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Short communication

Mixed ion-pair high-performance liquid chromatography of uridine 5′-diphospho-α-D-glucuronic acid and its hydrolysis products

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Abstract

Although conventional ion-exchange HPLC and ion-pair reversed-phase HPLC using tetrabutylammonium hydroxide allowed ready determination of uridine 5′-diphospho-α-D-glucose and its hydrolysis products, neither method was suitable for the determination of uridine 5′-diphospho-α-D-glucuronic acid (α-UDPGA). However, mixed ion-pair reversed-phase HPLC, using a combination of tetrabutylammonium hydroxide and tetraethylammonium hydroxide (1:1), has been found to offer a convenient and effective means of simultaneously determining α-UDPGA and its hydrolysis products, UDP, UMP and cUMP.

1. Introduction

During our structure activity studies of glucose transfer [1], it became necessary to develop a method of analysing the glycosylating coenzymes, uridine 5′-diphospho-α-D-glucose (α-UDPG) and uridine 5′-diphospho-α-D-glucuronic acid (α-UDPGA) in the presence of their hydrolysis products, uridine 5′-diphosphate (UDP) and uridine 5′-phosphate (UMP) (Fig. 1). At first, we investigated ion-exchange HPLC [2,3] and ion-pair reversed-phase HPLC [4,5]. As this was only partly successful, mixed ion-pair HPLC [6] was studied.

2. Experimental

2.1. Materials

α-UDPG, α-UDPGA, UDP, uridine 3′,5′-cyclic phosphate (cUMP) and UMP were obtained as sodium salts from Sigma. Tetrabutylammonium hydroxide and tetraethylammonium hydroxide were obtained as aqueous solutions from BDH. All other reagents were of analytical-reagent grade.

2.2. HPLC

The analytical HPLC system was composed of a Laboratory Data Control (LDC) gradient solvent-delivery system, a Rheodyne injection valve fitted with a 20-μl loop, a Pye LC3 spectrophotometric detector operating at 262 nm and a

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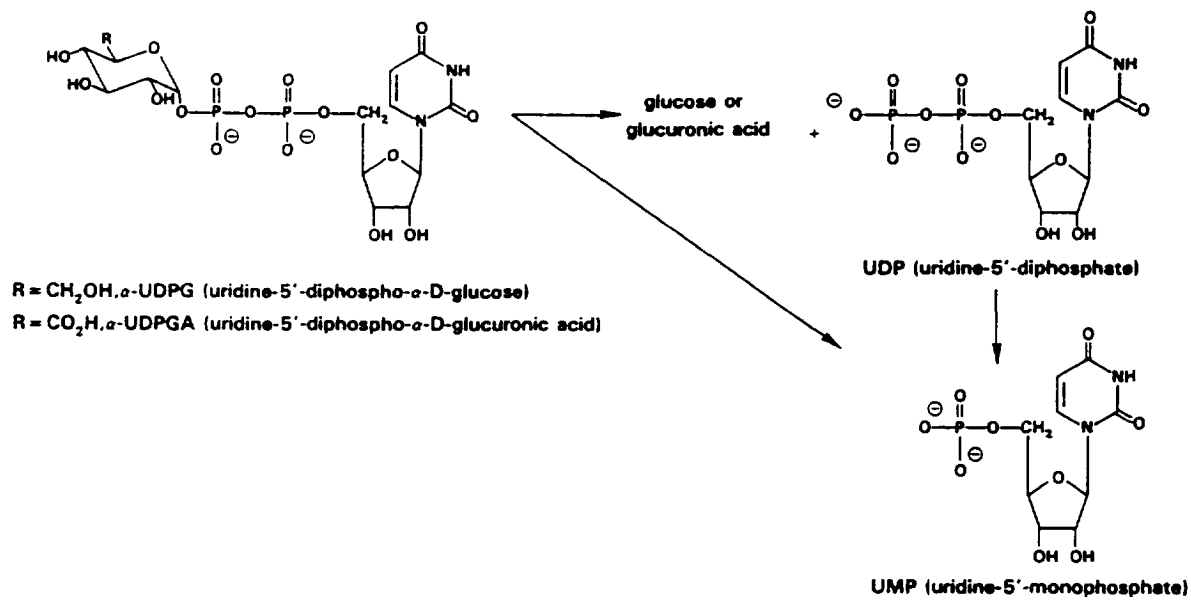


Fig. 1. Hydrolysis of α-UDPG and α-UDPGA.

Hewlett-Packard 338S integrator. The columns (200 × 4.5 mm) were either Whatman Partisil 10 SAX for ion-exchange or Partisil 10 ODS2 for ion-pair reversed-phase chromatography. Ion-exchange analyses were run at room temperature, but ion-pair analyses were run with the column maintained at 30°C by means of a Magnus Scientific water jacket and a Churchill water pump and heater. The flow-rate was 2 ml/min.

3. Results

3.1. Ion-exchange chromatography

Ion-exchange HPLC using a strong anion exchanger was found to give good separation of all the compounds when the sample solution was close to neutrality, as shown in Fig. 2. However, acidic samples (pH > 3) gave poor, and often merged peaks. This, coupled with the degradation of the silica in the columns by the mobile phase, led us to consider ion-pair reversed-phase HPLC.

3.2. Ion-pair reversed-phase chromatography

Employing a C_{18} column and using tetra-

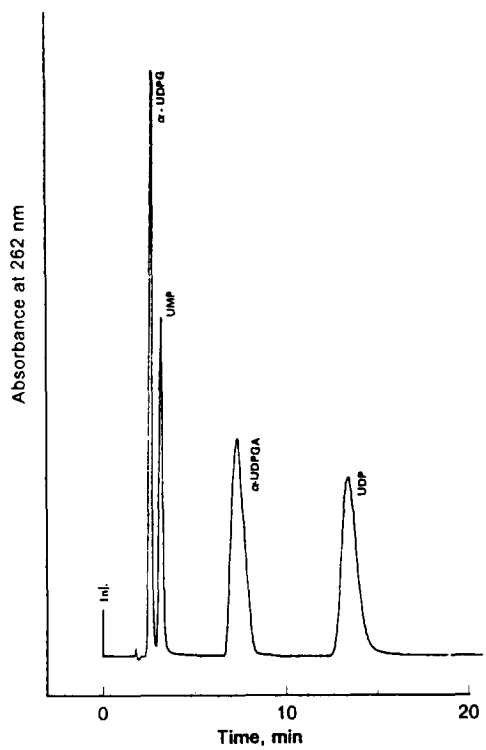


Fig. 2. Ion-exchange HPLC trace of a mixture of α-UDPG, UMP, α-UDPGA and UDP. Conditions: 20 μl from an aqueous solution of about 1 mg/ml injected by valve onto a 200 × 4.5 mm column packed with Partisil 10 SAX. The mobile phase was 0.05 *M* potassium dihydrogenorthophosphate, 0.05 *M* potassium chloride, adjusted to pH 6 with KOH.

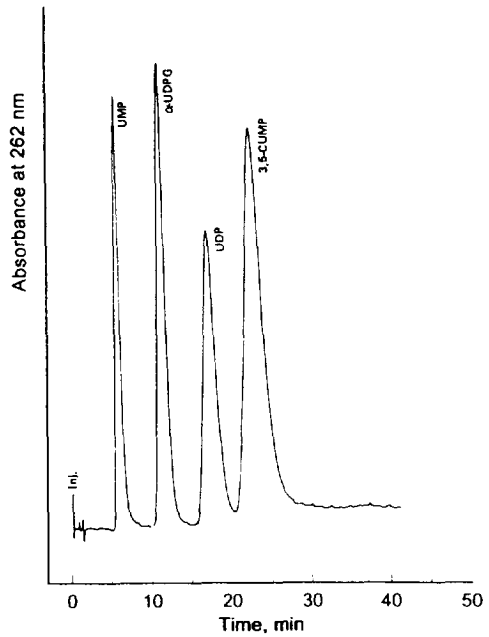


Fig. 3. Ion-pair reversed-phase HPLC trace of a mixture of UMP, α-UDPG, UDP and cUMP. Conditions: 20 μl from an aqueous solution of about 1 mg/ml injected by valve onto a 200 × 4.5 mm column packed with Partisil 10 ODS 2. The mobile phase was 0.0475 *M* tetrabutylammonium hydroxide, 0.0475 *M* ammonium dihydrogenorthophosphate, adjusted to pH 6 with KOH in 5% aqueous methanol.

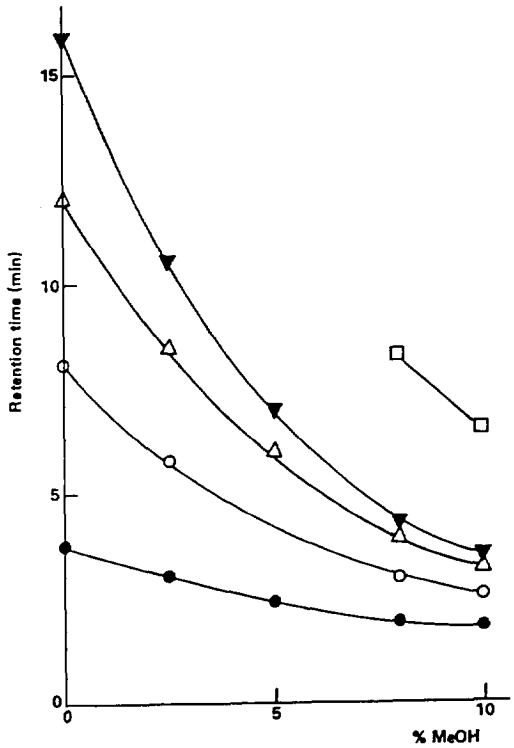


Fig. 4. Effect upon retention time of increasing methanol content of eluent buffer in ion-pair reversed-phase HPLC of UMP, α-UDPG, UDP, cUMP and α-UDPGA. Conditions: 20 μl from an aqueous solution of about 1 mg/ml injected by valve onto a 200 × 4.5 mm column packed with Partisil 10 ODS 2. The mobile phases were 0.0475 *M* tetrabutylammonium hydroxide, 0.0475 *M* ammonium dihydrogenorthophosphate, adjusted to pH 6 with KOH in water, or in 2.5, 5, 8 and 10% aqueous methanol. □ = α-UDPGA; ▼ = cUMP; △ = UDP; ○ = α-UDPG; ● = UMP.

butylammonium hydroxide as the ion-pairing reagent in a phosphate buffer at pH 6 [4,5], a good separation under isocratic conditions of α-UDPG, UDP and UMP —and of the possible hydrolysis product cUMP— was achieved, as shown in Fig. 3. It was necessary to use methanol (5%, v/v) in the eluent to decrease the retention times to below 30 min. However, α-UDPGA could not be analysed under these conditions, as it was indefinitely retained on the column. In order to reduce the retention time of α-UDPGA the proportion of methanol was increased to 10%. However, since this led to the merging of the peaks due to UDP and cUMP, as shown in Fig. 4, an alternative method was sought. When tetrabutylammonium hydroxide was replaced with tetraethylammonium hydroxide the retention time of α-UDPGA was lowered to 6 min with no methanol added. Unfortunately, this caused UMP and UDP to merge together at a retention time of 2.5 min. This led to the development of a system using a mixed ion pair, similar to that used by Au et al. [6] for the analysis of fluorinated nucleotides.

Using various ratios of tetrabutylammonium hydroxide and tetraethylammonium hydroxide in the eluent allowed the retention times of all the phosphates to be varied to different degrees, as shown in Fig. 5. The best mobile phase for an isocratic system, which utilised the two reagents in equal proportions, effected a relatively rapid and efficient separation of all five phosphates, as shown in Fig. 6.

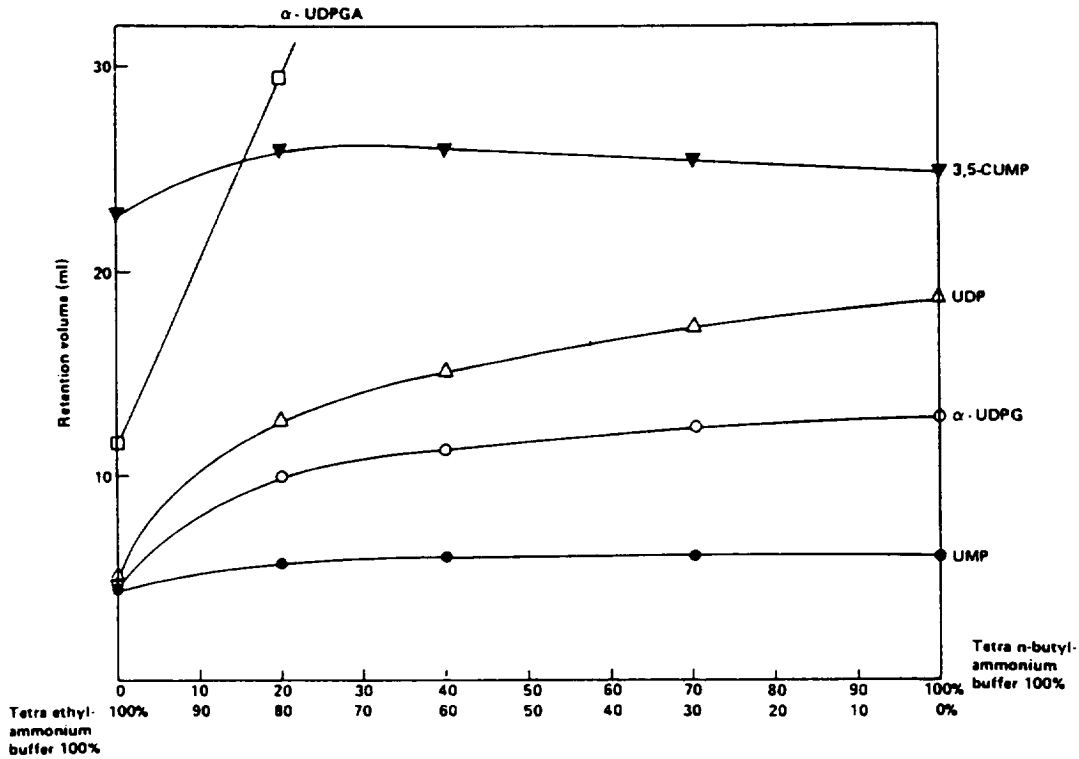
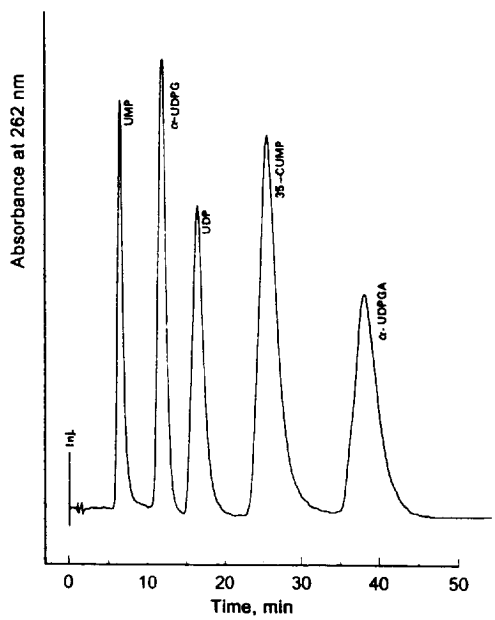


Fig. 5. Effect of the composition of mixtures of tetrabutylammonium hydroxide and tetraethylammonium hydroxide upon the ion-pair reversed-phase HPLC retention volumes of UMP, α-UDPG, UDP, cUMP and α-UDPGA. Conditions: 20 μl from a solution of about 1 mg/ml injected by valve onto a 200 × 4.5 mm column packed with Partisil 10 ODS 2. Mobile phase: various ratios (0:100, 20:80, 40:60, 70:30 and 100:0) of 0.002375 *M* tetrabutylammonium hydroxide and 0.002375 *M* tetraethylammonium hydroxide, in 0.0475 *M* ammonium dihydrogenorthophosphate, adjusted to pH 6 with KOH in 10% aqueous methanol.



4. Discussion

Three methods of separating α-UDPG and α-UDPGA and their hydrolysis products have been developed. The use of ion-pair reversed-phase HPLC proved to be most versatile, although not as rapid as ion-exchange HPLC. It was found that varying the ratio of two ion-pairing reagents, tetrabutylammonium hydroxide

Fig. 6. Mixed ion-pair reversed-phase HPLC trace of a mixture of UMP, α-UDPG, UDP, cUMP and α-UDPGA. Conditions: 10 μl from an aqueous solution of about 1 mg/ml injected by valve onto a 200 × 4.5 mm column packed with Partisil 10 ODS 2. The mobile phase was 0.002375 *M* tetrabutylammonium hydroxide, 0.002375 *M* tetraethylammonium hydroxide, 0.0475 *M* ammonium dihydrogenorthophosphate, adjusted to pH 6 with KOH in 10% aqueous methanol.

and tetraethylammonium hydroxide, from 0 to 100% (Fig. 5) allowed the relative retention times of all the phosphates to be varied to differing degrees. It is particularly interesting to note the very high sensitivity for α-UDPGA, the only carboxylic acid, to the ratio of the ion-pairing reagents compared to the relatively low sensitivity of all of the other phosphate compounds. At pH 6 this carboxylic group will exist as anionic carboxylate. The coupling of this extra anionic group of UDPGA with tetrabutylammonium clearly, as could be expected, provides highly efficient binding to the C_{18} stationary phase and thereby dramatically lengthens the retention time.

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