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Note

Reversed-phase high-performance liquid chromatographic separation of ribosyl, 2′-deoxyribosyl and arabinosyl nucleosides of adenine and hypoxanthine*

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One area of analysis which has lent itself extremely well to high-performance liquid chromatographic (HPLC) methods is the separation of nucleic acid components. Because of the importance of these compounds in biological systems many investigations have been conducted in order to find rapid and reproducible methods for their separation[1].

In connection with our studies on the metabolism of arabinosyl nucleosides possessing antiviral activity, we required a quantitative separation of the ribosyl, 2′-deoxyribosyl and arabinosyl derivatives of adenine and hypoxanthine. A further requirement was that the separated components be readily collectable, free from interfering materials and easily analyzed by liquid scintillation spectrometry.

Early methods of separating nucleosides were based on experiences acquired with nucleotides, namely ion exchange chromatography. However, recent work has involved the more rapid process of reversed-phase high-performance liquid chromatography (RP-HPLC)[2–7]. We now have examined this process to accomplish the required separation.

Previous work has focused on the separation of ribosyl nucleosides from 2′-deoxyribosyl nucleosides[2,3], pyrimidine ribosyl nucleosides from purine ribosyl nucleosides[4,6] and the arabinosyl derivative of adenine from the arabinosyl derivative of hypoxanthine[5]. Previously reported separations of the three carbohydrate classes by means of thin-layer chromatography (TLC) of nucleotides[8], mixtures of nucleotides, nucleosides and bases[9] and anion exchange HPLC[10] were not suitable for our needs.

We wish to report two methods found suitable for the separation of the title compounds. One method involves an isocratic separation while the second utilizes a gradient system.

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EXPERIMENTAL

Chemicals and reagents

Ammonium dihydrogen phosphate (Fisher), sodium borate (J. T. Baker), boric acid (E. Merck) and sodium periodate (Eimer and Amend) were all of reagent grade and used without further purification. The methanol (Burdick & Jackson) and acetonitrile (Fisher) were HPLC grade. The mixed borate–phosphate buffer used in the final analyses contains 80 parts 0.075 *M* $Na_2B_4O_7$ and 20 parts 0.01 *M* $NH_4H_2PO_4$ with the pH adjusted to 6.03.

Adenosine (Ado) and inosine (Ino) were from Nutritional Biochemicals; 2′-deoxyadenosine (dAdo) and 2′-deoxyinosine (dIno) were from Sigma; 9-β-D-arabinofuranosyladenine (ara-A) and 9-β-D-arabinofuranosylhypoxanthine (ara-H) were obtained through the courtesy of Dr. H. Machamer, Warner-Lambert/Parke-Davis Co., Detroit, MI, U.S.A.

Apparatus

The HPLC system consisted of a dual piston pump and controller (Nester Faust) and an Altex Model 153 fixed UV detector set at 254 nm with instrument attenuation at 0.02 a.u.f.s. The column was either a Partisil PXS 10/25 C_8 (25 × 3 cm) or a Partisil PXS 10/25 ODS-2 (25 × 3 cm) (both from Whatman).

Procedures

The water used throughout was distilled and all solutions were passed through a Millipore filter (0.45 μm) and degassed prior to use. All runs were conducted at ambient temperature at a flow-rate of 1 ml/min and a chart speed of 0.25 cm/min. Adjustments to pH were made by adding crystalline boric acid to the buffered solutions prior to mixing with the organic solvents.

RESULTS AND DISCUSSION

Initial studies were conducted using the ODS-2 column in attempts to separate Ado, ara-A, Ino and ara-H. With this column good separation was achieved for the adenine derivatives. Ara-H and Ino were eluted first, but together, while ara-A and Ado were clearly separated from each other and from the hypoxanthine derivatives. Different ratios of phosphate buffer (0.01–0.10 *M*) to methanol and phosphate buffer (0.01–0.10 *M*) to acetonitrile covering a pH range of 3.07–7.40 were utilized but failed to separate ara-H and Ino.

In order to investigate the conditions necessary to separate Ino and ara-H, we explored chromatography on a reversed-phase (C_8) column. A slight separation of Ino and ara-H was obtained using a phosphate buffer–methanol solvent system at pH 7 but improvement in the degree of separation was desired. The technique of carbohydrate–borate complexation has been explored to effect the separation of ribosyl, 2′-deoxyribosyl and arabinosyl nucleosides on both anion and cation exchange systems[10]. Replacing the phosphate buffer with a borate buffer at pH 7 provided a baseline separation of these two nucleosides. However the baseline separation of ara-H and Ado was lost. In the borate system the elution order was reversed and the ribosyl derivatives were eluted before the corresponding arabinosyl derivatives. The

cis-diol functionality facilitated the formation of stable borate complexes which were readily eluted on the reverse phase medium. Complexing with the *trans*-diol system was less complete resulting in relatively longer retention times for the arabinosyl compounds. By a comparison of the retention times in these two systems it was noted that borate concentration had a greater effect on retention times than either the phosphate concentration or pH.

To obtain a larger difference in retention times of ara-H and Ado the borate buffer was modified by the addition of varying amounts of phosphate buffer. The retention times of ara-A and ara-H were very sensitive to changes in the borate/phosphate ratio. Increases in concentration of borate in these mixtures increased the retention times of ara-A and ara-H while the opposite effect was observed for both Ado and Ino. As a result, we were able to achieve a baseline separation of the four previously discussed nucleosides on the C_8 column using a mixed borate–phosphate (75:25) buffer at pH 7.16 and acetonitrile in a ratio of 98:2.

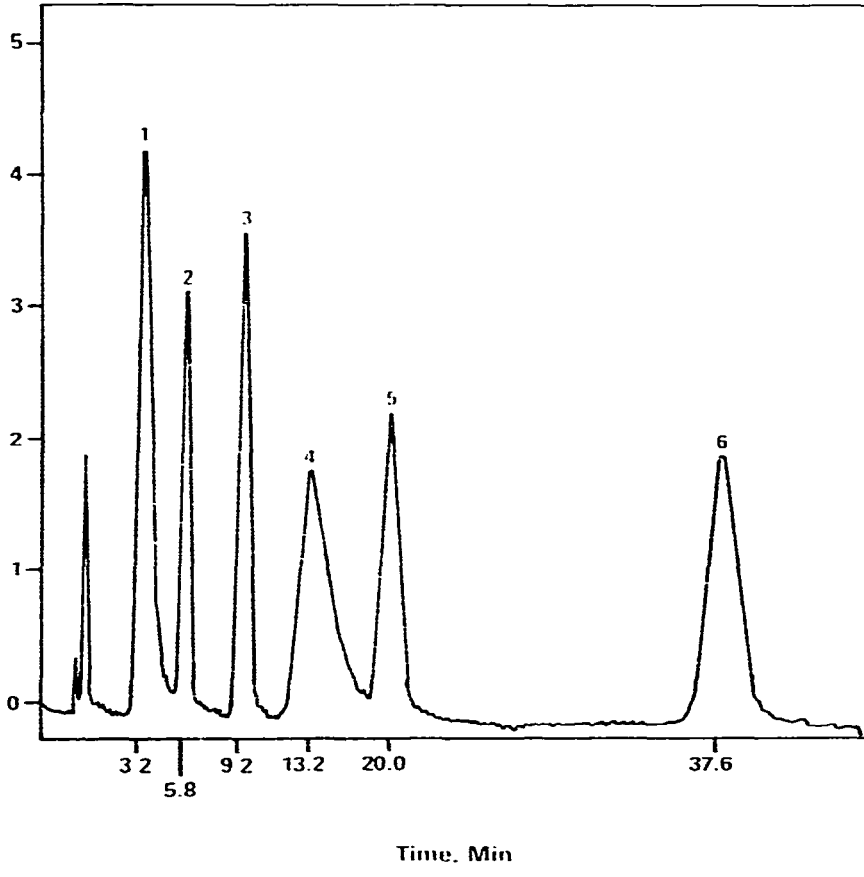

Fig. 1. Isocratic elution of ribosyl, 2′-deoxyribosyl and arabinosyl derivatives of adenine and hypoxanthine. The following conditions were employed: column, Partisil PXS 10/25 C_8; mobile phase, buffer–methanol (95:5); pressure, 900 p.s.i.; detection, UV at 254 nm (0.02 a.u.f.s.). The compounds eluted in the following order: 1 = Ino; 2 = ara-H; 3 = dIno; 4 = Ado; 5 = ara-A; 6 = dAdo.

When we included the deoxynucleosides, however, we were unable to separate dIno and Ado. Through a series of minor adjustments to the buffer system, pH and changing the organic solvent to methanol a complete separation of all six nucleosides was effected (see Fig. 1).

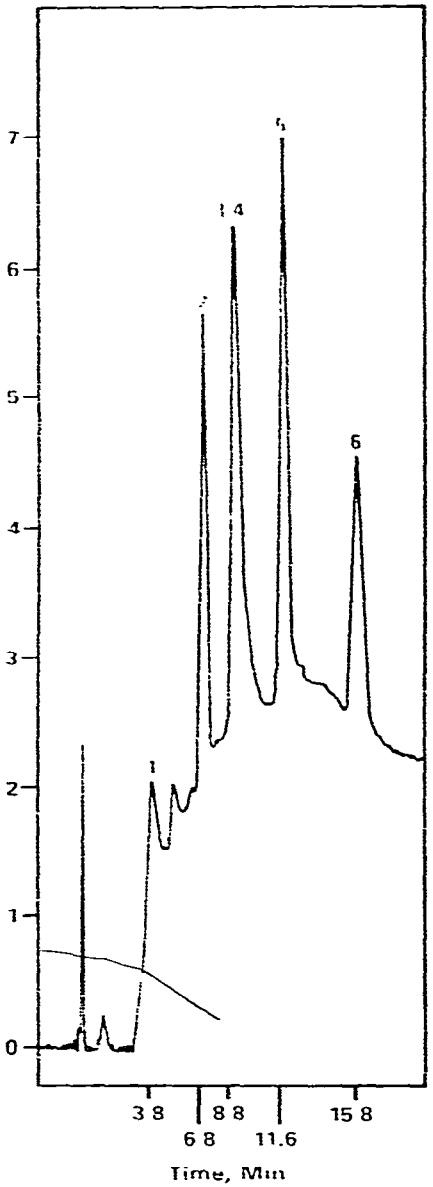

Fig. 2. Gradient elution of ribosyl, 2'-deoxyribosyl and arabinosyl derivatives of adenine and hypoxanthine. The following conditions were employed: column, Partisil PXS 10/25 C_8; mobile phase, linear gradient from buffer–methanol (99:1) to (75:25); pressure, 900 p.s.i.; detection, UV at 254 nm (0.02 a.u.f.s.). Compounds as in Fig. 1.

Even though this separation of the six nucleosides satisfied our immediate needs, the elaborate composition of the solvent system was seen as a potential problem in repetitive analyses. Consequently we undertook an examination of gradient conditions in hopes of achieving a simpler solvent system. Utilization of a mixed borate/phosphate buffer–methanol (from 99:1 to 75:25) failed to separate the most difficult pair, namely dIno and Ado, but was very effective in separating all of the other nucleosides (see Fig. 2). For most metabolic studies this particular difficulty was not seen to be important.

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