite of ddAdo, this separation is an important one for pharmacokinetic studies of ddAdo. In addition, as can be seen from Figs. 2 and 3, an increase in temperature can be used not only to decrease the retention time but also to improve peak shape of the adenosine analogues. Although higher temperature also improved the resolution of thymidine from

2′-deoxyguanosine, it did not affect the excellent resolution of the three ddN peaks which were eluted first (Figs. 2 and 3).

The effects of pH are largely dependent on the structures of the solutes. The most pronounced effects of structure were observed with deoxyribonucleotides (Fig. 2 of ref. 14) and least pronounced with the dideoxyribonucleosides (Fig. 5). Thus, even through all the deoxyribonucleotides have the same charge on the phosphate group, the extent of influence of this charged moiety is dependent on the structure of the base and the presence or absence of a 3′-hydroxyl group on the ribose ring.

An important advantage of the use of retention surfaces is that if we wish to hold k' constant for isochronal analyses, we can find two or more sets of conditions which will give good separation of the ddNs.

Our work to date involved deoxy- and dideoxyribonucleosides in which there was no substitution on either the heterocyclic base or ribose rings. To test whether these retention and selectivity surfaces together with our structure–retention guidelines were valid in predicting the conditions that would give us good separation of substituted ddNs, we applied our work to the separation of AZT from AZT-5′-P. This separation is needed since AZT-5′-P is a precursor used in the synthesis of new AZT analogues. Based on retention surfaces and our structure–retention guidelines, we predicted that AZT would be eluted after dThd and before ddAdo in the RPLC separation of dideoxynucleosides using the conditions given in Fig. 3. All five compounds were well resolved. The AZT eluted at 14 min and was separated cleanly from both neighboring peaks, dThd and ddAdo. We also predicted that AZT-5′-P would be eluted quickly prior to Thd. This prediction was also verified experimentally.

Using the retention surfaces generated for the deoxyribonucleotides, deoxyribonucleosides and the ddNs, the conditions for analytical, method development and preparative separations of AZT from AZT-5′-P were chosen. Although the column length or diameter and/or particle size accounted for differences in actual retention times of AZT from AZT-5′-P [16], the relative retention times and orders were analogous.

From the pH–methanol and pH–temperature surfaces, it was indicated that a pH of 5.0 gave optimal selectivity. Since our ultmate objective was to optimize the conditions for a preparative separation, we chose ambient temperature as it was easier to work at room temperature with a preparative system. According to our surfaces, at pH 5.0, 14% methanol and a temperature of 30°C, good selectivity would be obtained. However, since room temperature was 26°C, the surfaces indicated that a higher concentration of methanol would compensate for the lower temperature, thus it was predicted that 15% methanol in an eluent of pH 5.0 would give the desired separation at 26°C. Experimentally these conditions gave excellent separations of the AZT and AZT-5′-P both on the analytical scale (Fig. 10) and preparative scale.

In conclusion, using our structure–retention guidelines together with retention and selectivity surfaces of the parent compounds, the ddNs, we found that we could optimize the conditions for the separations of analogues of these compounds as illustrated by the analytical and preparative separations of AZT and AZT-5′-P. We are applying these predictions to separations of other analogues of AZT from precursors and impurities in synthetic mixtures and will report on this work in the near future.

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