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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF NUCLEOSIDE ANALOGUES

SIMULTANEOUS ANALYSIS OF ANOMERIC D-XYLO- AND D-LYXOFURANONUCLEOSIDES AND SOME OTHER D-PENTOFURANONUCLEOSIDES

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SUMMARY

Reversed-phase high-performance liquid chromatography using a C_{18} column was applied to the analysis of reconstituted mixtures of previously synthesized α, β D-xylo- and D-lyxofuranonucleosides as well as a number of commercially available D-ribo- and D-arabinofuranonucleosides. From a detailed study of various parameters (size of support particles, nature and pH of the mobile phase, temperature), optimized conditions were established. Correlations between the retention times and structures of the bases, the orientations of the secondary hydroxyl groups of the sugar moiety and the anomeric configurations of the nucleosides are also reported.

INTRODUCTION

We have previously reported the systematic synthesis of α and β D-xylo- and D-lyxofuranosyl nucleosides of the five naturally occurring nucleic acid bases and their biological evaluation as chemotherapeutic agents[1–3]. In these studies, difficulties were occasionally encountered in the purification of the final reaction products, particularly in the guanine series. This led us to the utilization of reversed-phase high-performance liquid chromatography (RPLC), which has been used extensively for the analytical separation and determination of natural or modified nucleosides[4,5]. Nevertheless, there has been no rationalization of the intrinsic structural factors which can influence the RPLC retention times of nucleosides. We have thus taken advantage of the fact that we possessed two homogeneous series (xylo and lyxo) of anomeric D-pentofuranonucleosides (Fig. 1) in order to study the RPLC behaviour of nucleosides. Since, in RPLC, compounds are retained on columns according to their hydrophobicities, we have exploited this technique to separate anomeric D-pentofuranonucleosides and to establish structure–retention rules.

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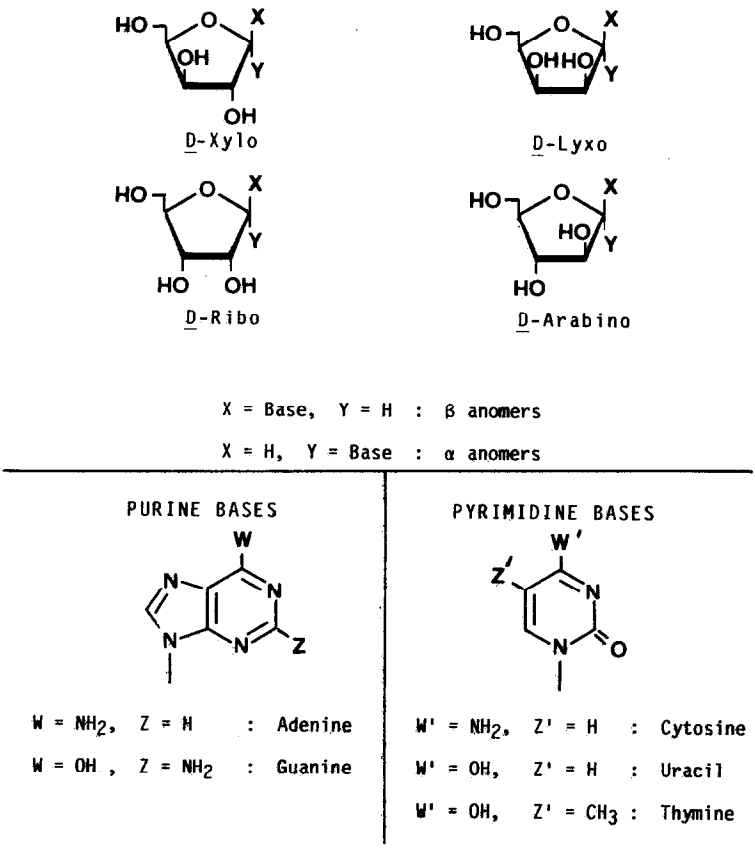


Fig. 1. Structures of D-pentofuranonucleosides.

EXPERIMENTAL

Instrumentation

HPLC was performed on a Waters Assoc. instrument, equipped with two Model 6000 A solvent-delivery systems, a Model 720 solvent programmer, a Model U6K sample injector, a Model 440 UV-absorbance detector operating at 254 nm, a Model R401 differential refractometer and a Model 730 integrator. Protected by prefilters and a precolumn C_{18} Guard-Park, the analytical reversed-phase column was a C_{18} Radial-Pak (100 mm × 8 mm I.D., particle size 5 or 10 μm) in a Waters Assoc. radial compression module RCM-100. Apart from the programmer and the integrator, the whole system was placed in a thermostatted chamber (Frigor-Kulmobel) which could be controlled between 13 and 24°C with a precision of 0.25°C. Because of this feature, reproducible results ($\Delta t_R/t_R < 1\%$) could be obtained during an interval of several weeks despite significant variations of the ambient temperature (15–31°C).

Chemicals

The water used for the buffer was distilled water purified in a Milli-Q system to a resistivity of 18 MΩ/cm (Millipore). Methanol was of HPLC grade (Fisons). Sodium dihydrogenphosphate of Rectapur grade was obtained from Prolabo. The control solution of phosphate (2 l, 1.0 *M*) was filtered through a Millipore membrane (Type HA, pore size 0.45 μm) and degassed in an ultrasonic bath. For the studies of pH, this control solution was diluted (2 l, 0.1 *M*) and the pH was adjusted by means of phosphoric acid or sodium hydroxide solutions. For temperature studies, the control solution was diluted (solvent A, 10 l, 0.1 *M*), the pH was adjusted to 4.73 and the container was aseptically closed and placed in the thermostatted chamber. After about 3 weeks, some bacterial colonies appeared but did not affect the reproducibility. Connections were fitted with filters (20 and 5 μm) which were changed daily. Solvent B (2 l) was water–methanol (1:1, v/v), degassed in an ultrasonic bath.

The twenty samples of the lyxo and xylo series were synthesized in the laboratory[2,3]. The nine samples of the ribo and arabino series were obtained from Sigma (St. Louis, MD, U.S.A.) or Ajinomoto (Tokyo, Japan). Each pure sample was dissolved in water and filtered (HV-4, Millipore). The different solutions were mixed in various proportions in order to identify each compound on the chromatogram. For the chromatograms illustrated (Figs. 4, 6 and 10), the mixtures were adjusted so that the heights of the different peaks were approximately the same. The standard solutions of β-ribosides were stable, but when mixed with other commercial compounds their peaks rapidly decreased, probably due to enzymatic degradations. This obliged us to prepare fresh mixtures containing the β-ribosides before each analysis.

Abbreviations

To simplify the nomenclature for the aldofuranonucleosides we have used the following abbreviations. The nucleobase is symbolized by its initial letter: C = cytosine; U = uracil; G = guanine; T = thymine; A = adenine. The sugar moiety is symbolized by its initial (small) letter: l = lyxose; x = xylose; r = ribose; a = arabinose. The anomeric configuration is represented by α or β. Thus uridine is indicated not by the usual Urd, but by βrU. Additionally, the secondary hydroxyls at positions 2′ and 3′ are denoted by *endo* if they are on the same side of the plane of the sugar as the 5′ primary hydroxyl, and *exo* if they are on the opposite side.

RESULTS AND DISCUSSION

Optimization of chromatographic analysis

Preliminary work following literature procedures[5–8] showed that isocratic elution on a reversed-phase C_{18} column with sodium phosphate buffer allowed resolution of C and U derivatives, but addition of methanol was necessary to elute the G, T and A derivatives within a reasonable retention time. Pure water, acetate buffers or acetonitrile were less selective. The resolution changed only slightly with buffer concentration (0.05–0.2 *M*)[4] but was strongly dependent on the pH, particle size of the stationary phase and temperature.

pH. Nucleosides are relatively weak acids and bases. Among the usual ones, cytidine (βrC) has the highest pK_{ab} (4.15), uridine (βrU) and guanosine (βrG) have the lowest pK_{aa} (9.2). Since they are neutral between their pK_{ab} and pK_{aa} values, they can be analysed by reversed-phase HPLC.

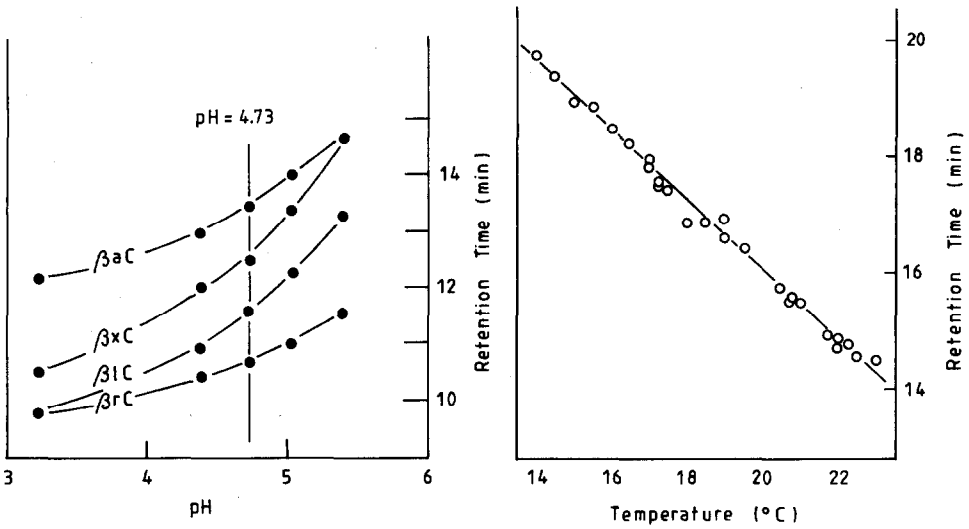


Fig. 2. Variation of the retention times of the β-anomers of cytosine derivatives with pH. Column: Radial-Pak C_{18}, 10 μm. Phosphate buffer: 0.1 *M*; flow-rate 1.0 ml/min.

Fig. 3. Variation of retention time of the β-lyxo-uracil derivative (βlU) with temperature. Column: Radial-Pak C_{18}, 5 μm. Solvents: A = 0.1 *M* phosphate buffer, pH 4.73; B = water–methanol (1:1, v/v). Program A: solvent A during 12 min, then linear gradient until 80% A, 20% B during 38 min; flow-rate 1.0 ml/min. Twenty-six measurements at 21 different temperatures between 14 and 23°C.

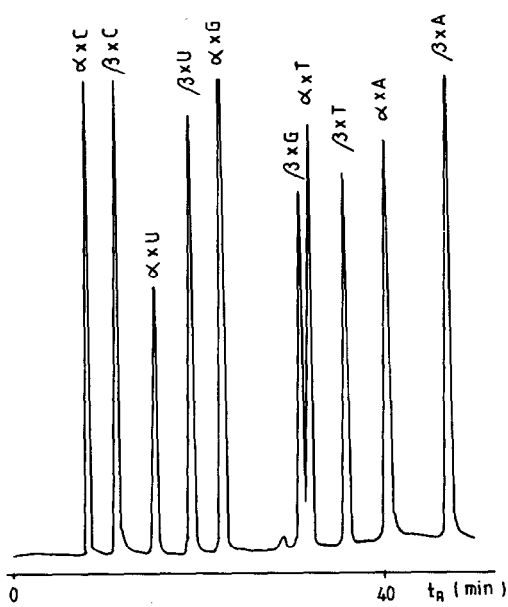
Cytosine derivatives were eluted first by phosphate buffer but were difficult to resolve. Fig. 2 shows the variation of the retention time of their β-anomers with pH: the βrC and βlC signals were superimposed at pH 3.24, and the same pattern was observed for the βxC and βaC signals at pH 5.40. Analysis at pH 4.38 and 5.04 revealed the possibility of resolving these four isomers and the optimum separation was obtained at pH 4.73.

Particle size of the stationary phase. A 10-μm C_{18} column was insufficient to separate certain derivatives, such αxC and βrC or βaU and αrG. The resolution was improved by using a 5-μm phase.

Effect of temperature. Controlled temperature variation allowed chromatographic studies in the range 13–24°C. Fig. 3 shows the variation of the retention time, t_R, of the βlU derivative with temperature. Quasi-linearity was observed for all compounds eluted isocratically, but not for the compounds eluted by a methanol gradient. After optimization of the chromatographic conditions (program A), the plot of $t_R = f(T)$ was quasi-linear for all compounds studied. The theory predicts linear Van 't Hoff plots of log k' vs. $1/T$ under isocratic conditions. The observed linearity of t_R *vs.* T was due to the very narrow range of temperature studied.

Xylose derivatives

Fig. 4 shows an example of the chromatograms obtained for a mixture of the ten xylo derivatives, and Fig. 5 shows the variation of their retention times with temperature. For the β-anomers, t_R increased with the lipophilicity of the nucleobase:



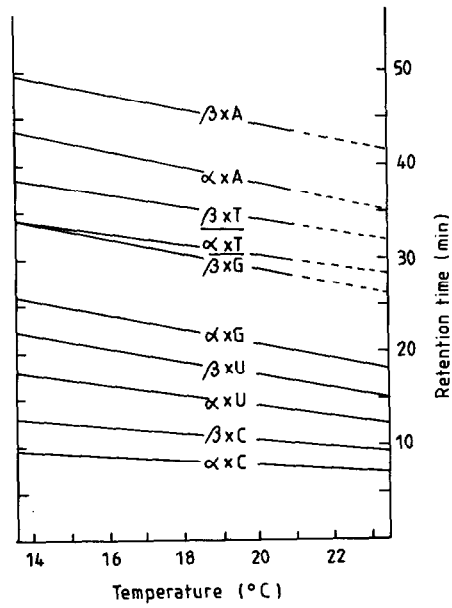
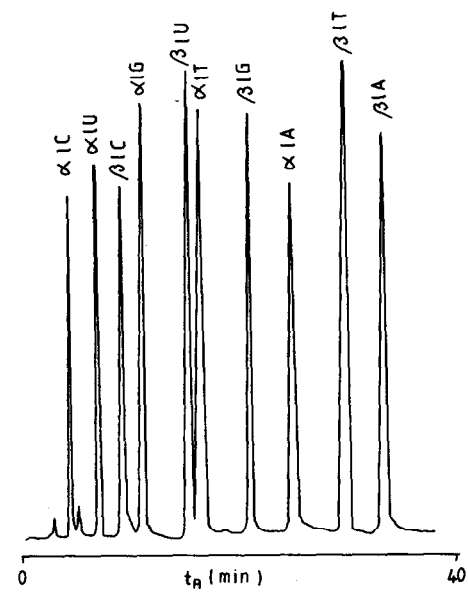


Fig. 4. Separation of the ten xylo derivatives studied. Chromatographic condition as in Fig. 3. Temperature: 17.5°C.

Fig. 5. Variation of the retention times of the ten xylo derivatives with temperature. Chromatographic conditions as in Fig. 3.



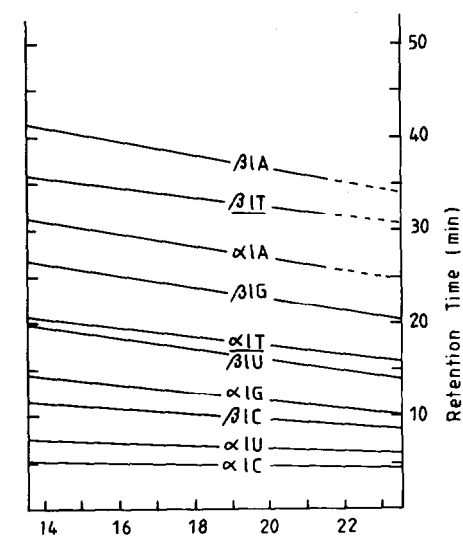


Fig. 6. Separation of the ten lyxo derivatives studied. Chromatographic conditions as in Fig. 3. Temperature: 17.5°C.

Fig. 7. Variation of the retention times of the ten lyxo derivatives with temperature. Chromatographic conditions as in Fig. 3.

C < U < G < T < A. The same order was observed for the α-anomers. For a given nucleobase, t_R was greater for the β-anomer than for the α-anomer. In the xylo series, the effect of the anomeric configuration on t_R was relatively weak so that the following elution order was observed: αxC < βxC < αxU < βxU < αxG < βxG < αxT < βxT < αxA < βxA. For the thymine derivatives, the variation of t_R with temperature was smaller than the variation observed with other compounds.

Lyxose derivatives

The elution order C < U < G < T < A was found for both α- and β-anomers, and the order was α < β for each nucleobase. The difference in retention times between the α- and β-anomers was greater than in the xylo series, so that the observed elution order was: αlC < αlU < βlC < αlG < βlU < βlG < αlA < βlT < βlA (Figs. 6 and 7). As in the xylo series, retention times decreased more slowly with increasing temperature for thymine derivatives than for the others.

Comparison of xylo and lyxo derivatives

The resolution of the twenty derivatives of these two series is optimal at 17.5°C. Because of the particular pattern of thymine derivatives, αxT and βxG are unresolved

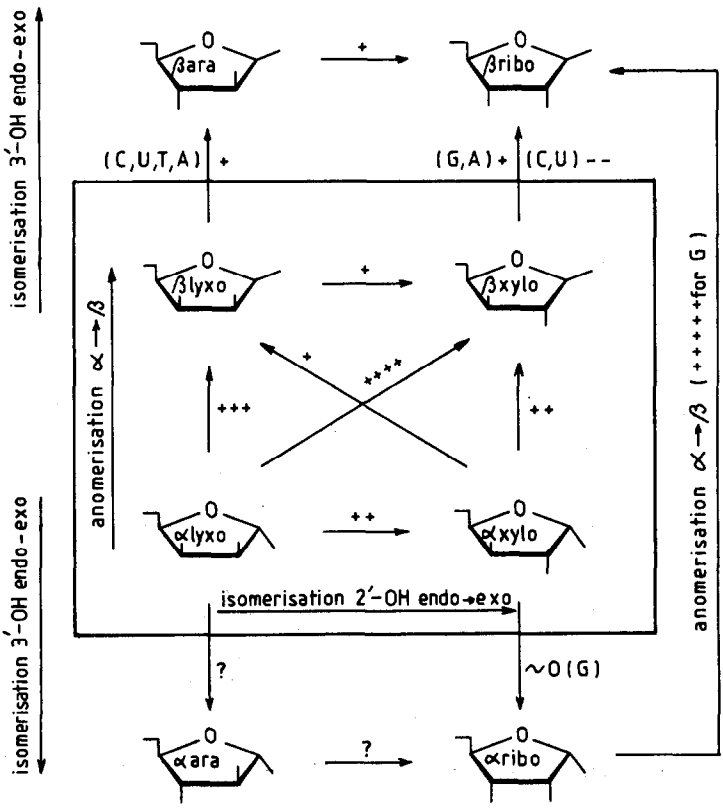


Fig. 8. Order of magnitude of the variation of the retention time according to the different isomerizations of the sugar moiety.

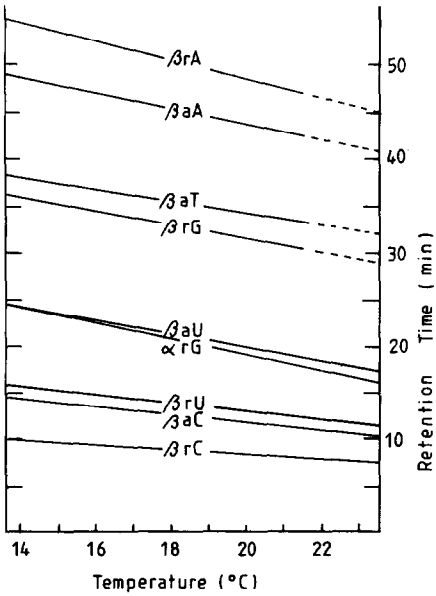


Fig. 9. Variation of the retention times of the five ribo derivatives and of the four arabino derivatives with temperature. Chromatographic conditions as in Fig. 3.

at low temperature (14°C), while αlT and βxU are unresolved between 19 and 21°C. Independent of the nature of the base and the anomery, all the lyxo derivatives were eluted before their xylo isomers.

These results are summarized in Fig. 8: for a given base moiety, the variation of retention time is presented as a function of the sugar moiety. The partition coefficient of the solute between the stationary organic phase and the mobile aqueous phase is lowest for the α-lyxo derivatives. It is increased by either anomerization ($\alpha \rightarrow \beta$), or by isomerization of the 2'-hydroxyl (*endo* → *exo*). These two contributions are additive, so that the β-xylo derivatives have the greatest partition coefficients.

To determine the effect of isomerization of the 3'-hydroxyl on retention times, studies of available compounds in the ribose and arabinose series were undertaken.

Ribo and arabino derivatives

Fig. 9 shows that the elution orders were the same as in the lyxo and xylo series: C < U < G < A for ribo derivatives; C < U < T < A for arabino derivatives and $\alpha < \beta$ for the ribo derivatives of guanine. Fig. 10 shows a chromatogram obtained under optimized conditions for a mixture of the 29 derivatives studied.

Rationalization

These data allow completion of the scheme in Fig. 8. Results obtained for the four β-arabinose derivatives show that the isomerization *endo* → *exo* of the 3'-hydroxyl increases the partition coefficient by the same order of magnitude as that by isomerization of 2'-hydroxyl. If these contributions are additive, the β-ribose derivative should display the greatest retention times. The effect is indeed observed for the

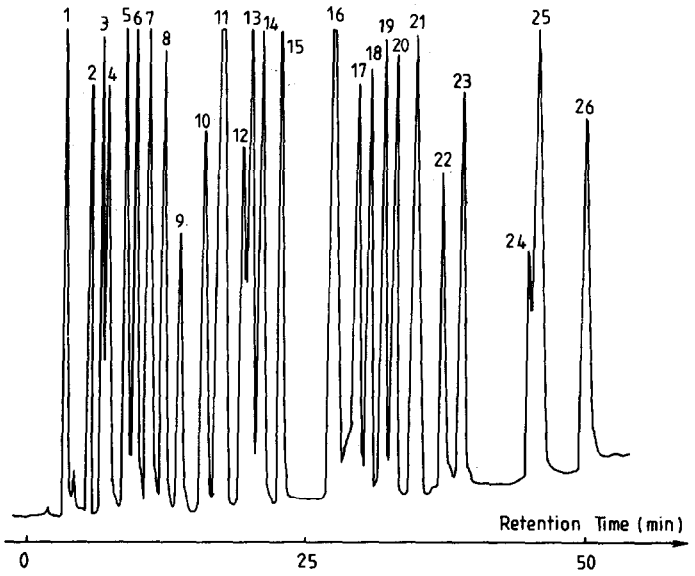


Fig. 10. Chromatogram obtained for a mixture of the 29 derivatives studied. Because of overlappings, only 26 peaks were observed. Chromatographic conditions as in Fig. 3. Temperature: 17.5°C. Elution order: 1 = αlC; 2 = αlU; 3 = αxC; 4 = βrC; 5 = βlc; 6 = βxC; 7 = βaC + αlG; 8 = βrU; 9 = αxU; 10 = βlU; 11 = αlT + βxU; 12 = αrG; 13 = βaU; 14 = αxG; 15 = βlG; 16 = αlA; 17 = βxG; 18 = αxT; 19 = βrG; 20 = βlT; 21 = βxT + βaT; 22 = βlA; 23 = αxA; 24 = βaA; 25 = βxA; 26 = βrA.

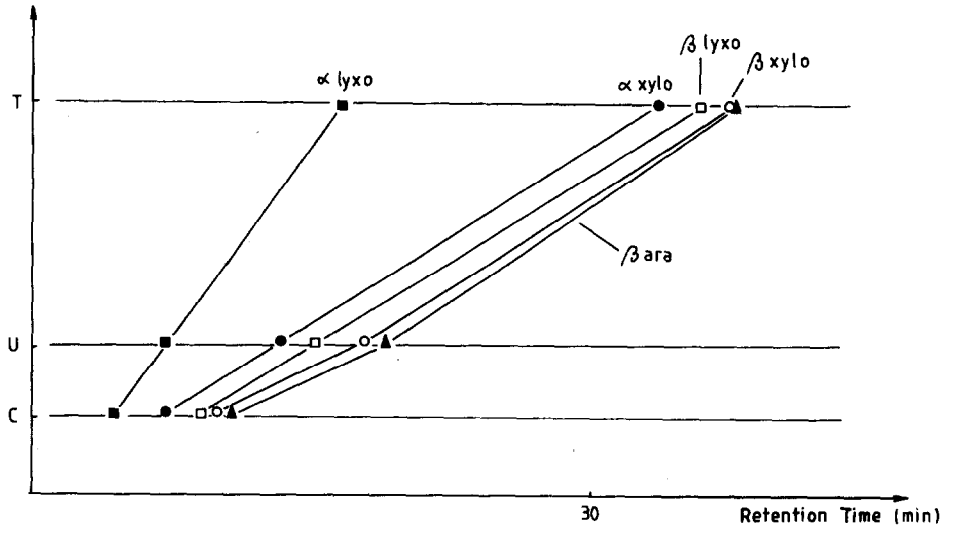


Fig. 11. Plot of the retention times of the pyrimidine derivatives according to the relative lipophilicity of the bases and the structure of the sugar moiety. Data from Fig. 10. See text for explanation.

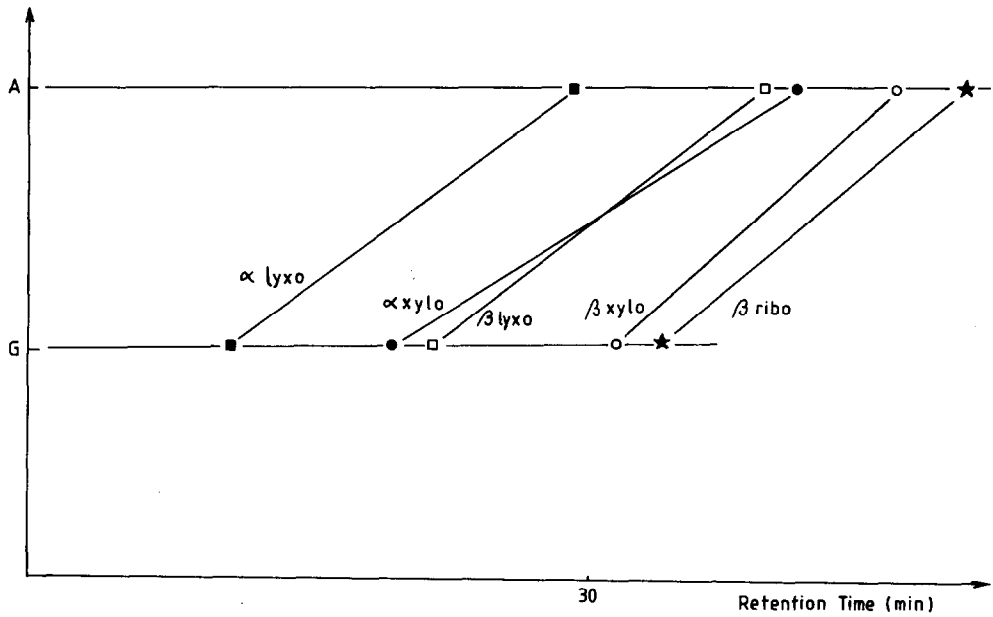


Fig. 12. Plot of the retention times of the purine derivatives according to the relative lipophilicity of the bases and the structure of the sugar moiety. The ordinate was chosen so that the points corresponding to the α-xylo derivatives line up with those of their homologues in the pyrimidine series.

purine derivatives βrG and βrA. On other hand, the behaviour of the pyrimidine derivatives βrC and βrU is quite different. It is likely that this is due to a particular conformation of these derivatives, or to a specific interaction between the base and 5'-hydroxyl. The retention time of the αrG compound is compatible with this scheme; the anomeric effect for the α- and β-ribo-guanine derivatives is of the same magnitude as in the lyxo derivatives.

This rationalization in terms of the contributions due to the nature of the base and the isomerization of the different groups (base, 2'-OH, 3'-OH) allows a comparison between the retention times of the 29 compounds studied. It appears that a quantitative difference exists between the pyrimidine derivatives (C, U, T) and the purine derivatives (G, A). Fig. 11 presents a plot of the retention times of the pyrimidine derivatives *vs.* the relative lipophilicities of the different bases. The ordinate was arbitrarily chosen so that the points corresponding to the α-xylo derivatives line up; for the other derivatives, there is a similar alignment of the corresponding points. This plot allows the prediction of the retention time of a given compound in the series. Its validity was supported in the case of the αlU derivative, a compound first synthesized at the end of the study. The intersection of the straight lines linking the points corresponding to αlT and αlC with the axis corresponding to the uracil derivatives allowed prediction of the retention time of αlU with an error of less than 1%.

A similar diagram was obtained for the purine derivatives G and A (Fig. 12). The quasi-parallelism of the straight lines shows the incremental character of the phenomena and its dependence on isomerism and the lipophilicity of the bases. Utilization of these diagrams should permit the prediction of retention times of missing

compounds in the series, and extension to the derivatives of other bases appears possible.

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