Supplemental Results and Discussion

In order to determine the efficiency of the labeling reaction and the possible generation of chemical by-products, such as de-sulfated or de-acetylated species, aniline labeling was carried out on individual HS and CS disaccharide standards and the products were subsequently analyzed by LC/MS. Supplemental Fig. S1 shows the results for three representative disaccharide standards that were subjected to aniline tagging at 65^{o}C and 37^{o}C. Analysis of the reaction products by LC/MS showed only negligible amounts of untagged disaccharides with greater than 98% conversion to aniline-tagged forms. While no sign of O-desulfation was found, some N-desulfation or N-deacetylation was detected for D2S6 and D2A6 when labeling was carried out at 65^{o}C. By reducing the labeling temperature to 37^{o}C, de-N-sulfation of D2S6 was reduced to undetectable levels and de-N-acetylation of D2A6 was significantly decreased. No decrease in the efficiency of the labeling reactions at the lower temperature was noted.

The reliability of GRIL-LC/MS quantitation of disaccharides within a biological sample was evaluated by measuring the recovery of individual disaccharides as a function of sample concentration using liver HS. GRIL-LC/MS analysis was performed at four different dilutions of the same sample. The results showed a strong linear correlation (R^2 values from 0.9954 to 1.000) between disaccharide concentration and the disaccharide/standard XIC ratio for all of the disaccharides detected in the sample (Supplemental Fig. S2). To estimate the analytical recovery of the GRIL-LC/MS technique, we assessed its ability to distinguish a small exogenous sample added to a larger biological sample before HS purification. In this experiment, 70 ng of heparin by-product (the lower sulfated HS fraction removed during heparin purification) was added to a heart tissue homogenate (containing approximately 250 ng of HS) and to an equal volume of PBS. These two samples along with an equal amount of unspiked heart homogenate were subjected to GAG purification, heparan lyase digestion and analysis by GRIL-LC/MS as described in the Experimental Procedures section. When the disaccharide profiles from the heparin by-product and the unspiked heart samples are combined, the average difference with disaccharides in the spiked sample was less than 5% (Supplemental Fig. S3). The heparin by-product is more highly sulfated than the heart HS (1.9 sulfates per disaccharide compared to 0.7 sulfates per disaccharide) and thus structurally distinct which caused the observed differential changes in the disaccharide profiles in the two heart samples. Overall, these results demonstrate the high degree of reliability provided by this technique to carry out comparative disaccharide analysis.

Due to the lack of any sulfates on D0H0, this species does not absorb to the C18 matrix and is thus not significantly retained by the reverse phase column. Its coelution with salts causes suppression of the ion current and attempts to quantify D0H0 failed. To circumvent this problem, disaccharides bearing free amino groups were amidated (Supplemental Fig. 4A) with propionic anhydride (20), which results in stronger interaction with the C18 matrix and separation from salts in the sample. Propionylation generates a product (D0R0) with a mass 56 amu larger than the corresponding free amino disaccharide (Supplemental Fig. 4B). Furthermore, aniline tagged D0R0 separates completely from aniline tagged D0A0 (Supplemental Fig. 4C). These two unsulfated disaccharides are structurally similar but vary in the length of the amide substituent.

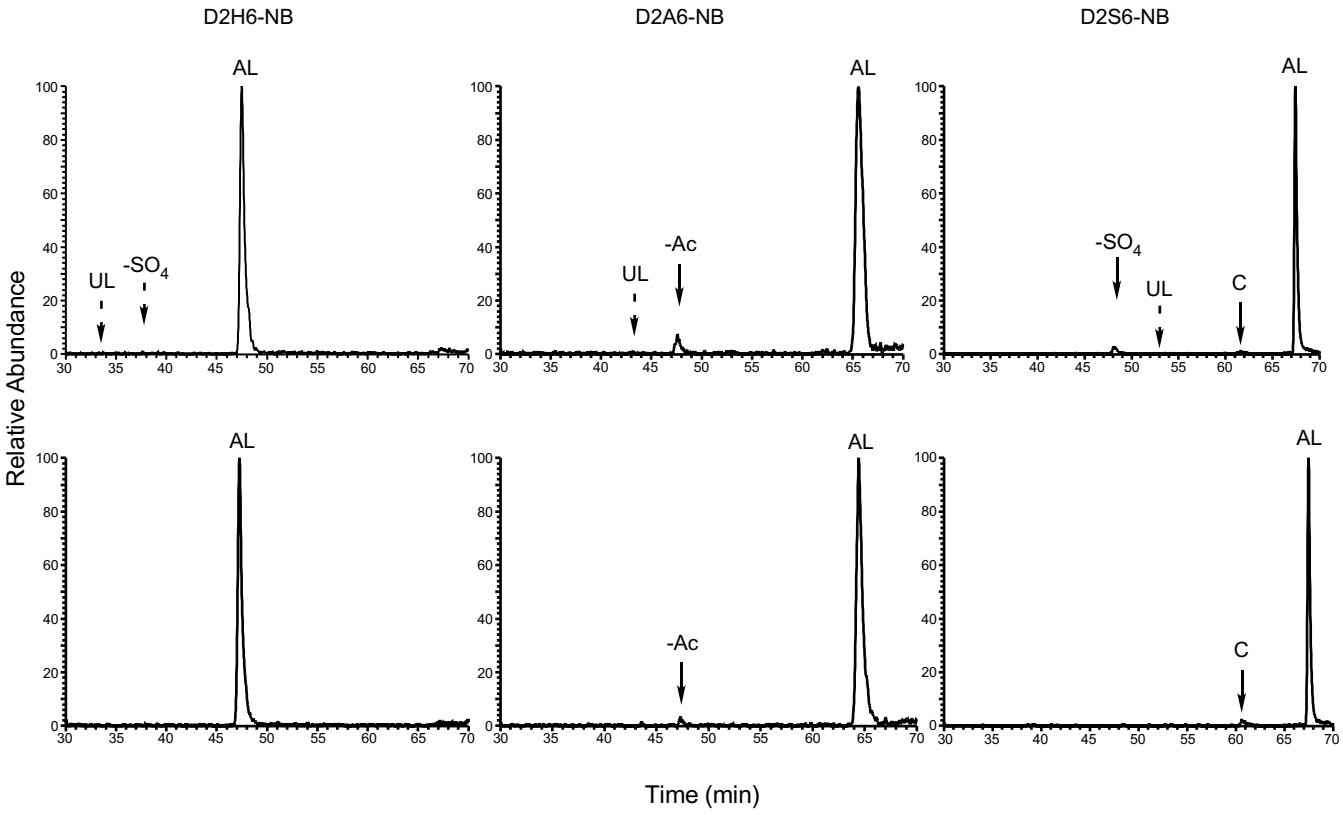
Supplemental Figure Legends

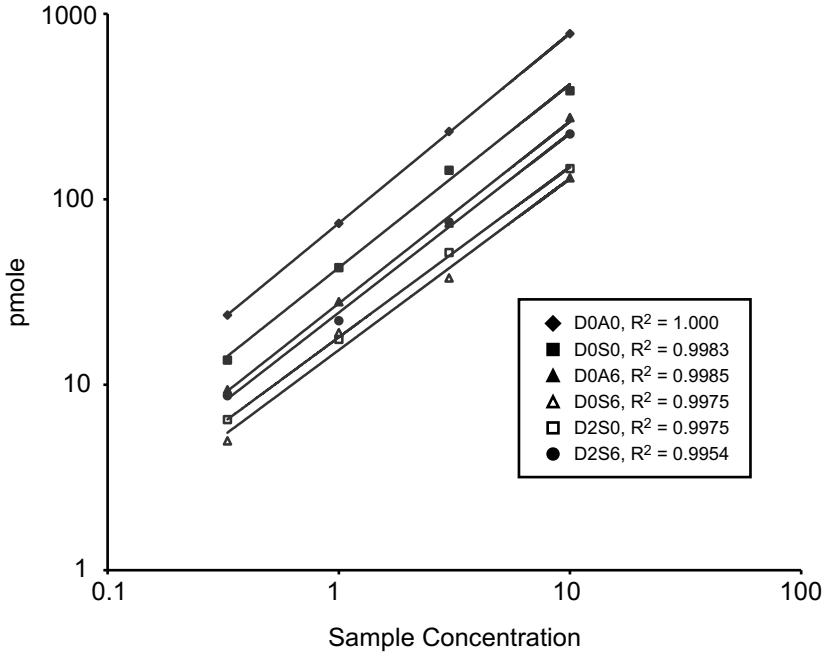
Supplemental Fig. S1. GRIL-LC/MS Analysis of HS Disaccharides at Two Different Temperatures. Aniline labeling of three representative HS disaccharides was carried out on 250 pmole of each disaccharide at either 65°C for 4 hr (top) or 37°C for 16 hr (bottom) and subsequently analyzed by LC/MS as described in the Experimental Procedures. Shown are the accumulative XIC traces for the ions corresponding to unlabeled (UL), aniline-labeled (AL), de-N-sulfated (-SO_3) or de-N-acetylated (-Ac) byproducts for each of the labeling reactions. For D2H6 the *m/z* values defining the XIC traces shown are: 573 (AL), 496 (UL), 493 (-SO_3); for D2A6 the *m/z* values defining the XIC traces shown are: 744 (AL), 667 (UL), 573 (-Ac), 535 (-SO_3); and for D2S6 the *m/z* values defining the XIC traces shown are: 653, 782 and 911 (AL), 576, 705 and 833 (UL), 573 (-SO_3). The peak (C) is an unknown contaminant.

Supplemental Fig. S2. GRIL-LC/MS Analysis of Murine Liver Sample Performed at Different Concentrations. HS was extracted from whole liver excised from $Ndst1^{f/f}$ transgenic mice and subjected to disaccharide compositional analysis using GRIL-LC/MS. The sample was analyzed at 4 different dilutions (x axis; 0.33X, 1X, 3X and 10X) and the number of pmoles recovered for all disaccharides detected in all sample were compared (y axis). In addition, the R^2 values calculated from the linear regression for each recovery curve are given.

Supplemental Fig. S3. Recovery of samples using GRIL-LC/MS. Heparin by-product (70 ng) was added to PBS and to a sample heart homogenate (containing approximately 250 ng HS). The samples and an equal portion of unspiked heart homogenate were subjected in parallel to HS purification, enzymatic depolymerization and GRIL-LC/MS to access the effect of a biological matrix on analytical recovery. The results are presented as pmole detected for each disaccharide comparing the results for heparin by-product (Spike) to those of heart alone and heart plus heparin by-product (Heart + Spike).

Supplemental Fig. S4. Propionylation of D0H0 enables detection after aniline tagging. (A) Propionic anhydride reacts with unsubstituted glucosamine to form the corresponding amide derivative D0R0, which can be detected by LC/MS. (B) The mass spectra shows the presence of the $[M-H]^{-1}$ ion (m/z = 469.13) which corresponds to the addition of both $[^{12}C_6]$aniline and propionyl moieties. (**C**) The elution positions of aniline-tagged D0R0 and D0A0 obtained from *Hydra* HS are shown for comparison.


D2H6-NB
D2A6-NB
D2S6-NB
AL
UL
-SO4
-Ac
C
Relative Abundance
Time (min)


1000
100
10
1
pmole
0.1
1
10
100
Sample Concentration
D0A0, R2 = 1.000
D0S0, R2 = 0.9983
D0A6, R2 = 0.9985
D0S6, R2 = 0.9975
D2S0, R2 = 0.9975
D2S6, R2 = 0.9954

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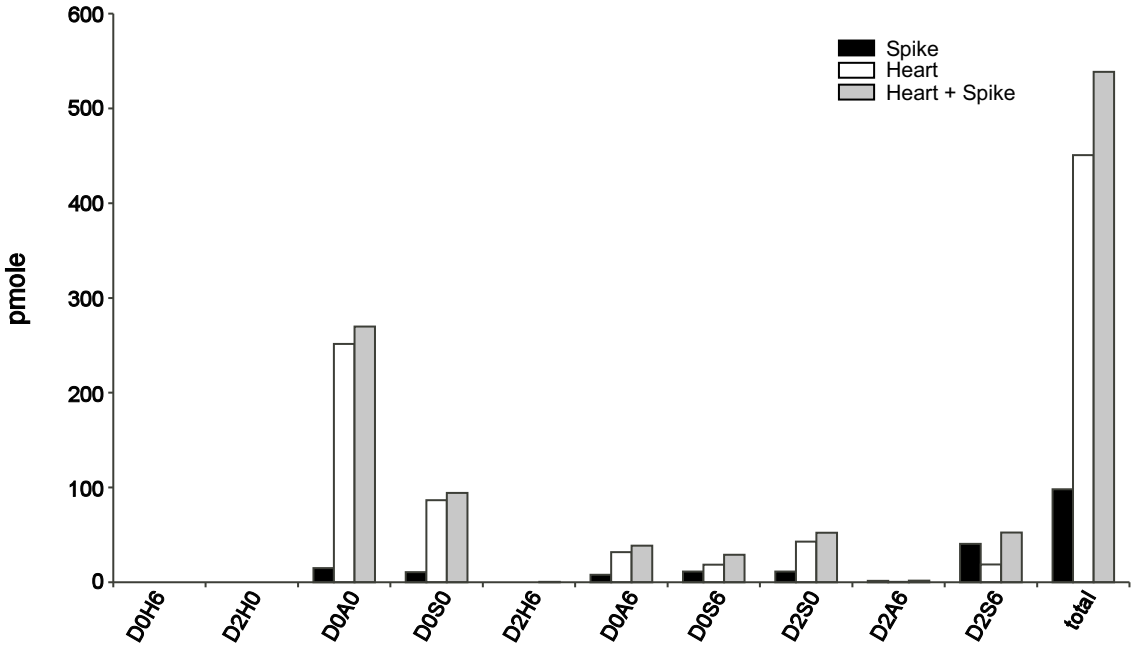

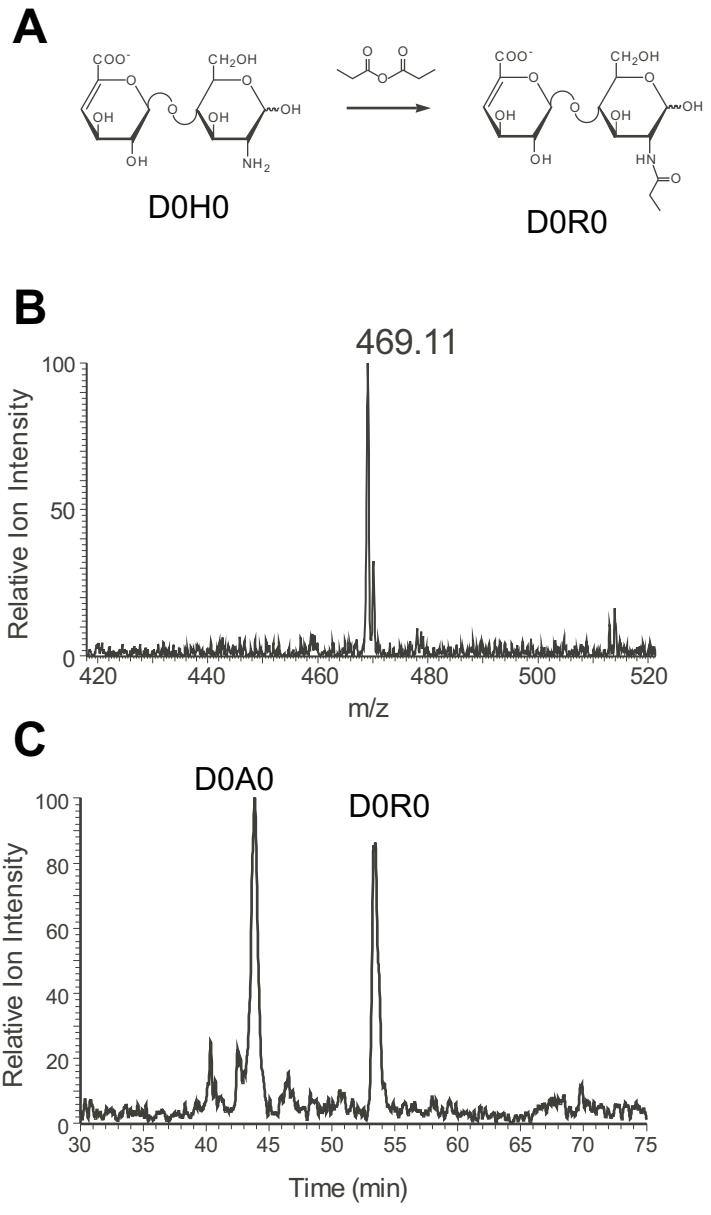
A
D0H0
D0R0
B
469.11
Relative Ion Intensity
m/z
C
D0A0
D0R0
Relative Ion Intensity
Time (min)