



Laboratory of the Government Chemist

LGC
Queens Road
Teddington
Middlesex
TW11 0LY
24 October 2012

A Review of Methods Available for the Determination of Chondroitin Sulphate in Supplements

Summary

Following a recent request, the Government Chemist has prepared the following note summarising the currently available methods for the determination of chondroitin sulphate in supplements as an update to the previously published report 'Analysis of chondroitin in supplements – ad hoc project 3¹'. This note is a brief review based on literature and web searches: no practical evaluation of any methods have been carried out.

Chondroitin Sulphate

Chondroitin sulphate is often sold as a dietary supplement. It is a natural product isolated from either land animals, for example, bovine trachea, or from avian or marine species, for example, shark cartilage. Chemically, chondroitin sulphate is a polysaccharide consisting of repeating disaccharide units; β -glucuronic acid linked to N-acetylgalactosamine. The disaccharide is sulphated on the N-acetylgalactosamine residue in either the 4 or 6 position. Chondroitin A, the predominant form in land animals, is sulphated on the 4 position and chondroitin C, predominant in marine animals, is sulphated on the 6 position. Other forms of chondroitin sulphate containing di- and tri- sulphated disaccharides also exist and appear

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http://www.governmentchemist.org.uk/dm_documents/Analysis%20of%20chondroitin%20in%20supplements_Wubgx.pdf

to be found only in marine animals. Chondroitin sulphate polymers range from 5,000 to 100,000 Daltons in size although most preparations for supplements are reported to contain polymers in the region of around 17,000 Daltons. Chondroitin sulphate therefore is a term which covers a range of differently sulphated polymers of varying sizes and is not a single defined chemical entity. This makes accurate analysis and quantification challenging.

Methods of Analysis

Dietary supplements can be appraised against the standards set out in the appropriate Pharmacopeia. The United States Pharmacopeia (USP) allows chondroitin sulphate isolated from either mammalian or avian species whereas the British Pharmacopeia (BP) allows chondroitin sulphate from both terrestrial and marine species to be used. The method described by both Pharmacopeia for chondroitin sulphate quantification is cetylpyridinium chloride (CPC) titration. The principle behind this method is the binding of the cationic CPC molecules with the negatively charged chondroitin sulphate to form insoluble ion pairs seen as a precipitate. The absorbance of the solution is monitored and by comparing with a chondroitin standard solution of known concentration, the amount of chondroitin in the test solution can be determined. Since the method is based on binding of CPC to a negatively charged macromolecule it is not specific for chondroitin sulphate and other large molecules, such as protein, may also give a positive response. A Standard Operating Procedure (SOP) for this method is available on the NSF International website² – it notes that this method is suitable only for pure chondroitin sulphate and also requires previous demonstration of purity by electrophoresis (for example as described in the pharmacopeia) or near infra-red spectrometry. The NSF SOP recommends the use of a specific chondroitin sulphate available from Sigma (C8529, Chondroitin sulphate A sodium salt, from bovine trachea) claiming that this material is among the best characterised available and is ultra pure. Other work³ has shown that for accurate quantification it is necessary to use an appropriate chondroitin sulphate standard i.e. one that is similar to the chondroitin sulphate in the test sample. This paper also notes that the method may be prone to giving inaccurate results, particularly at low concentrations, unless care is taken to blank correct the standard curve.

²Total chondroitin sulphate sodium by cetylpyridinium chloride method INA Method 120.002 <http://www.nsf.org/business/ina/cs.asp?program=INA> NSF International (formerly the national sanitation foundation).

³Tyler *et al.* 2002 Determination of chondroitin sulfate in raw materials by liquid chromatography. *Journal of AOAC International* **85** 567-571

There are other methods which have been used for the determination of chondroitin sulphate, for example, the carbazole method. In this method, the sample is acid hydrolysed and then the glucuronic acid present measured using a chromogenic reaction. This method is not specific for chondroitin sulphate and will also give a positive response with other glycosaminoglycans that contain uronic acid residues. The carbazole method has also been shown to give false positives response due to the interference of NaCl⁴ which suggests it may not be suitable for analysis of chondroitin sulphate present in supplement preparations as many appear to contain salts. There is no evidence in the scientific literature that this method has been formally validated for the determination of chondroitin sulphate in supplements. The carbazole method is therefore not recommended for the accurate determination of chondroitin sulphate in supplements in the absence of appropriate validation on a product specific basis.

A range of chromatographic methods have been used to try to quantify chondroitin sulphate; these include high performance liquid chromatography (HPLC) methods based on size exclusion where samples containing chondroitin sulphate are analysed without any pre-treatment and other methods using amine columns or strong ion exchange HPLC where samples containing chondroitin sulphate are pre-treated with a chondroitinase enzyme. Several of these HPLC methods were evaluated in our previous project¹.

Since our previous work, two methods with single laboratory validation data have been published; one based on enzymatic pre-treatment of the sample followed by ion-pairing reversed phase liquid chromatography with UV detection⁵ and the other based on acid hydrolysis of the chondroitin sulphate in the sample to its constituent monosaccharides followed by derivatisation with ortho-phthalaldehyde and reversed phase liquid chromatography with UV detection⁶. The method which has been most extensively

⁴ Frazier *et al.*, 2008 the Quantification of glycosaminoglycans: A comparison of HPLC, Carbazole and Alcian Blue methods *Open Glycosci* **1** 31-39

⁵ Ji *et al.*, 2007 Determination of chondroitin sulphate content in raw materials and dietary supplements by high performance liquid chromatography with ultraviolet detection after enzymatic hydrolysis: Single-Laboratory validation. *Journal of the AOAC International* **90** 659-669

⁶ Gatti *et al.*, 2010 A simple and validated LC method for the simultaneous analysis of glucosamine and chondroitin sulphate equivalent in dietary products. *Journal of Liquid Chromatography and Related Technologies* **33** 1760-1775

validated is the enzymatic HPLC method which has been single laboratory validated by the AOAC⁵. In this method, chondroitin sulphate in the sample is digested using Chondroitinase AC which breaks down the molecule to its component disaccharides. Chondroitinase AC is specific for chondroitin sulphate. The disaccharides obtained by digestion are then separated using ion-pairing reversed phase liquid chromatography with UV detection. Different disaccharides produce separate peaks and the amount of each disaccharide in the sample is quantified against an appropriate disaccharide standard curve. The amount of chondroitin sulphate in the sample is calculated by summing the amounts of each disaccharide present. One advantage of this approach is that since the standard used for quantification is the disaccharide rather than a chondroitin sulphate, the method overcomes some of the problems seen previously when trying to quantify a sample containing a chondroitin sulphate of unknown origin. The AOAC paper states that once further data has been obtained it may be possible to use the disaccharide profiles to give an indication of the source of the chondroitin sulphate.

The AOAC validated method has been shown to be specific for chondroitin sulphate. Glucosamine, methyl sulfonylmethane, dermatan sulphate and carrageenan are all potential co-ingredients in supplements and were shown not to interfere in the assay. A number of salts e.g. calcium sulphate and magnesium chloride likely to be found in supplements were also tested and again no interference was seen. Hyaluronic acid did give a positive response in the assay but the disaccharide profile obtained was considered unique to hyaluronic acid. Since this compound is expensive it was not thought likely to be used as a substitute for chondroitin sulphate.

The published validation study shows good recovery demonstrated from raw material (chondroitin sulphate added to heparin) and a supplement tablet (chondroitin sulphate added to a ground tablet). Repeatability data was obtained by analysing the chondroitin sulphate present in a range of commercial supplements (capsules, chewable tablets, tablets, liquid supplement); the RSDs ranged from 1.60% to 4.72%. The ruggedness of the method was demonstrated by a Youden ruggedness trial. The method was shown to be applicable to chondroitin sulphate isolated from a range of sources. As an outcome of the positive single laboratory validation study, the method was recommended for collaborative trial. This was expected to commence in 2008, however, to our knowledge, the results of such a trial, if it has taken place, are not yet publically available.

Conclusions

- The USP and BP stipulate that chondroitin sulphate should be quantified by the CPC titration method. It is advised that this method is to be used for the determination of chondroitin sulphate in pure samples of chondroitin sulphate whose purity has been demonstrated by electrophoresis.
- There are other methods available for the determination of chondroitin sulphate such as the carbozole method and HPLC methods based on size exclusion; these are not recommended for the determination of chondroitin sulphate as they are not specific for chondroitin sulphate and/or do not appear to have been formally validated.
- An enzymatic-HPLC method has been single laboratory validated by the AOAC. This method has been shown to be specific for chondroitin sulphate, suitable for the determination of chondroitin sulphate from a range of animal sources and in a range of matrices such as capsules, tablets and liquids.

This report was prepared by Dr Joanna Topping.

**** October 2012

For further information contact Selvarani Elahi, Selvarani.elahi@lgcgroup.com

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