Determination of Chondroitin Sulfate in Raw Materials by Liquid Chromatography

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A rapid method for the determination of chondroitin sulfate in raw materials was developed. The samples were finely powdered, dissolved in water, and injected directly into the liquid chromatograph. The method used a C_{18} column, a wavelength of 195 nm, and a mobile phase containing octane sulfonic acid. The method gave results that were slightly different from those generated by a titrimetric method using cetylpyridinium chloride.

Chondroitin sulfate is a highly water-soluble polymer with a molecular weight varying from 23 000 to 45 000 daltons. It is a mucopolysaccharide for which each monomer is a disaccharide containing one sulfate group. Different forms of chondroitin sulfate have the sulfate group in different positions. Chondroitin sulfate A is sulfated over the hydroxylic group in the 4-position and is the predominant form for chondroitin sulfate from bovine sources. Chondroitin sulfate C is sulfated over the hydroxylic group in the 6-position and is the predominant form for chondroitin sulfate from shark sources. Chondroitin sulfate is negatively charged because of the presence of these sulfate groups.

Chondroitin sulfate, along with glucosamine and methylsulfonylmethane (MSM), is currently being sold as a product to promote healthy joint function. The finished product is usually in the form of tablets containing 400 mg chondroitin sulfate and 500 mg glucosamine sulfate 2KCl. Other products may contain approximately 500 mg MSM in addition to the chondroitin and the glucosamine.

Many methods with biomedical applications are available (1–9) for determining chondroitin sulfate. Most of these methods involve enzyme digestion of the polymer into the individual disaccharide monomers. These disaccharides are then quantitated by liquid chromatography (LC).

Few methods are available for assaying raw materials and tablets with good precision. Recently, a method was proposed for determination of chondroitin sulfate in nutritional supplements (10). The method used UV detection at 207 nm after separation by size exclusion chromatography. Quantitation was by peak height to increase analytical precision.

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The current USP proposed method (11) uses a cetylpyridinium chloride (CPC) titration with turbidimetric end point detection. The method has good specificity because there are few molecules that will bind with the CPC to form precipitates other than large, negatively charged polymers.

The proposed LC method uses octane sulfonic acid in an acidic mobile phase. Chondroitin sulfate is excluded from the column and elutes considerably before the solvent front. Other molecules, such as glucosamine and proteins, elute near or considerably after the solvent front. Thus, the LC method, like the CPC method, shows good specificity. An LC scan of a sample containing chondroitin sulfate and glucosamine HCl is shown in Figure 1. The small peak following the chondroitin sulfate is due to chloride contributed by the glucosamine HCl.

METHOD

Reagents

(**a**) *LC buffer concentrate*.—Dilute 100 mL LC triethylamine to ca 900 mL with water in 1000 mL solvent reservoir. Add 80 mL reagent-grade 85% phosphoric acid, and mix cautiously. Cool to room temperature, bring to 1000 mL with water, and mix well.

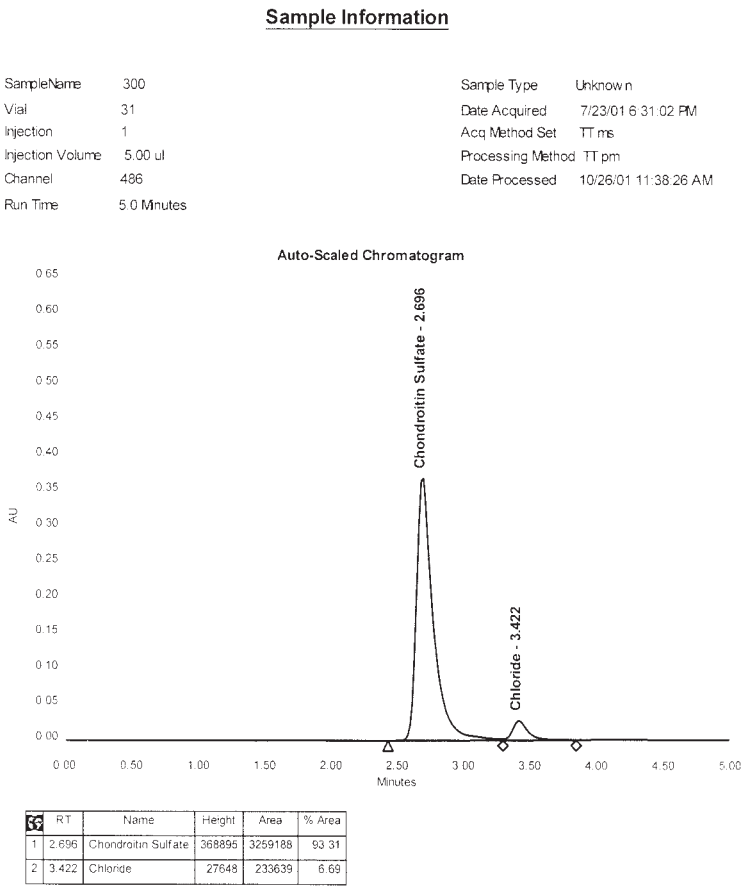
(**b**) *Mobile phase*.—Weigh 0.50 g LC quality octanesulfonic acid sodium salt into small beaker and quantitatively transfer to 1000 mL solvent reservoir with water. Add 5.0 mL LC buffer concentrate, 40 mL acetonitrile, dilute to volume with water, mix well, and de-gas by sparging or by filtration through 0.45 μm filter.

(**c**) *Chondroitin sulfate standard*.—Chondroitrin Sulfate (A; Sigma Chemical Co., St. Louis, MO).

Apparatus

(**a**) *Liquid chromatograph*.—A Waters Associates (Milford, MA) Millennium 32 system with a Waters Xterra column, C_{18}, 250 × 4.6 mm, was used. The system was operated at room temperature, the injection volume was 5.00 μL, the flow rate was 0.6 mL/min, and the run time was ca 6 min.

(**b**) *Titrimetric method*.—A Metrohm (Westbury, NY) 751 GPD Titrino equipped with Metrohm 730 Sample Changer and 759 Swing Head, along with a Brinkmann (Westbury, NY) PC 700 colorimeter, was used to study the CPC titration at room temperature.



	RT	Name	Height	Area	% Area
1	2.696	Chondroitin Sulfate	368895	3259188	93.31
2	3.422	Chloride	27648	233639	6.69

Figure 1. Liquid chromatographic scan of tablet sample containing chondroitin sulfate and glucosamine HCl.

Standard Preparation

Accurately weigh a sufficient quantity of chondroitin sulfate standard into 100 mL volumetric flask to contain 100–120 mg chondroitin sulfate. Add ca 60 mL water and dissolve by swirling. Dilute to volume with water, and mix well. Dilute 10/100 and 25/100 with water for the low concentrations on the standard curve. Calculate actual concentrations based on assay of the standard. Store aqueous chondroitin sulfate solutions at refrigerator temperature for no more than 2 days, as these solutions are microbiologically unstable.

Sample Preparation

Accurately weigh sufficient powdered sample to contain ca 75 mg chondroitrin sulfate into 100 mL volumetric flask. Add ca 60 mL water and dissolve by swirling and/or a few minutes sonication. Dilute to volume with water, and mix well. If solutions show signs of turbidity, clarify by centrifugation or by filtration through 0.45 μm filter.

Procedure

Wash LC column with methanol at 0.6 mL/min for ca 15 min and then equilibrate with the mobile phase at the same flow rate. Equilibrate the column for 30 min and put system in recycle until solutions to be injected are prepared. Recycle for a minimum of 1 h.

Prepare solutions for the standard curve. Inject 100 mg/100 mL standard solution a minimum of 3 times and use these injections to optimize integration parameters. When the instrument has been optimized, inject this solution 5 times. The relative standard deviation (RSD) should be <2%. Calibrate the instrument to calculate the concentrations in units of mg/100 mL.

Inject solutions of the standard curve. Force the curve through zero. The correlation should be >0.9998.

When it has been established that the instrument performs satisfactorily, prepare the sample solutions, terminate recycle, and begin injecting sample solutions. The instrument should yield a printout of the sample chondroitin sulfate concentrations in terms of mg/100 mL.

Calculation, “as is” basis:

$$\text{Chondroitin sulfate, \% w/w} = 100 \times (\text{mg/100 mL})/\text{sample weight}$$

If dry basis results are required, dry a portion of the sample for 3 h at 105°C, cool, and weigh promptly to obtain total solids.

Table 1. Calibration curve parameters

Linearity equation, $y = mx + b$		
y^a	$x1^b$	$x2^c$
10.04	1.212	10.64
25.10	2.114	26.09
100.40	6.397	100.20
150.10	9.362	148.00
Correlation	0.999947	0.999972
m	17.25919	1.01994
b	–10.93792	–1.24268
b/m	–0.63	–1.22

[a] y = Concentration of chondroitin sulfate standard solutions in mg/100 mL.
[b] $x1$ = mL CPC titrant.
[c] $x2$ = LC response in mg/100 mL.

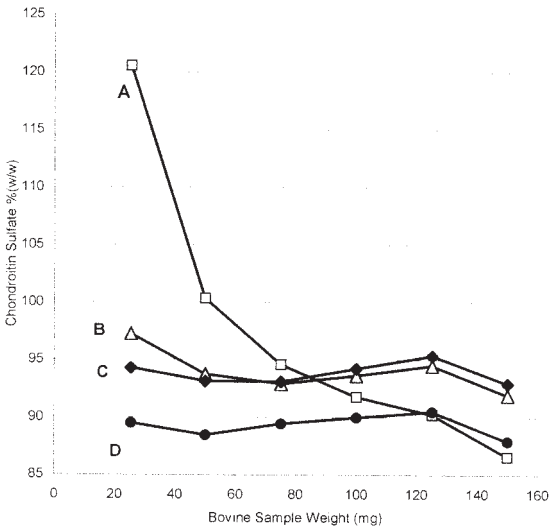


Figure 2. Assay of bovine chondroitin sulfate sample using 4 methods: CPC (A); LC (B); LC-corrected (C); and CPC-corrected (D).

(*Note*: Dry chondroitin sulfate absorbs water very quickly, so a delay in weighing can lead to incorrect results.)

Results and Discussion

To obtain a close comparison between the proposed LC method and the proposed CPC titrimetric method, we used the same standard solutions and the same sample solutions for the CPC titration and the LC method. Specifically, 5.00 mL primary solution was used for titration, and then 5.00 µL of the same primary solution was used for the LC assay.

All results were calculated relative to the same batch of Sigma standard. Thus, any error in the standard will shift all calculated results in the same proportion. To form the standard curves, a quantity of standard to contain approximately 100 mg CS was taken to 100 mL and was then diluted 25/100 and 10/100 to form the low concentrations. An additional weighing of standard was made to give a high concentration of 150 mg/100 mL. The standard curve data is shown in Table 1.

Graphing of the calibration curves indicated that both curves were remarkably linear. The correlation was in excess of 0.9999 for both the LC and the CPC data; however, the LC curve had an intercept very close to zero, while the CPC curve had an intercept which departed significantly from zero. The data were put in $y = mx + b$ form to give corrected standard curves for both the LC and the CPC methods. To obtain the uncorrected forms, the single calibration points of zero and 100 mg/100 mL were used. Thus, for each set of sample data, 4 sets of calculated results were generated: CPC and LC uncorrected, and CPC and LC corrected.

Bovine Chondroitin Sulfate

The LC and CPC data are shown in Table 2 and the calculated results from that data are shown in Figure 2. The graph shows that the LC method gave very nearly the same values, corrected and uncorrected, whereas the titrimetric method gave good values only if the correction was made. When the bovine sample was run using a bovine standard curve, the RSD for

Table 2. Bovine sample data

y^a	$x1^b$	$x2^c$
25.4	1.951	24.7
50.0	3.196	46.87
75.1	4.525	69.78
100.0	5.847	93.61
125.1	7.193	118.18
150.1	8.278	137.96

[a] y = Sample weight in mg.
[b] $x1$ = mL CPC titrant.
[c] $x2$ = LC response in mg/100 mL.

Table 3. Shark sample data

y^a	$x1^b$	$x2^c$
25.0	1.851	24.5
50.2	3.223	49.19
75.0	4.606	74.64
100.6	6.097	100.48
125.1	7.471	126.34
150.0	8.891	149.96

[a] y = Sample weight in mg.
[b] $x1$ = mL CPC titrant.
[c] $x2$ = LC response in mg/100 mL.

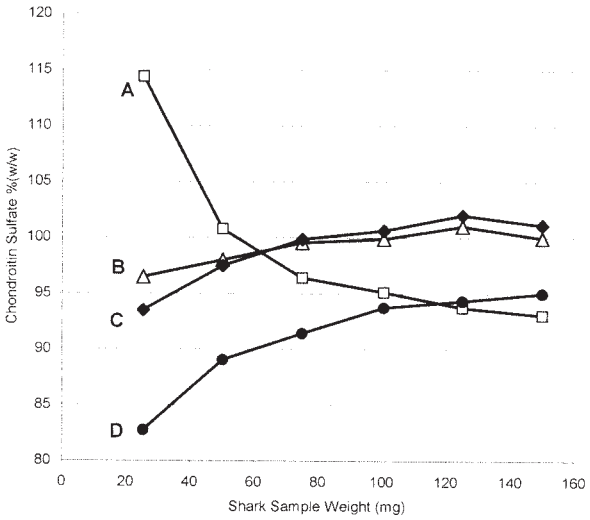


Figure 3. Assay of shark chondroitin sulfate sample using 4 methods: CPC (A); LC (B); LC-corrected (C); and CPC-corrected (D).

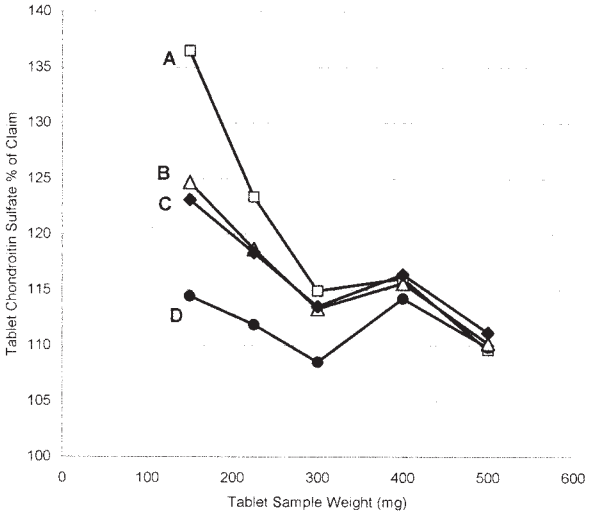


Figure 4. Assay of tablet containing chondroitin sulfate and glucosamine HCl using 4 methods: CPC (A); LC (B); LC-corrected (C); and CPC-corrected (D).

both the LC and the CPC method was approximately 1.0%. The LC uncorrected method gave an RSD of 1.93%, and the CPC uncorrected method gave data that were not usable.

Shark Chondroitin Sulfate

The LC and CPC data are shown in Table 3, and the calculated results from that data are shown in Figure 3. Like the bovine sample, the LC data show better consistency than the CPC data. In addition, the bovine standard curve is not appropriate for a shark sample. The curvatures of the corrected and uncorrected CPC analytical results suggest that the shark standard curve would have less of a departure from zero than the bovine standard curve; therefore, for analysis of shark samples, a shark standard must be used to assay those samples. The LC uncorrected method gave the lowest RSD of 1.20%.

Chondroitin Sulfate/Glucosamine HCl/MSM Tablet

The data for this assay are shown in Table 4, and the calculated results from that data are shown in Figure 4. The graph suggests that the source of the chondroitin sulfate is bovine. These results show a large standard deviation not found in the 2 raw material samples, suggesting that the sample was of nonuniform composition and that additional grinding of these mixed products would be required. The CPC corrected method gave the lowest RSD of 2.34%.

Table 4. Tablet sample data (average tablet weight = 978 mg)

y[a]	*x*1[b]	*x*2[c]
150.1	2.669	38.27
225.1	3.618	54.63
301.4	4.492	69.50
400.4	6.048	94.57
500.1	7.141	112.7

[a] *y* = Sample weight in mg.
[b] *x*1 = mL CPC titrant.
[c] *x*2 = LC response in mg/100 mL.

LC and CPC Comparison

In the CPC method, the only components quantitated will be the large negatively charged polymers. Uncharged components or inorganic sulfate will not form a precipitate with the CPC. In the LC method, only large negatively charged molecules will be excluded from the LC column and appear significantly before the solvent front. Inorganic sulfate does not absorb at 195 nm.

The main difference between the methods is in the detection mechanisms. The CPC method bases its quantitation on the ability of chondroitin sulfate to ion-pair with the CPC reagent and form a precipitate. The LC method bases its quantitation on the ability of chondroitin sulfate to absorb light at 195 nm. By using the Sigma A standard, the bovine sample assays about 4% higher with the LC method than with the CPC method. If the raw material were used as the standard and the Sigma A standard were used as the sample, the results would be quite different. Thus, the numerical values that are generated using either method are strongly dependent on the nature of the particular standard used.

Acknowledgments

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