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Analytical method of heparan sulfates using high-performance liquid chromatography turbo-ionspray ionization tandem mass spectrometry

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Abstract

We established a highly sensitive quantitative analytical method of heparan sulfates (HS) by LC–MS–MS. It became possible to determine the unsaturated disaccharides produced by the enzyme digestion of HS, and to perform the whole analyses on one sample within 3 min by use of a short column of CAPCELL PAK NH_2 UG80 (35 mm×2 mm I.D.). The assay method was validated and showed the satisfactory sensitivity, precision and accuracy, which enabled the quantitation up to picomol level. By employing this method, we performed the analyses of HS in mouse brain and liver, and tumor tissues of tumor-bearing mouse transplanted subcutaneously with Meth A fibrosarcoma cells. The compositions of the unsaturated disaccharide units derived from HS were found to be somewhat different among those tissues. It is assumed that the site of sulfation in HS may be controlled by certain regulatory mechanisms. The quantitative method developed in this study is believed to be a very useful method for the determination of compositional profiles of constitutive disaccharide units of tissue HS.

Keywords: Heparan sulfate

1. Introduction

Heparan sulfates (HS) is a kind of glycosaminoglycans and exists on various mammalian cell surface membranes [1,2] as a proteoglycan, and has been purified from a variety of different tissues [3]. HS is a straight chain polysaccharide with structural multiplicity and basically composed of two kinds of disaccharide units. Namely, one is *N*-acetylated [→4-α-D-GlcNpAc-(1→4)-β-D-GlcAp-(1→], and the other is *N*-sulfated disaccharides [→4-α-D-GlcNpS-(1→4)-β-D-GlcAp or -α-L-IdoAp-(1→] (where GlcAp is glucuronic acid, IdoAp is iduronic acid, GlcNp is glucosamine, S is sulfate and Ac is acetate) [4]. As particular characteristics of HS, the ratio of GlcNpAc residues to GlcNpS residues is approximately 3.0 or less, containing about 20% sulfate groups, and the content of D-GlcAp residues is more than twice the content of L-IdoAp residues [5]. *O*-sulfate groups are always found on proximity to *N*-sulfate groups, which enhances the clustering of the sulfate residues and heterogeneity in chemical

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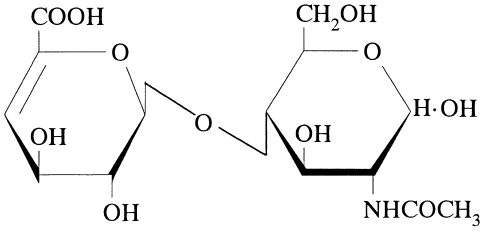
composition and charge density of HS [6]. Recently, it has been revealed that there exists two types of HS differing from each other in the degree of *N*- and *O*-sulfation [7]. These two types of HS show each characteristic biological property depending on their degree of the sulfation [8]. As a novel analytical method for HS, mass spectrometry has recently been reported to be very effective. Actually, disaccharides and tetrasaccharides derived from HS can be analyzed in the amount of less than 100 μg by fast atom bombardment mass spectrometry (FAB-MS) which can provide information not only on molecular ions but also on certain sugar sequences [9,10]. Furthermore, we can determine the positions of sulfate groups by means of FAB–MS–MS [11]. Matrix assisted laser desorption ionization (MALDI)-MS can detect molecular ions on a very small amount of sample of less than 1 μg and can be used for the study of oligosaccharide–peptide complexes [12,13]. Electrospray ionization-MS (ESI-MS) can also be used for the study of oligosaccharide–peptide complexes on a very small amount of sample of 1–10 ng [14,15]. We established a highly sensitive quantitative LC–MS–MS method for the unsaturated disaccharides produced from HS by a specific enzyme. We applied this method to the analyses of HS in mouse brain and liver, and tumor tissues of the tumor-bearing mouse transplanted subcutaneously with Meth A fibrosarcoma cells.



ΔDiHS-0S : $R^1 = COCH_3$, $R^2 = H$
ΔDiHS-NS : $R^1 = SO_3H$, $R^2 = H$
ΔDiHS-6S : $R^1 = COCH_3$, $R^2 = SO_3H$

Fig. 1. The chemical structure for disaccharides of ΔDiHS-0S, ΔDiHS-NS and ΔDiHS-6S.

2. Experimental

2.1. Materials

Unsaturated standard disaccharides (2-acetamido-2-deoxy-4-*O*-(4-deoxy-α-L-threo-hex-4-enopyranosyluronic acid)-D-glucose (ΔDiHS-0S), 2-deoxy-2-sulfamino-4-*O*-(4-deoxy-α-L-threo-hex-4-enopyranosyluronic acid)-D-glucose (ΔDiHS-NS), 2-acetamido-2-deoxy-4-*O*-(4-deoxy-α-L-threo-hex-4-enopyranosyluronic acid)-6-*O*-sulfo-D-glucose (ΔDiHS-6S), (Fig. 1), chondrosine, heparinase (Flavobacterium heparinum), heparitinase I (Flavobacterium heparinum) and heparitinase II (Flavobacterium heparinum) were purchased from Seikagaku Kogyo (Tokyo, Japan). All other chemicals were of reagent grade. Tumor tissues were isolated from the tumor-bearing mouse as follows: Balb/c male mice, 7-week-old, were transplanted subcutaneously with Meth A fibrosarcoma (1×10^6) cells. Subcutaneous tumor tissue obtained from 8-week-old mice were used for analyses.

2.2. Apparatus

The chromatographic system consisted of an Alliance system (Waters, Milford, MA, USA) and a CAPCELL PAK NH_2 UG80 column (35 mm×2 mm I.D.) (Shiseido, Tokyo, Japan). The column temperature was kept at 60°C. The mobile phase was an isocratic elution of acetonitrile–0.01 *M* ammonium formate (pH 9.4) (1:9, v/v). The flow-rate was 0.2 ml/min. API-365 mass spectrometer (Perkin-Elmer Sciex, Concord, Canada) equipped with a turbo-ionspray ion source was used. The mass spectrometer was operated in the negative-ion detection mode. The turbo-ionspray temperature was optimized at 450°C. The collision energy was optimized at −50 V (Q0-RO2), and the nitrogen gas was used to obtain collision induced detection (CID). During analyses, the indicated vacuum was 1.4×10^{-5} Torr.

2.3. Preparation of heparan sulfates from tissues

Heparan sulfates were isolated from tissues as follows: tissues were homogenized in 4×vol of acetone with ice cooling. After centrifuging at 1000 *g* for 15 min, the precipitate was washed with ether

and dried in vacuo. The defatted dry sample was suspended in 0.25 ml of 1.0 *M* NaOH and kept overnight at 4°C. After neutralizing with 1 *M* HCl, the solution was boiled for 2 min. The solution was mixed with 0.1 ml of 10 mg/ml actinase E solution in 0.01 *M* Tris–acetate buffer (pH 8), and the reaction mixture was heated at 45°C for 24 h. To the reaction solution, 0.7 ml of 50 m*M* acetic acid containing 15% NaCl was added, and then the solution was boiled for 5 min. After centrifuging at 2300 *g* for 15 min, the supernatant was neutralized with 0.25 *M* NaOH. To the solution, 5 ml of cold ethanol was added and then the solution was allowed to stand overnight at 4°C. After centrifuging at 2300 *g* for 15 min, the precipitate was washed with 1.0 ml of 80% ethanol, and then the precipitate was dried in vacuo.

2.4. Enzyme digestion of heparan sulfates

A 0.02 ml portion of heparan sulfates solution, 0.01 ml of 0.01 *M* tris–acetate buffer (pH 7.0) containing 0.01 *M* calcium acetate and 0.01 ml of an aqueous solution of heparinase (1 U), heparitinase I (0.1 U) and heparitinase II (0.1 U) were mixed, and then the solution mixture was incubated at 37°C for 24 h. This solution was applied to the ULTRA FREE-MC (30 000 NWWL filter unit, Millipore, USA) and centrifuged at 4000 *g* for 4 h at 4°C. The filtrate solution was diluted with 25-fold purified water. A 0.02 ml portion of the sample solution was subjected to LC–MS or LC–MS–MS.

3. Results and discussion

3.1. LC–MS–MS conditions

The spectra of ΔDiHS-0S in the turbo-ionspray LC–MS negative analysis showed $[M-H]^-$ at m/z 378. Using this $[M-H]^-$ as a precursor ion, the spectra of the collision induced decomposition (CID)-LC–MS–MS negative analysis showed a fragment ion at m/z 87 (Fig. 2a). The spectra of ΔDiHS-NS in the turbo-ionspray LC–MS negative analysis showed $[M-H]^-$ at m/z 416. Using this $[M-H]^-$ as a precursor ion, the spectra of the CID-LC–MS–MS negative analysis showed $[OHCHCHNHOSO_3]^-$ at m/z 138 as a fragment ion (Fig. 2b). The spectra of ΔDiHS-6S in the turbo-ionspray LC–MS negative analysis showed $[M-H]^-$ at m/z 458, and the spectra of the CID-LC–MS–MS negative analysis using the above $[M-H]^-$ as a precursor ion showed $[HSO_4]^-$ at m/z 97 as a fragment ion (Fig. 2c). The spectra of chondrosin used as an internal standard in the turbo-ionspray LC–MS negative analysis showed $[M-H]^-$ at m/z 354 and the spectra of the CID-LC–MS–MS analysis showed a major fragment ion at m/z 113 and 73 (Fig. 2d). As for the monitoring ions employed in the LC–MS–MS analytical system (Table 1), the ions at m/z 378 and at m/z 87 were selected as a precursor ion and its daughter ion, respectively, for the determination of ΔDiHS-0S. In the case of the determination of ΔDiHS-NS, we selected the ions at m/z 416 and m/z 138 as a precursor ion and its daughter ion, respectively. In the case of the determination of ΔDiHS-6S, we selected the ions at m/z 458 and m/z 97 as a precursor ion and its daughter ion. Furthermore, we selected the ions at m/z 354 and m/z 113 as a precursor ion and its daughter ion, respectively, in the determination of chondrosin as internal standard (IS). Since the precursor ions in the determination of these four compounds were different to each other, the separation of these compounds by HPLC was not necessary, and we succeeded in saving time required for analysis.

3.2. Validation of assay method

3.2.1. Calibration curves

The calibration curves were constructed by setting the peak area ratio of the analyte compound to I.S. to the *y*-axis and the amount of the unsaturated standard disaccharides to the *x*-axis, with the weight function of $1/x$, where x was the amount of the unsaturated disaccharides (Table 2). Individual calibration curves of ΔDiHS-0S constructed over the course of 5 days were linear over 3.1–80.0 pmol/injection and reproducible with mean±standard deviation values for the constants in the regression equation of $y=(0.003\pm0.001)x+(-0.0030\pm0.0017)$. The mean of coefficients of determination (r) was 0.9957 ($n=5$). Individual calibration curves of ΔDiHS-NS and ΔDiHS-6S constructed over the course of 5 days

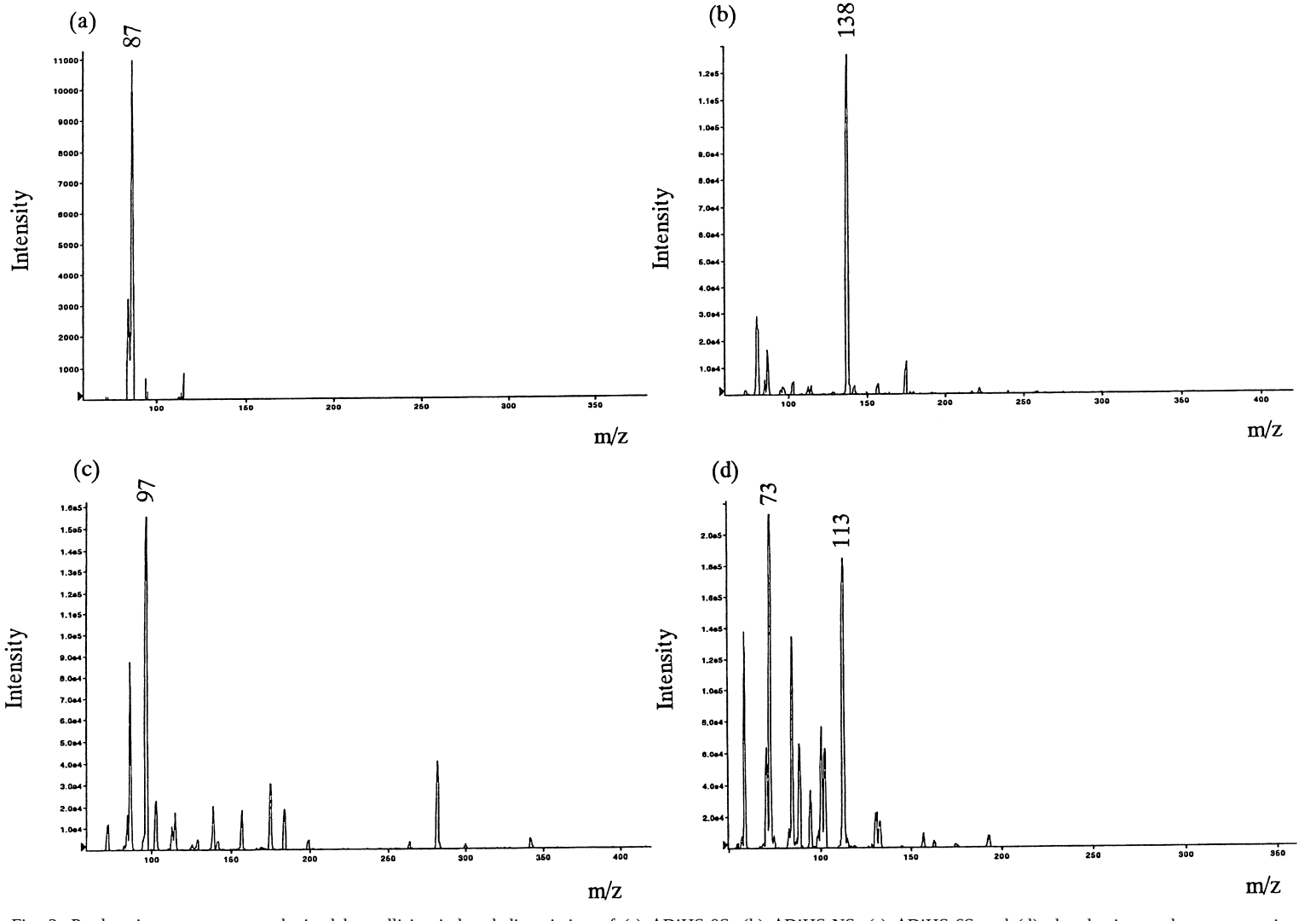


Fig. 2. Product ion mass spectra obtained by collision-induced dissociation of (a) ΔDiHS-0S, (b) ΔDiHS-NS, (c) ΔDiHS-6S and (d) chondrosine at the precursor ion of [M–H]. $[M-H]^-$.

Table 1
Precursor/daughter ions used for the multiple reaction monitoring of the unsaturated disaccharides of ΔDiHS-0S, ΔDiHS-NS and ΔDiHS-6S and IS

Unsaturated dissacharide	Precursor/daughter (m/z)
ΔDiHS-0S	378/87
ΔDiHS-NS	416/138
ΔDiHS-6S	458/97
IS (chondrosin)	354/113

were linear over 0.3–8.0 pmol/injection and reproducible with mean±standard deviation values for the constants in the regression equation of $y=(0.018\pm0.003)x+(-0.0016\pm0.002)$ and $y=(0.015\pm0.003)x+(-0.0011\pm0.0013)$. The mean of coefficients of determination (r) were 0.9958 and 0.9960 ($n=5$).

3.2.2. Precision and accuracy

The intra-day precision and accuracy were evaluated by analyzing the unsaturated standard disaccharides of ΔDiHS-0S (3.1–80.0 pmol/injection), ΔDiHS-NS and ΔDiHS-6S (0.3–8.0 pmol/injection) in five times over the course of 1 day (Table 3). Precision was based on the calculation of the relative error (RE) of the found concentration compared to the theoretical value. The intra-day precision (C.V.) of this method was <9.8%. The range of accuracy (RE) was 95.2–110.4%. The limit of quantitation of ΔDiHS-0S, ΔDiHS-NS and ΔDiHS-6S using this assay method was determined as 3.1 pmol/injection, 0.3 pmol/injection and 0.3 pmol/injection with acceptable precision and accuracy and the chromatograms were shown in Fig. 3. The inter-day precision and accuracy were evaluated by analyzing the unsaturated standard disaccharides of ΔDiHS-0S (3.1–80.0 pmol/injection), ΔDiHS-NS and ΔDiHS-6S (0.3–8.0 pmol/injection) over the course of 5 days (Table 4). The inter-day precision (C.V.) of this

Table 3
Intra-day validation of calibration standards for the determination of the unsaturated disaccharides of ΔDiHS-0S, ΔDiHS-NS and ΔDiHS-6S

Unsaturated disaccharides	Concentrations (pmol/injection)		C.V.[b] (%)	RE[c] (%)
	Added	Mean±SD[a] ($N=5$)		
ΔDiHS-0S	3.1	3.4±0.2	4.8	107.4
	6.3	6.7±0.4	5.2	107.2
	50.0	55.2±1.2	2.1	110.4
	80.0	84.3±1.1	1.3	105.4
ΔDiHS-NS	0.3	0.3±0.0	9.4	103.2
	0.6	0.6±0.0	3.3	95.2
	5.0	4.8±0.3	6.1	95.4
	8.0	7.8±0.2	2.5	97.1
ΔDiHS-6S	0.3	0.3±0.0	3.2	100.0
	0.6	0.6±0.1	9.8	96.8
	5.0	4.8±0.4	8.5	96.4
	8.0	7.9±0.4	5.6	98.1

[a] SD=Standard of deviation.
[b] C.V.=Coefficient of variation.
[c] RE=Relative error.

Table 2
Parameters of the calibration curves for the determination of the unsaturated disaccharides of ΔDiHS-0S, ΔDiHS-NS and ΔDiHS-6S

Unsaturated disaccharides	Regression coefficient						Mean±SD[a]
ΔDiHS-0S	Slope	0.004	0.003	0.004	0.003	0.003	0.0030±0.001
	Intercept	−0.0054	−0.0019	−0.0038	−0.0011	−0.0028	−0.0030±0.0017
	r	0.9878	0.9975	0.9989	0.9990	0.9953	0.9957±0.0047
ΔDiHS-NS	Slope	0.021	0.020	0.013	0.017	0.020	0.0180±0.003
	Intercept	−0.0022	−0.0011	−0.0002	−0.0007	−0.0040	−0.0016±0.002
	r	0.9899	0.9961	0.9979	0.9979	0.9972	0.9958±0.003
ΔDiHS-6S	Slope	0.018	0.016	0.011	0.014	0.016	0.0150±0.003
	Intercept	−0.0030	−0.0003	0.0005	−0.0017	−0.0008	−0.0011±0.0013
	r	0.9910	0.9971	0.9981	0.9984	0.9952	0.9960±0.0030

[a] SD=Standard of deviation.

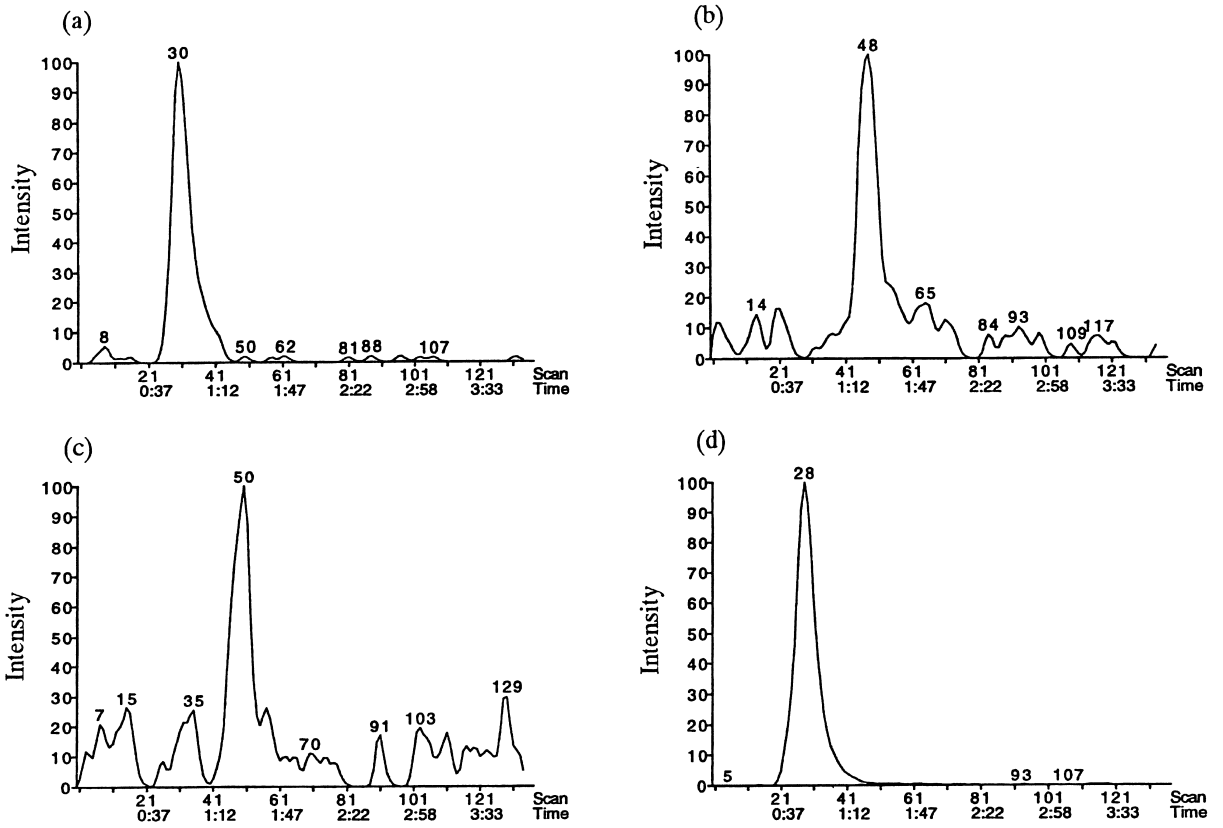


Fig. 3. Representative MRM chromatogram of (a) ΔDiHS-0S (3.1 pmol), (b) ΔDiHS-NS (0.3 pmol), (c) ΔDiHS-6S (0.3 pmol) and (d) chondrosine (56 pmol) by turbo-ionspray LC–MS–MS.

method was <12.5% (except for 20.0%: 0.3 pmol of ΔDiHS-NS). The range of accuracy (RE) was 92.6–112.9%. These results indicated that the present method has satisfactory precision, accuracy and reproducibility. It was revealed that the unsaturated disaccharides from HS could be determined with high accuracy up to picomol levels. Moreover, the time required for the analysis of a sample could be reduced to 3 min by employing a short column of CAPCELL PAK NH_2 UG80 column (35 mm×2 mm I.D.).

3.2.3. Analysis of heparan sulfates in brain, liver and tumor tissues

The compositional ratios of ΔDiHS-0S, ΔDiHS-NS and ΔDiHS-6S produced by the enzymatic digestion of HS derived from brain, liver and tumor tissue are listed in Table 5. As the sample solutions were diluted by purified water before injection to LC–MS–MS, these samples can be analyzed without ion suppression. The data were obtained using the calibration curves of the unsaturated standard disaccharides. The compositional ratios (0S/NS/6S) of the unsaturated disaccharides in HS from brain, liver and tumor tissue were 74.4/25.6/13.0(%), 73.9/26.1/7.5(%) and 61.4/36.2/2.3(%), respectively. Thus, the compositions of the unsaturated disaccharide units derived from HS were found to be somewhat different among those tissues. It is assumed that the site of sulfation in HS may be controlled by certain regulatory mechanisms. The

Table 4
Inter-day validation of calibration standards for the determination of the unsaturated disaccharides of ΔDiHS-0S, ΔDiHS-NS and ΔDiHS-6S

Unsaturated disaccharides	Concentrations (pmol/injection)		C.V.[b] (%)	RE[c] (%)
	Added	Mean±SD[a] (N=5)		
ΔDiHS-0S	3.1	3.5±0.2	4.6	110.9
	6.3	6.5±0.3	4.8	104.2
	50.0	49.5±4.0	8.1	98.9
	80.0	79.6±9.7	12.1	99.4
ΔDiHS-NS	0.3	0.4±0.1	20.0	112.9
	0.6	0.6±0.1	9.4	101.6
	5.0	4.8±0.3	6.7	95.4
	8.0	7.8±0.7	8.3	97.6
ΔDiHS-6S	0.3	0.3±0.0	12.5	103.2
	0.6	0.6±0.1	11.3	98.4
	5.0	4.6±0.3	5.4	92.6
	8.0	8.0±0.7	8.8	99.5

[b] C.V.=Coefficient of variation.
[c] RE=Relative error.
[a] SD=Standard of deviation.

Table 5
Disaccharide composition of heparan sulfates (molar percent)

Unsaturated disaccharides	Disaccharide composition (%)[a]		
	Mouse brain	Mouse liver	Tumor (tumor-bearing mouse)
ΔDiHS-0S	74.4±3.2	73.9±6.8	61.4±1.3
ΔDiHS-NS	25.6±3.2	26.1±6.8	36.2±1.4
ΔDiHS-6S	13.0±3.0	7.5±1.5	2.3±0.2

[a] Mean±SD (N=6).

quantitative method developed in this study is believed to be a very useful method for the determination of compositional profiles of constitutive disaccharide units of tissue HS.

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