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Microdetermination of hyaluronan in human plasma by high-performance liquid chromatography with a graphitized carbon column and postcolumn fluorometric detection

Hidenao Toyoda [a,b,*], Fumie Muraki [b], Toshio Imanari [b], Akiko Kinoshita-Toyoda [a]

[a] *Faculty of Pharmaceutical Sciences, Ritsumeikan University, 1-1-1 Nojihigashi, Kusatsu, Shiga 525-8577, Japan*
[b] *Graduate School of Pharmaceutical Sciences, Chiba University, 1-33, Yayoi, Inage, Chiba 263-8522, Japan*

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ABSTRACT

A chemical method for the determination of hyaluronan (hyaluronic acid, HA) has been developed and applied to the human blood plasma. Human blood plasma HA was converted to the ΔDi-HA by digestion with hyaluronidase SD and determined by a sensitive and selective high-performance liquid chromatography (HPLC). The HPLC includes the separation and detection of ΔDi-HA using a graphitized carbon column and fluorometric reaction with 2-cyanoacetamide in an alkaline eluent. The calibration graph for ΔDi-HA was linear over the range 0.2 ng–1 μg. It was revealed that the concentration of HA in normal human blood plasma is very low levels (about 24 ng/ml) in comparison to low-sulfated chondroitin 4-sulfate (about 13 μg/ml).

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1. Introduction

Glycosaminoglycans (GAGs) exist in various tissues as major components of the extracellular matrix, and small amounts of GAGs are reported in blood plasma and urine [1,2]. To elucidate the physiological roles of GAGs, many workers have studied analytical methods for determination of GAGs in plasma, serum and urine using chromatography and electrophoresis [3–5]. High-performance liquid chromatography (HPLC) is extensively utilized for the microdetermination of unsaturated disaccharides produced enzymatically from GAGs in biological and clinical fluids. The unsaturated disaccharides have been generally separated on chemical-bonded type silica columns (ODS-, amino- and amide-bonded type silica column) by several precolumn [6–8] and postcolumn [9–11] derivatization methods or mass spectrometric methods [12–15].

Hyaluronan (hyaluronic acid, HA) is a kind of GAGs composed of alternating β1,3-glucuronic acid and β1,4-*N*-acetylglucosaminidic bonds and ΔDi-HA is produced by enzymatic digestion (Fig. 1). HA has been postulated as playing important roles *in vivo*, based on the observations of the elevated HA levels in blood plasma and the excessive urinary HA excretion in the case of several diseases related to inflammation and cancer [16–19]. However, it is hard to study the biological functions of HA, because normal HA concentrations in blood plasma and urine is too low to determine precisely [20].

In blood plasma, low-sulfated chondroitin 4-sulfates (LSC) are major GAGs [3,21]. When plasma GAGs are digested enzymatically, large amounts of ΔDi-0S are produced and interfere with the measurement of HA. Furthermore, the chromatographic separation of ΔDi-HA and ΔDi-0S is very difficult, because ΔDi-HA is the C-4 epimer of *N*-acetylgalactosamine of ΔDi-0S (Fig. 1). In this paper, we established a sensitive and selective method for the determination of HA and demonstrated the application to human plasma samples.

2. Materials and methods

2.1. Reagents and materials

Standard unsaturated disaccharides of [2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-D-glucose (ΔDi-HA), 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-D-galactose (ΔDi-0S), 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4-*O*-sulfo-D-galactose (ΔDi-4S)], hyaluronidase SD from Streptococcus dysgalactiae (EC 4.2.2), chondroitinase ABC (EC 4.2.2.4) and chondroitinase ACII (EC 4.2.2.5) were obtained from Seikagaku (Tokyo, Japan). A

* Corresponding author at: Faculty of Pharmaceutical Sciences, Ritsumeikan University, 1-1-1 Nojihigashi, Kusatsu, Shiga 525-8577, Japan. Tel.: +81 77 561 5738; fax: +81 77 561 2659.
E-mail address: hidenao@ph.ritsumei.ac.jp (H. Toyoda).

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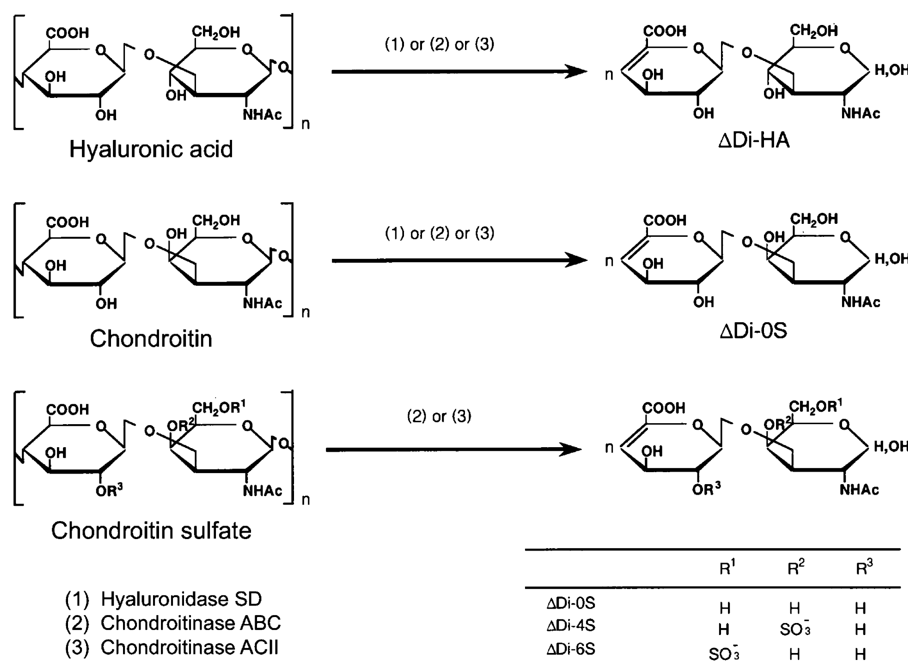

Fig. 1. Enzymatic digestion of HA and chondroitin sulfate. ΔDi-HA is the C-4 epimer of *N*-acetylgalactosamine of ΔDi-0S.

graphitized carbon column of Carbonex (3.5 μm, 100 mm × 4.6 mm I.D.) was purchased from Tonen (Tokyo, Japan). An amino-bonded silica column of TSKgel NH_2-60 (5 μm, 150 mm × 4.6 mm I.D.) was obtained from Tosoh (Tokyo, Japan). 2-Cyanoacetamide was obtained from Sigma. All other chemicals used were of analytical reagent grade.

2.2. Apparatus and chromatographic conditions

A flow diagram of liquid chromatography for the determination of HA is shown in Fig. 2. The chromatographic equipment included two high-pressure pumps (L-6000), a fluorescence detector (F-1050), a chromato-integrator (D-2500) from Hitachi Instruments (Tokyo, Japan), a sample injector with 20 μl loop (Model 7125) from Rheodyne (Rohnert Park, CA, USA), a column thermocontroller (Mini-80) from Taitec (Tokyo, Japan) and a dry reaction bath (DB-5) from Shimamura Instrument (Tokyo, Japan). Established HPLC conditions were as follows (Fig. 2): A Carbonex column was eluted at 40 °C with 25 mM sodium phosphate–NaOH buffer (pH 11) in 4% acetonitrile at a flow rate of 0.5 ml/min by using a L-6000 pump. To the effluent was added aqueous 0.5% 2-cyanoacetamide solution at the flow rate of 0.5 ml/min by using a L-6000 pump. The mixture passed through a polytetrafluoroethylene (PTFE) reaction coil (10 m × 0.5 mm I.D.) set in a dry reaction bath thermostated at 110 °C and a following PTFE cooling coil (2 m × 0.25 mm I.D.). The effluent was monitored fluorometrically (excitation 335 nm, emission 395 nm). A 10-μl portion of sample solution was loaded via a sample injector with a 20 μl loop.

2.3. Preparation of human plasma GAGs

Blood was collected from the healthy volunteers. Ethylenediamine tetra acetic acid disodium salt was added to the blood as an anticoagulant. The blood was separated into plasma and cells by centrifugation at 1200 × *g* for 15 min. Plasma GAGs were separated by our modified method reported previously [9]. To 250 μl of human plasma, 100 μl of 0.05 M Tris–HCl buffer (pH 8.0) containing 1% actinase E was added, and the mixture was incubated at 45 °C for 3 h. To the solution, 1 ml of 0.1 M acetic acid containing 10% sodium chloride solution was added. Then the mixture was heated in a boiling water bath for 5 min. After being cooled in a water bath, the solution was centrifuged at 2300 × *g* for 15 min. To 1 ml of the supernatant, 100 μl of 0.1 M sodium hydroxide solution and 4 ml of cooled ethanol saturated with sodium acetate were added. The mix-

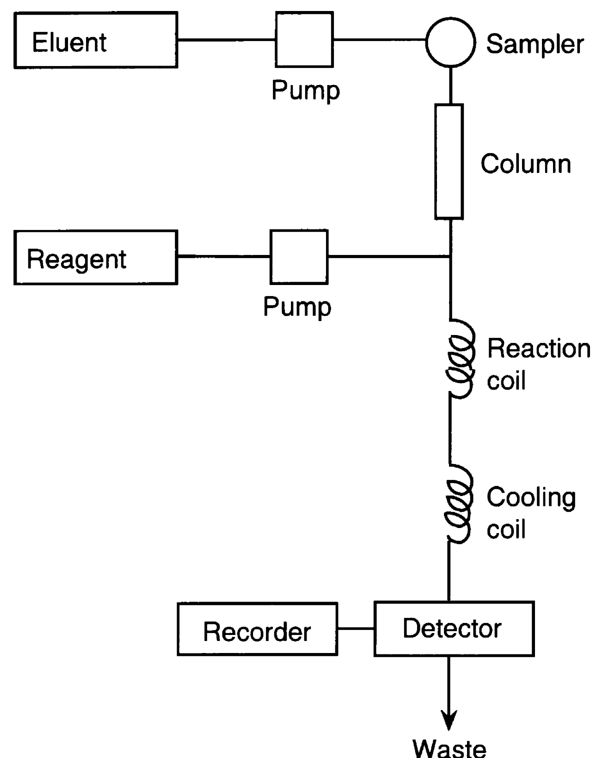

Fig. 2. Flow diagram of the postcolumn HPLC system for determination of HA with a graphitized carbon column. Column, Carbonex (3.5 μm, 100 mm × 4.6 mm I.D.) with a precolumn (3.5 μm, 10 mm × 4.6 mm I.D.) at 40 °C; eluent, 25 mM sodium phosphate–NaOH buffer (pH 11) in 4% acetonitrile; reagent, 0.5% 2-cyanoacetamide; reaction temperature, 110 °C; detection, Ex. 335 nm, Em. 395 nm. Other conditions as described in the text.

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