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SEPARATION AND QUANTIFICATION OF ADP-D-GLYCERO-D-MANNO-HEPTOSE AND ADP-L-GLYCERO-D-MANNOHEPTOSE BY BORATE LIQUID CHROMATOGRAPHY AND ITS USE IN THE ASSAY OF ADP-D-GLYCERO-D-MANNOHEPTOSE 6-EPIMERASE

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SUMMARY

A high-performance liquid chromatographic method using a borate–formate buffer system and an Aminex A-27 ion-exchange resin has been developed for the separation of heptose nucleotides from bacterial lipopolysaccharide biosynthesis. The main advantages of this system are isocratic elution with volatile buffer components, low working pressure, short analysis time, insensitivity to high salt contents in samples, stability of resin and adaptability to analytical and preparative separations.

INTRODUCTION

L-Glycero-D-mannoheptose (LD-heptose) is a typical component of the lipopolysaccharide core region of Gram-negative bacteria. Eidels and Osborn[1] have postulated a pathway for its biosynthesis. According to this scheme, starting from sedoheptulose-7-phosphate, a nucleotide-diphospho D-glycero-D-mannoheptose (DD-heptose) is synthesized which is then converted into a nucleotide-diphospho LD-heptose by the action of a 6-epimerase. We have recently isolated both types of nucleotide sugars from *Shigella sonnei* and *Salmonella typhimurium* R-mutants and the nucleotide moiety has been identified as ADP[2,3]. These results have been confirmed by Coleman[4], who found ADP-DD-heptose also in *Escherichia coli* K12.

The major problem in the determination of epimerase activity is the difficulty in separating the mixture of nucleotide sugars formed, because of the small differences between the structures of the substrate and products. Previously reported procedures for the separation of nucleotide sugar epimers such as paper[5] and thin-layer chromatography[6,7], paper electrophoresis[8] and lectin affinity chromatography[9,10] are cumbersome, time-consuming and hardly quantitative. In other cases, the nucleotide sugars are hydrolysed during the lengthy chromatographic procedure[11].

Weckbecker[12] and Keppler developed a method for the separation of UDP derivatives of galactose, glucose, N-acetylglycosamine, N-acetylgalactosamine, glucuronate and galacturonate as their borate complexes by high-performance liquid chromatography (HPLC) on a silica-based primary amine-containing anion exchanger.

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The main drawbacks of this system are the poor resistance of the silica matrix to even mildly alkaline solvents, and the use of glycerol in solvents which causes relatively high working pressures. Furthermore, the system is sensitive to the higher salt concentrations used in enzyme tests and needs a laborious desalting procedure. A gradient elution was applied and re-equilibration was necessary after each analysis.

To overcome all of these difficulties, we have developed an HPLC method for the separation of ADP-DD-heptose and ADP-LD-heptose which uses conventional anion-exchange resins with isocratic elution. It is less sensitive to high ion concentrations and buffer salts can easily be removed thus allowing application to preparative-scale separation of these nucleotide sugars. The method is able to separate NAD-, AMP-, ADP-, ATP- and ADP-heptoses in about 70 min and suitable for the quantitation of ADP-DD-heptose 6-epimerase.

MATERIALS AND METHODS

Chemicals and standard materials

Formic acid, boric acid and ammonium hydroxide (25% aqueous solution) were obtained in analytical grade from E. Merck (Darmstadt, F.R.G.). AMP, ADP, ATP and nicotiamide–adenine dinucleotide (oxidized, NAD) were obtained from Sigma (St. Louis, MO, U.S.A.). ADP-D-glycero-D-mannoheptose and ADP-L-glycero-D-mannoheptose were prepared in our laboratory as described elsewhere[2,3].

Preparation of buffer

Boric acid (18.55 g, 0.3 mol) was dissolved in 500 ml of 1 *M* formic acid and then concentrated ammonium hydroxide (about 43 ml) was added under constant stirring. The final pH was adjusted to 8.2 at room temperature to and the solution diluted to 1000 ml with distilled water. The buffer was filtered through a membrane filter (pore diameter: 0.45 μm) and deaerated before use.

Chromatographic procedures

Analytical columns (100 × 4.5 mm) stainless steel, fitted with 2-μm stainless-steel frits, were filled with Aminex A-27 anion-exchange resin (Bio-Rad Labs., Richmond, CA, U.S.A.) which is an 8% cross-linked styrene–divinylbenzene copolymer with quaternary ammonium functional groups. Before the packing procedure, the resin was slurried in two volumes of buffer and deaerated. The column was equilibrated with buffer (120 ml). Separations were performed at 70°C, with a flow-rate of 60 ml/h. Preparative separations were carried out at 60°C, with a flow-rate of 72 ml/h, on ANX4 anion-exchange resin (Hamilton, Bonaduz, Switzerland), in a jacketed glass column (250 × 9 mm; Glenco Scientific, Houston, TX, U.S.A.) heated with a water-circulation system. The sample volume was 1 ml.

For separations, a DuPont high-performance liquid chromatograph consisting of a Model 870 pump and thermostatic oven fitted with a Rheodyne Type 7125 injection valve (20-μl and 1000-μl sample loops) was used. The absorbance of the effluent was monitored at 254 nm with a DuPont Model 860 absorbance monitor (light paths 10 and 2 mm). The peak areas were determined with an electronic integrator built in our laboratory.

RESULTS AND DISCUSSION

Choice of buffer and pH

The only difference in the structure of ADP-DD- and ADP-LD-heptose is the steric configuration of the OH group on the C6 carbon atom of heptose. Therefore, the use of borate as the complexing buffer was crucial, because other systems available for nucleotide separation will not discriminate between structurally similar 6-epimeric ADP-sugars. Complete formation of the sugar borate complexes can be achieved with 0.3 *M* borate at pH 8.2. At this pH no serious decomposition of the pyrophosphate bond was observed, even at elevated temperature[13]. In this buffer, the nucleotides were strongly adsorbed onto the ion exchanger and it is necessary to advance the elution by addition of 0.5 *M* formate. A similar effect can be achieved with chloride salts as lithium, sodium or potassium chloride. The use of such salts, however, is not recommended because halogen ions are corrosive towards some types of stainless-steel components of HPLC pumps[14] and are non-volatile. Ammonium was chosen as the counter ion of the buffer system. Thus, the solvent contains only three ions which are easily removable in volatile form when necessary in preparative separations. Ammonium borate was used for the separation of ribo- and deoxyribonucleosides on Dowex 50 cation exchanger by Moran and Werkheiser[15]. These authors gave a detailed description of the method of removal of this buffer salt.

Effect of temperature

Separations at different temperatures in the range of 40–70°C were examined as shown in Fig. 1. With increasing temperature, a significant decrease in pressure and a considerable improvement in resolution were observed. From the resulting

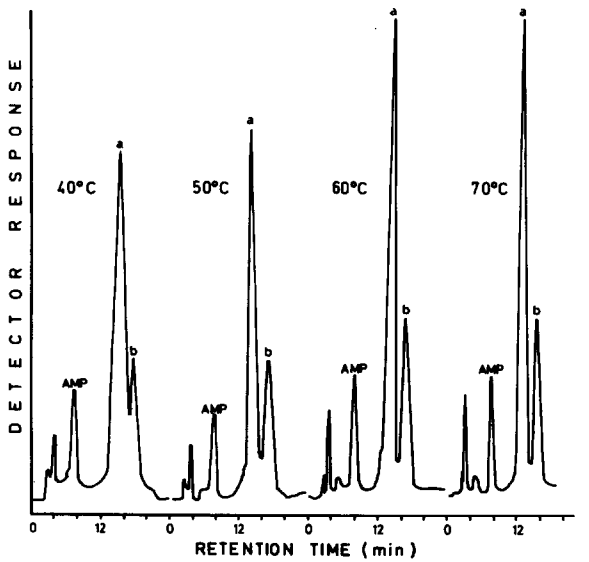


Fig. 1. Effect of temperature on the resolution and retention times of nucleotides. A standard mixture of nucleotides (20 µl) was assayed on the analytical column at the temperatures shown as described in Materials and methods. For other parameters see Table I. Peaks: a = ADP-LD-heptose; b = ADP-DD-heptose.

TABLE I

EFFECT OF TEMPERATURE ON THE CHROMATOGRAPHIC PARAMETERS OF HEPTOSE NUCLEOTIDES

For abbreviations see text.

	Working temperature (°C)			
	40	*50*	*60*	*70*
Pressure (MPa)	6.08	5.57	4.86	4.56
(p.s.i.)	882	808	705	661
Retention time (min)				
ADP-LD-heptose	15.03	14.24	13.68	12.95
ADP-DD-heptose	17.60	17.04	16.53	15.59
Δt (min)	2.57	2.84	2.85	2.64
$(W_{b1} + W_{b2})/2$ (min)	7.06	6.69	4.31	2.72
Resolution	0.36	0.42	0.66	0.97
Integrator counts				
AMP	90	69	71	55
ADP-LD-heptose	562	480	487	404
AMP/ADP-LD-heptose	0.16	0.14	0.15	0.14

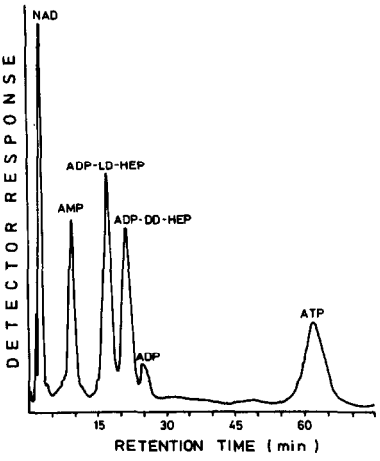
data (Table I), the optimum temperature for complete separation of ADP-sugars at pH 8.2 was established as 70°C. Retention times were not significantly influenced by the change in temperature. On the basis of the AMP to sugar nucleotide ratio, no deterioration was found even at 70°C. This is in accord with the observations of Khym[13], who separated nucleotides at the same pH and temperature as employed here. The resolution, R_s, of heptose nucleotides was nearly complete as established from the equation

$$R_s = \frac{\Delta t}{(W_{b1} + W_{b2})/2}$$

where Δt = the distance between the two peak maxima and W_{b1} and W_{b2} are the widths of the peaks at the base[16].

Analytical separation

To test the separation and quantitative recovery under the working conditions described in Materials and methods, standard mixtures of known concentrations of nucleotide components participating in heptose biosynthesis were examined. A typical chromatogram of NAD, AMP, ADP, ATP and of the corresponding sugar nucleotides is shown in Fig. 2. The separation of these components can be achieved in about 70 min and is sufficient for exact computing of the concentrations by the integrator. It is to be noted that normally ATP is not present in the reaction mixtures and quantitative analyses can be carried out within 30 min, in contrast to gas chromatographic determinations of heptose which need complicated derivatization procedures. The sensitivity of the method is in the range of 1–10 nmol of nucleotide sugar at 0.010 absorbance unit full scale. An examination of other nucleotide sugars, such as GDP-mannose, UDP-glucose and UDP-galactose on the same analytical



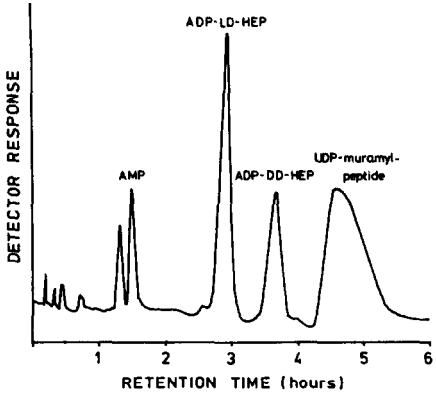


Fig. 2. Analytical separation of the mixture of nucleotides (20 μl) formed in heptose biosynthesis. For chromatographic conditions see Materials and methods.

Fig. 3. Preparative separation of heptose nucleotides. A 1-ml sample of the heptose nucleotide fraction extracted from 180 g (wet weight) of *Shigella sonnei* R-mutant bacteria and prepared by formate ion exchange[3] was loaded on the preparative column as described in Materials and methods.

column (not shown) indicated that, by alterations of the chromatographic parameters, *e.g.*, buffer concentration, use of gradient, etc., more complex mixtures of such components could similarly be resolved.

Preparative separation

Anion-exchange chromatography of a nucleotide extract from bacteria in the ammonium formate buffer system resulted in the separation of a peak containing both of the heptose nucleotides coeluted with an UDP-muramyl peptide[3]. To separate these components and to obtain in pure form, paper chromatography in which chromatograms were developed for 6 days or more and further rechromatography was needed. In such cases, the yield of the heptose nucleotides was low because of extensive hydrolytic degradation of pyrophosphate bonds.

In contrast, preparative separation in the borate–formate system described here yields high recovery and complete separation in 6–7 h, even in the presence of UDP-peptide contaminant (Fig. 3). About 1–1.5 μmol in 1 ml (calculated from the specific absorption of adenosine) can be loaded. In order to obtain high recovery and to avoid hydrolysis of nucleotide sugars, immediately after the appearance of the desired fractions in the effluent it is advisable to adjust the pH to 5.0 by the addition of 1 *M* formic acid and recover the nucleotides by adsorption on charcoal as described[2]. When solutions at pH 8.2 in borate buffer were left to stand even at −20°C, hydrolytic degradation and loss of heptose nucleotides resulted.

Assay of ADP-D-glycero-D-mannoheptose 6-epimerase

ADP-LD-heptose is formed from ADP-DD-heptose by the action of a 6-epimerase enzyme[3]. To test the activity of this enzyme during its preparation and to study the parameters influencing the enzyme kinetics, the analytical separation tech-

nique was adapted to replace the previously used gas chromatographic method, *i.e.*, the determination of heptose derivatives obtained from hydrolysates of nucleotide-containing reaction mixtures. The activity of the 6-epimerase was tested by incubation of a mixture of the enzyme preparation and ADP-DD-heptose as described previously[3,17]. 100-μl Aliquots were taken at given intervals and the enzyme was inactivated in a bath of boiling water. Precipitated protein was removed by centrifugation (15 000 *g*, for 5 min). The supernatant (20 μl) was immediately injected onto an analytical column and eluted as described in Materials and Methods.

Through the action of the 6-epimerase, the peak of ADP-DD-heptose was diminished, with a concomitant increase in the ADP-LD-heptose peak, as shown in Fig. 4. The relatively large peak of AMP was attributed to the activity of a phosphodiesterase coextracted in the enzyme preparation. In a control test mixture without enzyme preparation (see Fig. 4A), no deterioration of the heptose nucleotide was observed. Retention time and resolution were not influenced by buffer and other salts (Tris, magnesium chloride etc.). The ratio of the concentrations of both nucleotides when plotted against elapsed time is characteristic of the kinetics of enzyme action (see Fig. 4, inset). It is obvious that the effect of DD-heptose 6-epimerase reaches an equilibrium at 67% of ADP-LD-heptose. A shift of this reaction to the right takes place when LD-heptose nucleotide is consumed in lipopolysaccharide biosynthesis by transferases.

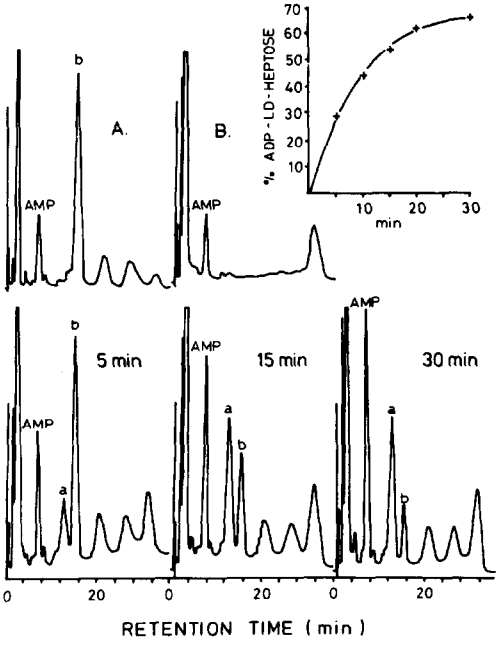


Fig. 4. Assay of ADP-DD-heptose 6-epimerase on the analytical column. After incubation with enzyme, samples were taken at given intervals and 20-μl inactivated aliquots were injected. (A) Reaction mixture without enzyme preparation; (B) reaction mixture without DD-heptose nucleotide substrate. Peaks: a = ADP-LD-heptose; b = ADP-DD-heptose. Inset: per cent of ADP-LD-heptose formed at different times during incubation of ADP-DD-heptose with 6-epimerase.

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