Journal of Pharmaceutical and Biomedical Analysis 51 (2010) 969–972

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Short communication

Determination of molecular mass values of chondroitin sulfates by fluorophore-assisted carbohydrate electrophoresis (FACE)

Dania Buzzega, Francesca Maccari, Nicola Volpi*

Department of Biologia Animale, University of Modena and Reggio Emilia, Via Campi 213/d, 41100 Modena, Italy

ARTICLE INFO

Article history:
Received 10 July 2009
Received in revised form 19 October 2009
Accepted 20 October 2009
Available online 30 October 2009

Keywords:
Chondroitin sulfate
FACE
Electrophoresis
Molecular mass
Glycosaminoglycans

ABSTRACT

Fluorophore-assisted carbohydrate electrophoresis (FACE) was applied to determine the molecular mass (M) values of various chondroitin sulfate (CS) samples. After labeling with 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS), FACE was able to resolve each CS sample as a discrete band depending on the M value. After densitometric acquisition, the migration distance of each CS standard was acquired and the third grade polynomial calibration standard curve was determined by plotting the logarithms of the M values as a function of migration ratio. Purified CS samples of different origin and the European Pharmacopeia CS standard were analyzed by both FACE and conventional high-performance size-exclusion liquid chromatography (HPSEC) methods. The molecular weight value on the top of the chromatographic peak (M_p), the number-average M_n, weight-average M_w, and polydispersity (M_w/M_n) were examined by both techniques and found to be quite similar. This study demonstrates that FACE analysis is a suitable, sensitive and simple method for the determination of the M values of CS macromolecules with possible utilization in virtually any kind of research and development such as quality control laboratories.

1. Introduction

Chondroitin sulfate (CS) is a natural complex polysaccharide belonging to glycosaminoglycans (GAGs) composed of alternate disaccharide sequences of differently sulfated residues of D-glucuronic acid (GlcA) and of *N*-acetyl-D-galactosamine (GalNAc) linked by β(1–>3) bonds [1]. CS is currently recommended by EULAR as a SYSADOA (symptomatic slow acting drug for OA) drug in Europe in the treatment of knee and hand osteoarthritis (OA) based on research evidence and meta-analysis of numerous clinical studies [1–3]. Furthermore, recent clinical trials demonstrated its possible structure-modifying effects [4,5].

Besides the charge density and disaccharide nature heterogeneity, the number of disaccharide units forming the CS polymer is another key factor influencing its biological and pharmacological activities [1,2]. In fact, CS is polydisperse with a molecular mass (M) range depending on the source generally of 15,000–>50,000, with an average M of approx. 15,000–30,000 [1,2]. As a consequence, molecular mass parameters are of paramount importance for CS properties.

In a previous research [6], we reported the use of fluorophore-assisted carbohydrate electrophoresis (FACE) to determine the M values of various purified and pharmaceutical heparin samples. Heparins were labeled with 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) and FACE was able to resolve each fraction as a discrete band depending on their M values. After densitometric acquisition, the migration distance of each heparin was acquired and their M values calculated versus a third grade polynomial calibration standard curve determined by plotting the logarithms of the M values as a function of migration ratio. In this study, we evaluate the possibility to apply this analytical approach to the M values determination of CS samples of various origins generally used for pharmaceutical purposes and known to posses M values greater that those of heparin samples, approx. 12,000–14,000 [7]. The determination of the M_p, M_n, M_w and p values of various CS samples by FACE was obtained and compared with those determined by conventional high-performance size-exclusion liquid chromatography (HPSEC).

2. Experimental

2.1. Materials

CS samples from bovine trachea, porcine trachea, and chicken trachea were extracted and purified according to standardized pro-

Abbreviations: ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid; CS, chondroitin sulfate; EULAR, European League Against Rheumatism; FACE, fluorophore-assisted carbohydrate electrophoresis; GAG(s), glycosaminoglycan(s); GlcA, D-glucuronic acid; GalNAc, *N*-acetyl-D-galactosamine; HPSEC, high-performance size-exclusion liquid chromatography; MALLS, multiangle laser light scattering; mm, migration distance; M, molecular mass; M_n, number-average mean molecular weight; M_p, molecular weight value on the top of the chromatographic peak; M_w, weight-average mean molecular weight; OA, osteoarthritis; p, polydispersity; RA, relative Absorbance; SYSADOA, symptomatic slow acting drug for OA.

* Corresponding author. Tel.: +39 059 2055543; fax: +39 0 59 2055548.
E-mail address: volpi@unimo.it (N. Volpi).

0731-7085/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2009.10.015

tocols [9,10]. Shark cartilage CS was donated by IBSA (Institute Biochimique SA, Lugano, Switzerland). CS reference standard produced from bovine cartilage, CSPh, manufactured by Bioiberica (www.bioiberica.com/) and approved in 2004 as chemical reference substance (CRS) by the European Pharmacopeia Commission was from Bioiberica. CS standards were prepared from bovine trachea CS by gel-permeation chromatography and characterized with respect to *M* values by means of multiangle laser light scattering (MALLS) detection [8].

8-Aminonaphthalene-1,3,6-trisulfonic acid disodium salt (ANTS, >90%, M_w 427.34), glacial acetic acid, dimethylsulfoxide (DMSO, 99.9%), sodium cyanoborohydride (95%) were from Sigma–Aldrich. Polyacrylamide (>99%), N,N′-methylenebisacrylamide (>99%), N,N,N′,N′-tetramethylethylenediamine (TEMED, 99%) and ammonium persulfate (98%) were purchased from Sigma–Aldrich. Polaroid black-and-white print film, Polaroid Type 667, was from Sigma. All other reagents, of the purest grade available, were from Sigma–Aldrich.

Densitometric acquisition was performed with a densitometer composed of a Macintosh IIsi computer interfaced with Microtek Color Scanner from Microtek International Inc., Hsinchu, Taiwan. The photographs were scanned and saved in gray scale. Image processing and analysis program, from Jet Propulsion Lab., NASA, FL, U.S.A., were used for densitometry.

2.2. HPSEC analysis

HPLC mod. LC-1500 was from Jasco. The mobile phase was composed of a 125 mM Na_2SO_4 and 2 mM NaH_2PO_4 adjusted to pH 6.0 with 0.1N NaOH at a flow rate of 0.9 ml/min. Standards or samples were solubilized in the mobile phase at a concentration of 10 mg/ml and 10 μl (100 μg) were injected in HPLC. Columns were Protein Pak 125 (Waters, cod. 84601, 7.8 mm × 30 cm) and Protein Pak 300 (Waters, cod. T72711, 7.5 mm × 30 cm) assembled in series [6]. The retention times were plotted against the logarithm of *M* for standard CSs. The curve that fits the experimental data is a third grade polynomial with the formula $y(fx) = -ax^3 + bx^2 - cx + d$ performed using the Jasco Borwin program. The molecular weight value on the top of the chromatographic peak (M_p), the number-average molecular weight (M_n), the weight-average molecular weight (M_w), and the polydispersity index (p) were calculated using the Jasco Borwin GPC software ver 4.1.

2.3. Fluorophore-ssisted carbohydrate electrophoresis (FACE) of CS-ANTS derivatives

Derivatization of various CS samples with ANTS was essentially performed as previously described [6,11]. 400 μg of the various CS samples were lyophilized and reconstituted with 10 μl of a 30 mM ANTS solution in 15% glacial acetic acid. After incubation at room temperature for 15 min, 10 μl of a freshly prepared solution of 1 M sodium cyanoborohydride in DMSO was added. Derivatization was performed by incubating at 45 °C for 4 h. Finally, 30 μl of 40% (v/v) glycerol was added to the samples and aliquots were taken for FACE analysis.

A Miniprotean II cell vertical slab gel electrophoresis apparatus (Bio-Rad) was used for FACE analysis. The stock buffer solutions were Tris–borate (pH 8.8; 1.5 M) and Tris–HCl (pH 8.8; 1.5 M). Acrylamide solution T 50%/C 7.5% for the resolving gel and T 50%/C 15% for the stacking gel were used as stock solutions [%T refers to the total concentration (w/v) of acrylamide monomer (i.e., acrylamide plus methylenebisacrylamide); % C refers to the concentration (w/v) of cross-linker relative to the total monomer]. A 10 ml volume of T 25%/C 3.75% resolving gel solution, in Tris–HCl (pH 8.8; 0.375 M) was prepared and degassed. A 5 μl volume of TEMED and 50 μl of a freshly prepared 10% (w/v) ammonium persulfate were added. The solution was mixed rapidly and then placed between the glass plates, eliminating the air bubbles. The non-polymerized gel was overlaid with butanol. The resolving gel surface was rinsed with stacking gel buffer (Tris–HCl diluted from the stock solution). A 5 ml volume of T 50%/C 1.5% acrylamide stacking gel in Tris–HCl (pH 8.8; 0.36 M) was prepared, followed by an addition of 5 μl of TEMED and 50 μl of 10% ammonium persulfate. The solution was immediately poured on the top of the resolving gel and the well-forming comb was inserted. The height of the stacking gel was 5 mm.

Immediately before electrophoresis, the wells were rinsed with electrophoresis buffer and a pre-run of the gel was performed at 400 V for 5 min at 4 °C. A 5 μl volume of each sample was loaded in each well. Electrophoresis was performed at 400 V for 10 min at 4 °C.

The gels were illuminated with UV light from a Transilluminator and imaged with a CCD camera. Black-and-white negatives were obtained for each gel and the photographs (see for example Fig. 1A) were prepared for densitometry scanning. Each lane was scanned at a rate of 1.0 cm/min, and the relative absorbance (RA) was plotted with a Epson printer. The scans produced by this method were digitized with the Image Analysis Software. The *x* value obtained corresponded to migration distance (mm) from the origin, and the *y* value corresponded to the RA. Finally, *x*,*y* pairs (of approx. equal

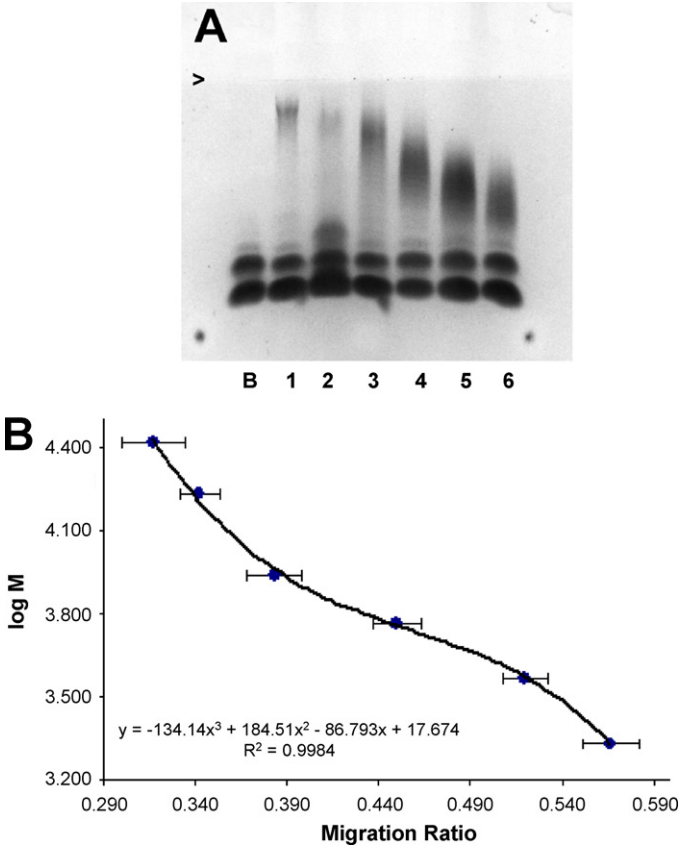

Fig. 1. (A) FACE analysis and separation of the six CS (20 μg) standards (from 1 to 6). B = blank derivatization reagent. > indicates the top of the running gel. The two bands at the bottom of the gel are formed of residual ANTS reagent as demonstrated by the blank derivatization reagent. (B) The FACE third grade polynomial standard curve having the formula $y(fx) = -ax^3 + bx^2 - cx + d$ performed by means of CS standards. The logarithm of the CS *M* values is plotted as a function of migration ratio (calculated as migration distance of the band divided by the total length of the gel). The equation of the curve and the correlation coefficient (R^2) are also reported along with the coefficient of variations (CV%) of the migration distance (expressed as absolute value of migration ratio) found to be lower than approx. 15%. *Mp* values of the six CS standards are: (1) = 26,140, (2) = 17,020, (3) = 8,700, (4) = 5,830, (5) = 3,700, (6) = 2,130.

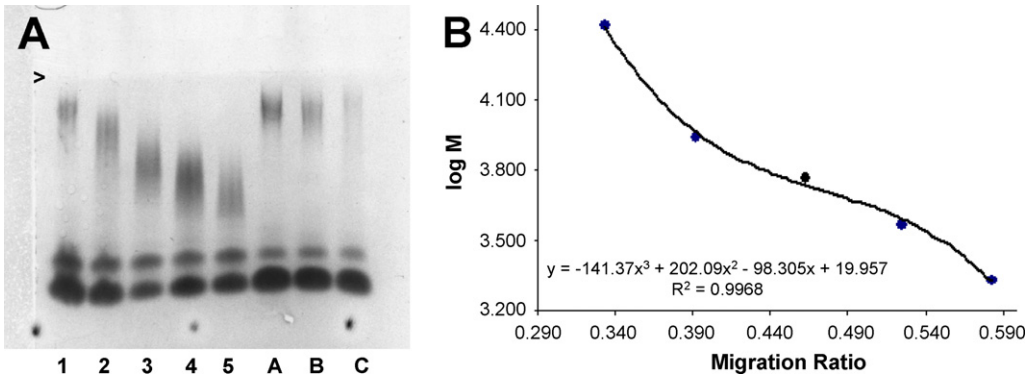

Fig. 2. (A) FACE analysis and separation of CS bovine (A), CS porcine (B) and CS avian (C) along with five CS standards and (B) the related calibration curve. > indicates the top of the running gel.

spacing of x points) were assembled as an ASCII file, imported into Excel, and processed to obtain values of M and p.

According to the previous study [6], after densitometric evaluation, a collection of x,y pairs corresponding to a scanned gel track was selected. Each x point (i.e., x_i) was converted from distance (mm) to M (M_i) by using the standard curve obtained on the same gel. Each y point was used directly as RA (i.e., RA_i). The absorbance at any position on the gel is directly proportional to the mass of the sample at that point:

$$RA_i = KW_i \tag{1}$$

where W_i (equal to N_iM_i) is the mass of sample at point i, K is the proportionality constant, and N_i is the number of molecules at point i:

$$N_iM_i = W_i = \frac{RA_i}{K}. \tag{2}$$

By definition:

$$M_n = \frac{\sum N_iM_i}{\sum N_i} \tag{3}$$

and

$$M_w = \frac{\sum N_iM_i^2}{\sum N_i}. \tag{4}$$

Substituting (2) into Eqs. (3) and (4) gives:

$$M_n = \frac{\sum(RA_i/K)}{\sum(RA_i/KM_i)} = \frac{\sum RA_i}{\sum(RA_i/M_i)} \tag{5}$$

$$M_w = \frac{\sum[(RA_i/K)M_i]}{\sum(RA_i/K)} = \frac{\sum(RA_iM_i)}{\sum RA_i} \tag{6}$$

and

$$p = \frac{M_w}{M_n}. \tag{7}$$

Eqs. (5)–(7) were used to analyze the FACE results.

3. Results

Six CS standards having M values from 26,140 to 2130 were prepared from purified bovine trachea CS by preparative gel-permeation chromatography and characterized with respect to M values by means of MALLS detection [8]. These standards were labeled with ANTS and their electrophoretic behaviour evaluated by FACE. As illustrated in Fig. 1A as an example, FACE is able to resolve each CS fraction as a discrete band depending on their M values. After densitometric acquisition, the migration distance (mm) for each CS standard was acquired and the calibration standard curve was determined. The logarithms of the M values were plotted as a function of migration ratio (calculated as migration distance of the band divided by the total length of the gel) to prepare the standard curves (Fig. 1B). The correlation coefficient (R^2) was found to be very high and the variation coefficient (CV%) of the migration distance (expressed as the absolute value of migration ratio) was found to be lower than approx. 15%.

The M values of different origin CS samples were obtained with a standard curve (Figs. 2B and 3B) performed every time for each gel (see Fig. 2A for CS from bovine, porcine and avian, and Fig. 3A for CS Pharmacopoeia and CS from shark cartilage). Table 1 illustrates the

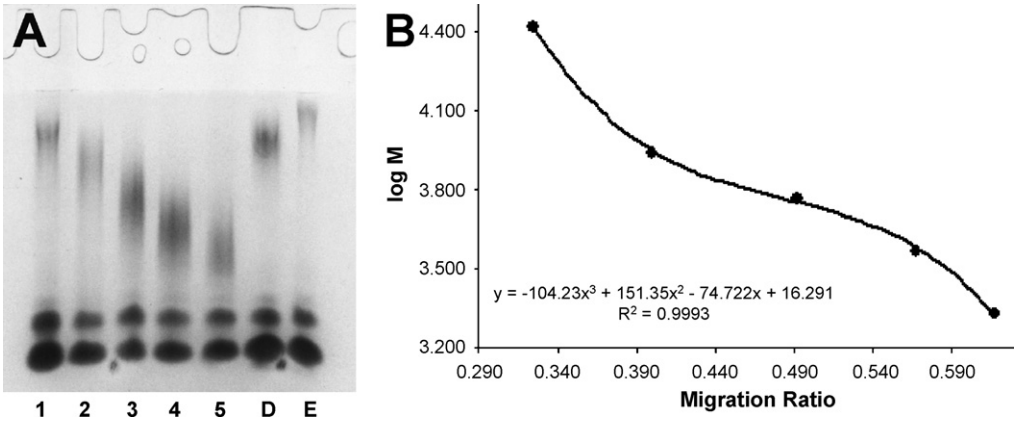

Fig. 3. (A) FACE analysis and separation of CS pharmacopoeia (D) and CS shark (E) along with five CS standards and (B) the related calibration curve. > indicates the top of the running gel.

Table 1
M and *p* values of the various CS samples calculated by means of HPSEC and FACE. M_p = molecular weight value on the top of the chromatographic peak, M_n = number-average mean molecular weight, M_w = weight-average mean molecular weight, *p* = polydispersity.

	HPSEC				FACE			
	M_p	M_n	M_w	$p\ (M_w/M_n)$	M_p	M_n	M_w	$p\ (M_w/M_n)$
CS bovine	19,990	13,960	25,280	1.811	20,860	14,210	25,750	1.812
CS porcine	16,180	11,140	17,930	1.609	17,600	12,020	18,110	1.507
CS avian	19,860	11,910	20,540	1.725	21,760	12,400	23,630	1.906
CS shark	59,180	25,400	68,220	2.686	59,390	26,110	72,370	2.772
CS pharmacopoeia	18,650	11,320	21,410	1.891	20,030	12,400	22,890	1.844

M values of each CS determined by FACE and compared with more common HPSEC showing quite similar results for the different CS samples.

4. Discussion

CS is currently used as a drug in Europe in the treatment of knee and hand OA [1–5] and it is utilized as a nutraceutical in Europe and the United States [1]. As it is well known, CS, like other natural polysaccharides, is derived from animal sources by extraction and purification processes [1]. As a result of the biosynthetic processes related to specific tissues and species, CSs with different grades of polymerization may be biosynthetized producing macromolecules having various *M* and *p* values. Furthermore, extraction and purification processes may introduce modifications of the structural characteristics and properties. Finally, the evaluation of the CS *M* parameters is a key factor related to pharmacological activity as degraded products are unable to produce comparable biological effects [2,3].

In a previous study [6], we were able to resolve various heparin samples on the basis of their *M* values by using FACE after labeling with ANTS. Due to the great difference in the *M* values between heparins, average *M* of approx. 12,000–14,000 [8], and CS samples, average *M* from 15,000 to >50,000 [1], we tested in this research the feasibility of FACE analysis to be able to separate also CS depending on *M* values besides heparins. As evident, a very good agreement was found between *M* values evaluated by FACE approach as compared with more common HPSEC determination. Moreover, no influence of oversulfation related to CS heterogeneity was observed. In fact, no differences were found for CS from shark cartilage, known to possess a sulfation grade greater of about 25–30% than CS from bovine/porcine/avian [2], determined by FACE and compared with HPSEC. As a consequence, FACE analysis after labeling with ANTS is a sensitive and accurate method for the determination of the *M* values of CS samples of various origin having *M* parameters very different each other. Furthermore, as also observed with heparins [6], FACE is a very sensitive method as it requires approx. 10–20 μg of CSs, about 50–100-fold lower than samples and standards used in HPSEC evaluation. Finally, the utilization of mini-gels allows the use of very low amounts of reagents with no expensive equipment nor any complicated procedures having to be applied.

References

[1] N. Volpi (Ed.), Chondroitin Sulfate: Structure, Role and Pharmacological Activity, Academic Press, Amsterdam/Boston/Heidelberg/London/New York/Oxford/Paris/San Diego/San Francisco/Singapore/Sydney/Tokyo, 2006.
[2] N. Volpi, Analytical aspects of pharmaceutical grade chondroitin sulfates, J. Pharm. Sci. 96 (2007) 3168–3180.
[3] N. Volpi, Quality of different chondroitin sulfate preparations in relation to their therapeutic activity, J. Pharm. Pharmacol. 61 (2009) 1271–1280.
[4] D. Uebelhart, R. Knols, E.D. de Bruin, G. Verbruggen, Treatment of knee osteoarthritis with oral chondroitin sulfate, Adv. Pharmacol. 53 (2006) 475–488.
[5] A. Kahan, D. Uebelhart, F. De Vathaire, P.D. Delmas, J.Y. Reginster, Long-term effects of chondroitins 4 and 6 sulfate on knee osteoarthritis: the study on osteoarthritis progression prevention, a two-year, randomized, double-blind, placebo-controlled trial, Arthritis Rheum. 60 (2009) 524–533.
[6] D Buzzega, F. Maccari, N. Volpi, Fluorophore-assisted carbohydrate electrophoresis for the determination of molecular mass of heparins and low-molecular-weight (LMW) heparins, Electrophoresis 29 (2008) 4192–4202.
[7] F.A. Ofosu, I. Danishefsky, J. Hirsh (Eds.), Heparin and Related Polysaccharides. Structure and Activities, vol. 556, N.Y. Acad. Sci., New York, 1989.
[8] U.R. Desai, R.J. Linhardt, Molecular weight of heparin using 13C nuclear magnetic resonance spectroscopy, J. Pharm. Sci. 84 (1995) 212–215.
[9] N. Volpi, R. Fregni, T. Venturelli, Activity of chondroitin ABC lyase on dermatan sulfate partially degraded by cupric-ion-mediated free-radical treatment, J. Chromatogr. Biomed. Appl. 669 (1995) 197–205.
[10] N. Volpi, Disaccharide analysis and molecular mass determination to microgram level of single sulfated glycosaminoglycan species in mixtures following agarose-gel electrophoresis, Anal. Biochem. 273 (1999) 229–239.
[11] Y. Oonuki, Y. Yoshida, Y. Uchiyama, A. Asari, Application of fluorophore-assisted carbohydrate electrophoresis to analysis of disaccharides and oligosaccharides derived from glycosaminoglycans, Anal. Biochem. 343 (2005) 212–222.