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Fast separation and determination of free *myo*-inositol by hydrophilic liquid chromatography

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ABSTRACT

A fast liquid chromatography method for separation and determination of *myo*-inositol is reported. Determination of the biologically important isomer of inositols, *myo*-inositol, was optimized to avoid overlapping to possible interferents according to *European Pharmacopoeia* (glycerol, D-mannitol) and saccharose. The method in HILIC mode is extremely selective to other carbohydrates which allows to separate *myo*-inositol from *allo*- and D-*chiro*-inositol with resolution 12.3 and 5.2, resp. and this way it enables to separate *myo*-inostiol from contingent carbohydrates present in a sample matrix. Retention time of *myo*-inositol was 12 min at 10 °C, though higher temperatures (25 °C or 40 °C) or higher water content in the mobile phase could speed up the separation and determination to four minutes. LOD of the method was 9 mg/L at 10 °C, and 5 mg/L at 25 °C, resp.

1. Introduction

Inositol (cyclohexane-1,2,3,4,5,6-hexol) is an isomer of glucose widely distributed in plant and animal tissues. It is found in food, for example cereals with high bran content (buckwheat), nuts, beans, and fruit.[1] The biologically important isomer of inositols, myo-inositol plays an important role as the structural basis for a number of secondary messengers in eukaryotic cells, including inositol phosphates (phytic acid), phosphatidylinositol and phosphatidylinositol phosphate lipids. New data point to a new perspectives of inositol phosphates, with functions in many diverse aspects of cell biology, such as ion-channel physiology, membrane dynamics and nuclear signaling.[2]

Inositol itself is not considered as a vitamin because it can be synthesized by the human body. On the other hand, *myo*-inositol was classified as a member of the vitamin B-complex (often called vitamin B8). Inositol plays also interesting roles in human medicine. Patients suffering from clinical depression generally have decreased levels of inositol in their cerebrospinal fluid. Some preliminary results of studies on high dose *myo*-inositol supplements show promising results for people suffering from bulimia, epilepsy, panic disorder, obsessive–compulsive disorder, and unipolar and bipolar depression.[3–5] Several inositol isomers, in particular *myo*-inositol and D-*chiro*-inositol, were shown to possess insulin-mimetic properties and to be efficient in lowering postprandial blood glucose.[6]

Myo-inositol is a compound necessary for infants, and when this substance cannot be administered through breast milk, it must be supplied in the diet by inositol-enriched milk or formula.[7] Nowadays, the maximum limits permitted for *myo*-inositol in infant formulas range from 4.0 to 40.0 mg/100 kcal (1.0–9.5 mg/100 kJ), and the requirements for premature ranged from 27.0 to 67.5 mg/100 kcal (*Codex Alimentarius*, FAO WHO, 2007). Furthermore, there are currently increasing number of food products enriched with inositol both for human and animals (food supplements and feed additives).

Thus, determination of *myo*-inositol in a mixture with other inositols is of great interest in several areas, such as biochemistry, medical cell biology, and biotechnology research or nutrition. The advocated stationary phase for separation of polyols (incl. inositols) by HPLC is an amino silica-bonded phase (aminopropylsilyl, APS) working in mode of hydrophilic interaction liquid chromatography (HILIC). It suffers, however, from (i) a short life-time due to the formation of glycosylamines between the stationary phase amines and reducing sugars (column deactivation) and also (ii) bleeding of the amino-propyl ligand. Using a sensitive detector (evaporative light-scattering detector, ELSD) instead of typical refractive index detector, we also observed an unstable baseline that probably reflects the above mentioned effects. Alternative phases with a good selectivity have been described—a diol-bonded silica or vinyl pyridinium polymers.[8] Reviews on carbohydrate separations can be found in[9], review on HILIC separation in.[10]

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HILIC mode is an attractive alternative to reverse phase HPLC (RPHPLC), superior for separation of polar, hydrophilic compounds including carbohydrates. HILIC works well where traditional reverse phase methodology fails and offers higher flexibility than ion chromatography. The elution order is typically opposite to RPHPLC—with the most polar compounds eluting after the less polar compounds, which offers flexible selectivity. Partitioning mechanism in HILIC is explained by partitioning between a polar layer of water stagnant on the stationary phase and less polar (organic) mobile phase. Retention mechanism in the HILIC mode has been first labeled by Alpert who has described the mechanism of separation in HILIC by a combined model of 'contact groups' interacting in a stagnant mobile phase.[11] Hemström and Irgum[10] have agreed with Alpert that 'most of the real HILIC separations are in essence multimodal'. For separation of anomers, HILIC mode on a DIOL column has shown the highest selectivity.[12]

Methods for determination of (total) inositols in food or food supplements often concern phosphorylated inositols (phytates); such methods usually start with a laborious sample preparation: AOAC method for determination of total and free *myo*-inositol requires microwave extraction and enzymatic treatment with ion-exchange chromatography followed with pulsed amperometric detection (HPAEC-PAD)[13] (LOD was 3 or 10 mg/kg, resp.). *European Pharmacopoeia* (01/2008:1805) recommends for identification and test of (free) *myo*-inositol LC on a strong cation exchange resin in calcium form with refractometric detection. Another alternative for inositol and its derivatives' determination is HPAEC-MS[14] (LOD was 45 pg). HPLC methods with ELSD have been used for determination of D-*chiro*-inositol[15] (LOD was 0.1 μg) or several carbohydrates[16] (LOD was 8.3 mg/L). Sample derivatization has been applied for inositol determination by HPLC[17] in blood (LOD was 0.2 mg/L for plasma) with UV-detection or by GC–MS.[18,19] MS detection has been employed in a UHPLC method[20] (LOD was 0.2 mg/kg) or HILIC[21,22] (LOD were 27 ng or 5 μg/L). Clearly, above mentioned methods hyphenated with MS exhibit lower limits of detection.

This paper deals with the development of an analytical method in HILIC mode for separation and determination of free *myo*-inositol by HPLC with ELSD. Under optimized conditions, three isomers of inositols could be baseline separated in four minutes. Extreme selectivity of the method is demonstrated by experiments under varying experimental conditions. These experiments also suggested how to improve separation from other carbohydrates (monosaccharides, disaccharides, and polyols). Supplementary experiments with ammonium acetate in the mobile phase also showed the influence of a buffer addition to the mobile phase if ionizable carbohydrate derivatives should be separated from a real matrix. The method was applied to determination of inositol in a food supplement.

2. Materials and methods

The column used was Lichrosphere100 DIOL (Merck, Darmstadt, Germany), 150 × 4.1 mm, 5 μm, with void volume V_0 of 1.8 mL. The HPLC system YL9100 (Young Lin Instruments, Korea) consists of a degasser, a quaternary pump, a thermostated column compartment connected to ELSD (Agilent, Germany), and an autosampler. The system was controlled by software Clarity version 3.0.6 (DataApex, Czech Republic). ELSD parameters were as follows: the chamber temperature was set to 40 °C, gain factor was 6, and pressure of nitrogen in a standard nebulizer was 2.9 bar. Acetonitrile (Chromasolv gradient) was from Sigma Aldrich (Palo Alto, USA), water used was from a purification system AquaMax Standard 360 system (Young Lin Instruments, Korea).

Ammonium acetate, N-acetylglucosamine, D-(+)-glucosamine, *myo*-inositol, *allo*-inositol, D-(+)-galactose, L-(+)-arabinose, D-(+)-glucose, and glucose-6-phosphate sodium salt were purchased from Sigma Aldrich (Palo Alto, USA), D-*chiro*-inositol from Molekules (Dorset, UK). Acetic acid and glycerol were purchased from Lachner (Neratovice, Czech Republic). Saccharose and fructose were purchased from Dr. Kulich Pharma (Czech Republic), sorbose, dulcit, adonit, lactose, and D-mannitol were from Lachema (Czech Republic). Aqueous stock solutions of the standards with concentration 2 mg/mL were always prepared.

Evaluation of chromatograms (performance of the method) was calculated by the software Clarity, version 3.0.6 (DataApex, Czech Republic) according to common formulas: capacity factor $k' = (t_R - t_0)/t_0$, peak efficiency (the number of theoretical plates) $N = 5.545 \times (t_R/w_{05})^2$, resolution $R_{S1,2} = 1.18 \times (t_{R1} - t_{R2})/(w_{05,1} + w_{05,2})$, limit of detection $LOD = 3 \times SD^*/K$, where t_R is retention time, t_0 is void time, w_{05} is the peak width at half height, SD^* is the standard deviation of the signal noise corrected by the linearization exponent (0.6–0.9), and K is the slope of the linearized calibration response function.[12]

3. Results

Typical mobile phase in HILIC mode is a mixture of water with an excess of acetonitrile. Using this mobile phase, very selective separation of monosaccharides and disaccharides (even with resolution of anomers) on DIOL columns was already reported.[12] Because polarity of inositols is comparable to polarity of monosaccharides, similar conditions were utilized here as a starting point for the study—mobile phase of 90% acetonitrile with 10% water (v/v), flow rate 2.0 mL/min, injection volume 5 μL, detection by ELSD. Figure 1a, lower chromatogram, shows separation of isomers of inositols at 10 °C, where the elution order is clear from the peak labels, namely *allo*-inositol, D-*chiro*-inositol, and *myo*-inositol. Although separation efficiency of the peaks is low as typical for HILIC mode (N = 4400–6000), despite the observed resolutions of *myo*-inositol are 12.3 and 5.2, resp., due to higher selectivity of the system (k' = 4.25, 7.09, 9.76, resp.). The high selectivity can be explained by Alpert's theory of a contact region which is given by positions of the hydroxyl groups on the six-carbon ring of inositols.[11]

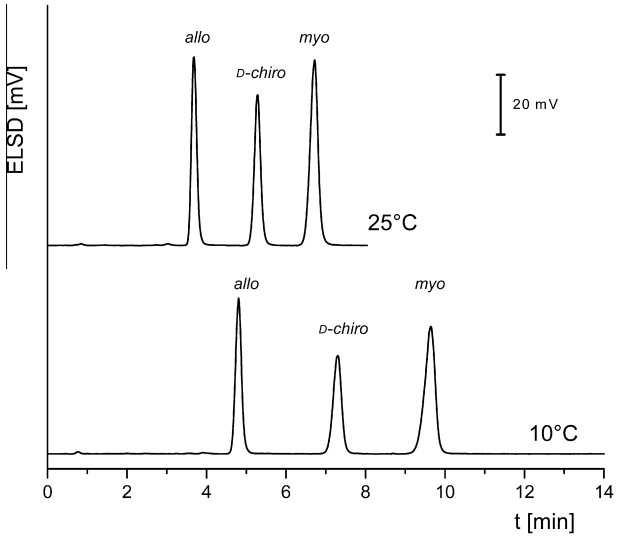

Figure 1a. Chromatograms of separation of a mixture of three inositol standards at temperatures 10 °C and 25 °C, resp. The peak identification was done by runs of respective standards. Experimental conditions: 90% acetonitrile with 10% water (v/v), flow rate 2.0 mL/min, and injection volume was 5 μL. The concentrations of *allo*-, D-*chiro*-, and *myo*-inositol in the mixture were 1.0, 1.0, and 1.4 mg/mL, resp. Corresponding temperatures are labeled at the chromatograms.