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SEPARATION OF EIGHT INOSITOL ISOMERS BY LIQUID CHROMATOGRAPHY UNDER PRESSURE USING A CALCIUM-FORM, CATION-EXCHANGE COLUMN

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ABSTRACT

All eight inositols were resolved by liquid chromatography under pressure using a Ca^{2+}-form cation-exchange resin as stationary phase and water as an eluting solvent. The method was successfully applied to the separation of the products formed when *myo*-inositol was boiled in water in the presence of Raney nickel. In this case, six peaks corresponding to inositols and at least seven unknown peaks were resolved, showing that this liquid chromatographic method is an efficient way to monitor the side reactions which occur during the ^1H–^2H exchange reaction of *myo*-inositol in deuterium oxide with Raney nickel catalyst.

INTRODUCTION

myo-Inositol is widespread in Nature. It is a component of membranes and may have very important roles in cellular signal transduction[1,2]. The roles of *myo*-inositol in plants have been reviewed[3,4]: Especially important in plants is its role as a precursor of uronic acid and pentose residues of cell-wall polysaccharides. *myo*-Inositol is converted into those sugar residues for the synthesis of new cell-wall polysaccharides in growing seedlings[5–7]. For further studies of those important roles of *myo*-inositol, the requirement for deuterium-labeled *myo*-inositol is increasing with the development of such analytical instruments as the gas–liquid chromatograph–mass spectrometer, nuclear magnetic resonance spectrometer, and so on.

The carbon-bound hydrogen atoms of hydroxymethyl groups undergo readily ^1H–^2H exchange in deuterium oxide in the presence of Raney nickel catalyst[8]. Extensive epimerization occurs during the ^1H–^2H exchange reaction, and this seriously hinders the preparation of deuterium-labeled *myo*-inositol[9]. Therefore, methods for the effective separation of inositol isomers are extremely important. We have reported the separation of inositols as their borate complexes by anion-exchange chromatography using Dowex 1 resin[9]. The method was successfully applied to the preparative-scale separation of perdeuterated inositol isomers which were produced during the ^1H–^2H exchange reaction of *myo*-inositol in deuterium oxide

in the presence of Raney nickel catalyst. However, *neo*- and *chiro*-inositols were only partially resolved by this method. To separate the two inositol isomers and to facilitate the entire separation procedure, we investigated the separation of inositols by liquid chromatography under pressure (l.c.).

scyllo-Inositol and *myo*-inositol were separated previously by l.c. using an Aminex HPX-87H organic acid column[10]. A silica-bonded stainless-steel column (Microsil) resolved *chiro*- and *myo*-inositols[11]. However, resolution of all eight inositol isomers by l.c. has not previously been reported.

Polyols containing two or three hydroxyl groups in a suitable arrangement form complexes with metal ions. Details of the mechanism of the complex formation were published by Angyal and Davies[12-14], and others[15]. The complex-forming ability of polyols allows their separation on metal-form cation-exchange resins[15-17]. Cation-exchange chromatography using Sr^{2+} as the cation, for instance, separated *epi*-inositol from *myo*- and *chiro*-inositols, but failed to resolve the latter two[18]. Paper electrophoresis using calcium acetate buffer was effective in resolving a number of the inositols[19]. Therefore, we have studied the chromatography of inositols on an l.c. column packed with Ca^{2+}-form cation-exchange resin, and now report efficient new methods for the separation of all eight inositol isomers in either standard mixture or in complex synthetic mixtures.

EXPERIMENTAL

Inositols. — *myo*-Inositol was purchased from Sigma Chemical Co. (St. Louis, MO). *cis*-Inositol was a gift from Professor S. J. Angyal (University of New South Wales, Australia), and other standard inositols were gifts from Professor L. Anderson (University of Wisconsin-Madison). *scyllo*-Inositol, *chiro*-inositol, and *neo*-inositol were also prepared from *myo*-inositol boiled in water with Raney nickel (Sigma) and purified by a combination of crystallization and Dowex 1 column chromatography[9].

Treatment of myo-*inositol with Raney nickel.* — *myo*-Inositol was boiled for 3 h under reflux in water with Raney nickel and filtered[9]. The filtrate was passed through a column (1 × 9.5 cm) of Amberlite MB-3, a mixed resin of Amberlite IR-120 (H^+) and IRA-410 (OH^-), and the column was washed with water (100 mL). The eluate and washings were combined and evaporated to dryness. The dried material was dissolved into water and analyzed by l.c. The recovery of standard *myo*-inositol from the column of Amberlite MB-3 was determined by l.c. on a calcium column with *scyllo*-inositol as an internal standard.

L.c. analyses. — The chromatographic system consisted of a Dupont 870 pump module equipped with a Bio-Rad HPX-87C column (Ca^{2+} form), a Dupont heated column compartment, a Rheodyne fixed-loop injector (20 μL), a Hewlett–Packard 3390A recording integrator, and an ERMA ERC-7510 refractive index detector. The HPX-87C column was packed with $\approx$7 μm spherical beads of sulfonated polystyrene that has been cross-linked with 8% of divinyl benzene. It was

TABLE I

RETENTION TIMES OF INOSITOL PEAKS

Inositols	*85°, 1 mL/min*[a]		*24°, 0.65 mL/min*[a]	
	Retention time (min)	R[b]	*Retention time (min)*	R[b]
scyllo-	6.91	2.3	10.09	3.0
myo-	8.13[c]	2.3	12.80	0.9
chiro-	9.51	1.5	13.72	3.2
muco-	10.52	9.8	17.48	2.0
neo-	20.30	0.7	20.29	16.5
epi-	21.26	25.8	64.66	4.0
allo-	95.08		82.01	
cis-			[d]	

[a]Oven temperature and flow rate. [b]Resolution between adjacent peaks. $R = (t_2 - t_1)/1/2(t_{w1} + t_{w2})$, where t_1 and t_2 refer to the peak retention times, and t_{w1} and t_{w2} are their peak widths at the baseline. [c]These two peaks were not resolved under the conditions. [d]Not determined.

produced and used in the Ca^{2+} form. The eluting solvent was distilled de-ionized water, filtered prior to use through 0.2-μm Nylon 66 filters, and the elution was performed at 0.65 to 1.0 mL per min. Depending upon temperature, the resulting pressures were from about 2 to 7 MPa.

RESULTS

Separation of standard inositols. — *chiro*-Inositol and *neo*-Inositol were clearly resolved within 11 min at 85° with a flow rate of 1 mL per min. Under the same conditions, almost all isomers were resolved except for *myo*- and *chiro*-inositols (Table I). When these two isomers were injected separately, the retention time of *myo*-inositol was slightly shorter than that of *chiro*-inositol. To find the best conditions for the separation of all eight inositols, the effects of changing temperature and flow rates on their retention times were investigated. As expected, the retention times of all eight isomers decreased as the flow rate increased (Fig. 1). However, the effects of temperature on their retention times varied among the inositols (Fig. 2). The retention times of *allo*- and *epi*-inositols decreased logarithmically with an increase in temperature. The retention times of *neo*- and *muco*-inositols decreased gradually with the temperature increase. A slight decrease was also observed in the retention time of *chiro*-inositol, but the decrease in the retention time of *myo*-inositol was negligible. On the other hand, the retention time of *scyllo*-inositol slightly increased with an increase in temperature. The retention time of *cis*-inositol is reported only at 85° with a flow rate of 1 mL per min, because its retention times were extremely long at the lower temperatures or flow rates. The best resolution of seven isomers (*cis* excluded) was achieved at room temperature (24°) with a flow rate of 0.65 mL per min (Fig. 3). The resolution values were

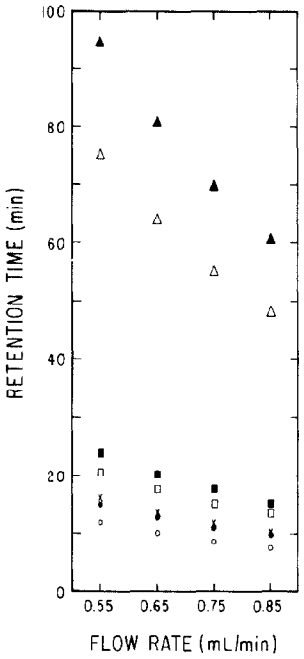

Fig. 1. Effect of flow rate on the retention times of inositol isomers. The mixture of inositols (0.25 mg/mL each) were separated by l.c. equipped with a HPX-87C column at 24° using water as a mobile phase. The injection volume was 20 μL, and the eluted peaks were detected by refractive index detection at ½X. Key: (▲—▲) *allo*-inositol, (△—△) *epi*-inositol, (■—■) *neo*-inositol, (□—□) *muco*-inositol, (×—×) *chiro-inositol,* (●—●) *myo*-inositol, and (○—○) *scyllo*-inositol.

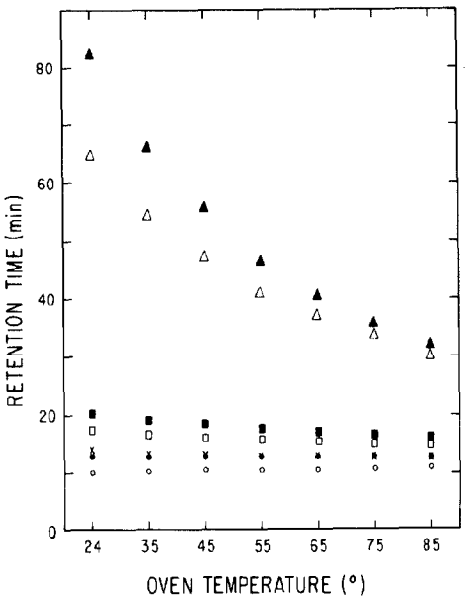

Fig. 2. Effect of temperature on the retention times of inositol isomers. The chromatography was performed at 0.65 mL/min. Other conditions were the same as in Fig. 1. Key: see legend to Fig. 1.

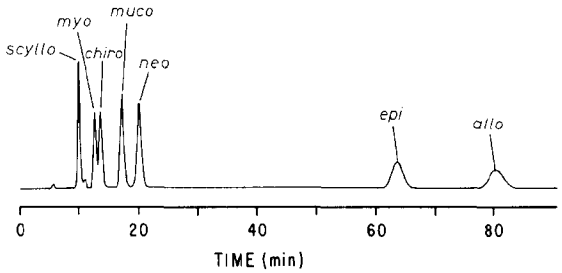

Fig. 3. Separation of seven inositol isomers by l.c. The mixture of seven inositol isomers was separated at 24° with the flow rate of 0.65 mL/min. Other conditions were the same as in Fig. 1.

large enough for accurate quantification of peak areas (Table I). The method was also quite sensitive; levels as low as 50 ng of *myo*-inositol could easily be detected. Lower flow rates than 0.65 mL per min caused unnecessarily longer retention times of *epi*- and *allo*-inositols.

There are two chiral isomers of *chiro*-inositol (D- and L-), which are expected to be formed during epimerization of *myo*-inositol. Our l.c. system did not separate the two chiral forms. The standard *chiro*-inositol from Dr. Anderson was the D form.

Application of the Ca^{2+}-form cation-exchange column to the separation and detection of products from myo-*inositol boiled in water with Raney nickel.* — Pretreatment of the Raney nickel-*myo*-inositol reaction products with Amberlite MB-3 resin completely removed colored materials present in the reaction solution. Although strongly basic anion-exchange resins adsorb and decompose some carbohydrates[20–23], we obtained a 96% recovery of standard *myo*-inositol after the Amberlite MB-3 resin treatment.

As seen in Fig. 4, all peaks were eluted from the column within 40 min at 85°, and at 0.6 mL per min. After a 3-h treatment of *myo*-inositol with Raney nickel in water at 100°, six inositol isomers (*scyllo*-, *myo*-, *chiro*-, *neo*-, *epi*-, and *allo*-inositols) were detected and characterized by retention-time comparison with standards. *chiro*-Inositol was resolved from *myo*-inositol at 24°, such as illustrated in Fig. 3. Though *muco*-Inositol was detected and identified by g.l.c.–m.s. using capillary

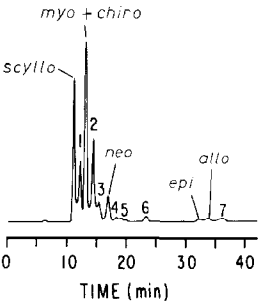

Fig. 4. Separation of the products from *myo*-inositol boiled with Raney nickel in water by l.c. The sample was prepared as described in the Experimental section. The chromatography was performed at 85° with the flow rate of 0.6 mL/min. Other conditions were the same as in Fig. 1.

columns of SE-30 and SE-54 (ref. 9), its presence was not confirmed by this l.c. system because the *muco*-inositol peak overlapped with an unknown peak 3; these were not separated even at 24°.

In addition to the inositol peaks, at least seven unknown peaks were resolved and detected by the RI detector. Unknown peaks 1 and 2 were major peaks found after only 3 h of boiling the water solution. Unknown peaks 1 and 3 in Fig. 4 corresponded with a single unknown peak that emerged faster than *scyllo*-inositol (ref. 9) in the water wash from a column of Dowex 1 (BO_2^-), showing that the calcium column resolved the two compounds which were not separated by anion-exchange chromatography. Unknown peak 2 corresponded to another unknown one (previously referred to as "I"[9]), which also emerged in a water fraction from a column of Dowex 1 a little later than *scyllo*-inositol.

DISCUSSION

Ion-exchange chromatography using Dowex 1 resin (borate form) is a useful method for the preparative scale separation of [^{2}H]inositol epimers which are produced during the ^{1}H–^{2}H exchange reaction of *myo*-inositol in deuterium oxide with Raney nickel[9]. However, the procedure is lengthy, and *chiro*- and *neo*-inositols were only partially resolved. Since these two inositols are major products of the epimerization reaction, better separation methods were required to resolve the two isomers. We clearly resolved the two inositol isomers within 11 min by use of a Ca^{2+}-form cation-exchange column and only water as a mobile phase. The separation method was successfully extended to the separation of all eight inositols.

The separation mechanism of polyols on a Ca^{2+}-form column has been studied and its applications to the separation of some carbohydrates have been reported[17]. This technique is often called ligand-exchange chromatography, because polyols passing through the column form transient coordination complexes with the column-bound cation. Different polyols will have differing affinities for the cation and, therefore, be eluted at different times thus allowing chromatographic resolution.

The ability of polyols to form coordination complexes depends on the geometrical arrangement of their hydroxyl groups, and details of the mechanism of the complex formation were also published by Angyal and Davies[12–14], and others[15].

For cyclic polyols such as cyclitols, the following generalizations have been drawn. A set of three adjacent hydroxyl groups in an axial–equatorial–axial arrangement provides the strongest complex with Ca^{2+} ions. A pair of adjacent hydroxyl groups in axial–equatorial sequence forms a weak, but still a significant coordination complex with Ca^{2+}. Sequences of adjacent hydroxyl groups with the equatorial–equatorial or axial–axial geometry provide very poor opportunities for coordination complexes. As shown in Fig. 5, *cis*-inositol has three potential *a–e–a* arrangements of hydroxyl groups, and hence forms the strongest complex with Ca^{2+}

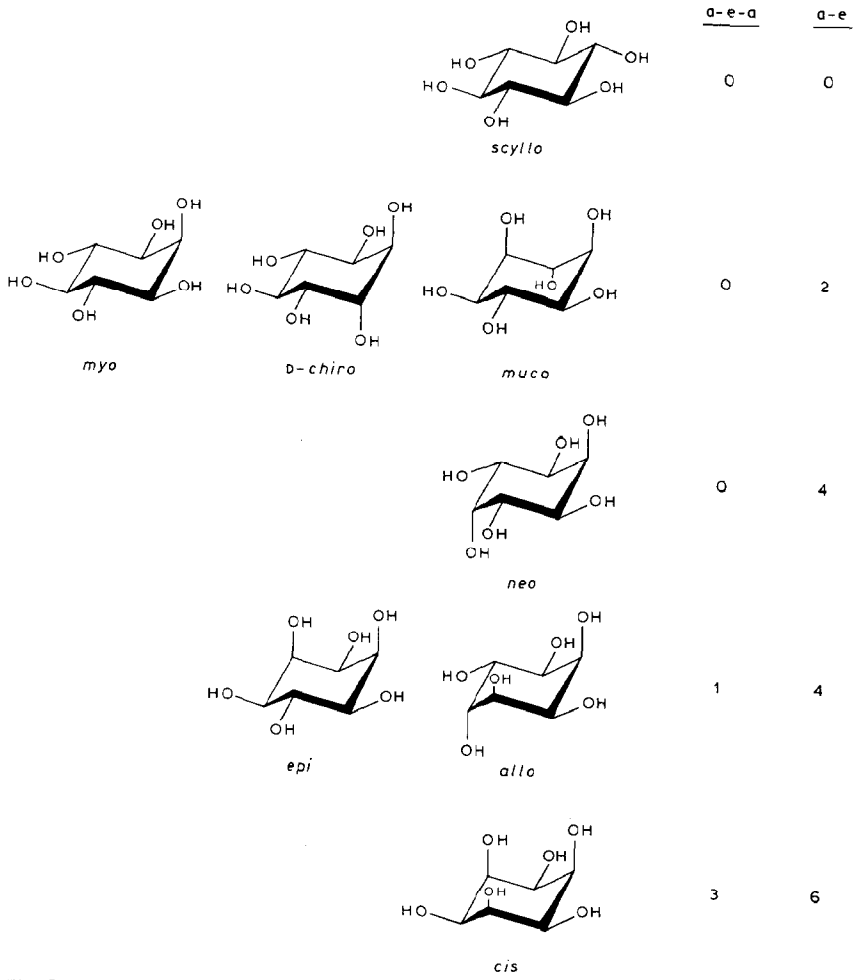

Fig. 5. Stereochemical features of the inositols.

of all the inositols[14]. Accordingly, *cis*-inositol has the longest retention time (Table I). Each of *allo*- and *epi*-inositols has one *a–e–a* arrangement and is also strongly retained on the column, although not nearly so much as the *cis* form. *allo*-Inositol has an additional independent *a–e* group that could form a complex, simultaneously with the coordination complex resulting from the *a–e–a* arrangement and is, therefore, retained slightly longer on the column than *epi*-inositol. Next in elution is *neo*-inositol. This molecule contains four *a–e* sites. All of *myo*-, *chiro*-, and *muco*-inositols possess only two possible *a–e* sites and, as expected, are retained less strongly than *neo*-inositol. *scyllo*-Inositol, all of whose hydroxyl groups are *trans*, has the weakest affinity for the column and emerges from the column rapidly.

The movement of a polyol down the chromatographic column depends on the stability constant of its calcium complex[17]. Increasing temperature would greatly decrease the stability of *allo*- and *epi*-inositol complexes with Ca^{2+}; therefore, their retention times sharply decreased with the increase of temperature. The

retention times of the inositols that form weaker complexes with Ca^{2+} were less affected by the temperature increase. It is noteworthy, however, that the retention time of only *scyllo*-inositol increased as temperature increased.

The sequence of the emergence of inositol isomers from the Ca^{2+}-form column corresponded well to the sequence of their migration on paper electrophoresis[19], in which *cis*-inositol showed the greatest migration and *scyllo*-inositol the least.

The separation of inositol isomers using a calcium column was applied to the separation of the products from *myo*-inositol boiled in water with Raney nickel. Seven inositol isomers were identified in the reaction mixture of *myo*-inositol and Raney nickel in water (or in deuterium oxide) by g.l.c.–m.s. analyses of their per(trimethylsilyl) derivatives[9]. Using our l.c. system with the Ca^{2+}-form column, we found six inositol peaks in the reaction mixture after 3 h of boiling in water with Raney nickel. In addition to the inositol isomers, at least seven unknown peaks were detected, showing that reactions other than epimerization also occurred[9]. Angyal and Odier[24] assigned the signals of cyclohexanepentols in the ^{13}C-n.m.r. spectra of the *myo*-inositol-Raney nickel reaction products. Some major peaks resolved from inositol peaks by our Ca^{2+}-form column may be cyclohexanepentols, the deoxygenation products of *myo*-inositol, which can also form complexes with calcium ions[19]. The identifications of the unknown peaks are in progress.

Results show that the l.c. separation of inositols using a Ca^{2+}-form cation-exchange column is an efficient way to monitor epimerization and other reactions that occur during the ^1H–^2H exchange of *myo*-inositol in deuterium oxide with Raney nickel. Most of the side reaction products other than inositol isomers can be easily removed by crystallization[9]. Our l.c. method combined with crystallization has promise for the quick and easy preparative-scale separation of deuterium-labeled inositol isomers. The use of water as a mobile phase also has an advantage for this purpose.

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