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Molecular and biochemical profiling of a heparin-derived oligosaccharide, C3

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Abstract

This study was designed to characterize a heparin-derived oligosaccharide (HDO), C3, using chemical and biochemical methods. Although previous studies have suggested C3 as a promising compound in the treatment of Alzheimer's disease (AD), its molecular and biochemical properties are still unknown. In this study, the molecular profiles and anticoagulant effects of C3 were investigated. To characterize the molecular and biochemical properties of C3, gel permeation chromatography (GPC), polyacrylmide gel electrophoresis (PAGE), radiolabeling and anticoagulant assays, such as activated partial thromboplastin time (APTT), Heptest, and anti-factor Xa assay, were used. The GPC profile revealed that C3 was an ultra-low-molecular-weight (MW) heparin mixture. The multiple components in C3 were studied with PAGE analysis. Tritium-labeled C3 exhibited similar biological properties as nonlabeled materials. The biological assays showed that C3 and its components exhibited weak anticoagulant effect. These results demonstrated the applicability of the combination of GPC, PAGE, and coagulation assays to characterize the molecular and biochemical profile of HDO. In addition, the low anticoagulant effect of C3 suggests that this compound could be a relatively low-risk adjunct in the treatment of AD.

Keywords: Heparin-derived oligosaccharide; Gel permeation chromatography; Polyacrylmide gel electrophoresis; Radiolabeling; Anticoagulant

1. Introduction

Heparin-derived oligosaccharides (HDO) are defined as oligosaccharides with ultra low molecular weight (MW) and narrow MW distribution, degraded from heparin by chemical or enzymatic methods. It has been shown that some low MW components of heparin can pass through the blood–brain barrier (BBB) [1], suggesting that these agents may modulate the functionality of the central nervous system [2]. Since glycosaminoglycans (GAGs), including heparins, have an important impact on the aggregation, breakdown, and toxicity of β-amyloid peptide (Aβ) [3–5] and on Aβ-induced tau-2 immunoreactivity [6], HDO were suggested as a useful adjunct in the treatment of Alzheimer's disease (AD) [7,8]. Moreover, HDO have been demonstrated to exhibit different biological properties from those of heparin, such as anti-inflammatory effect [9] and more predictable anticoagulant action [10].

During our in vitro studies on biological activities of HDO, an oligosaccharide mixture formed by γ-irradiation,

Abbreviations: HDO, heparin-derived oligosaccharide; MW, molecular weight; BBB, blood–brain barrier; GAGs, glycosaminoglycans; Aβ, β-amyloid peptide; AD, Alzheimer's disease; GPC, gel permeation chromatography; PAGE, polyacrylmide gel electrophoresis; APTT, activated partial thromboplastin time; UFH, unfractionated heparin; HPLC, high-pressure liquid chromatography; UV, ultraviolet; RI, refractive index; NRC, narrow range calibration; MMCO, molecular mass cut off

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C3, showed a profile of low anticoagulant effects but prominent neuronal action (unpublished data). Further comparative studies using amidolytic assays showed that C3 was devoid of antithrombin (factor IIa) activity and its anti-factor Xa activity was 85% lower than that of heparin.

As molecular profile is of importance in the determination of biological activity of any particular heparin [11,12], various methods have been developed to estimate this property. The techniques for molecular profiling such as measurement of MW by gel permeation chromatography (GPC) [13], determination of oligosaccharide components by polyacrylmide gel electrophoresis (PAGE) [14] and radiolabeling [15] have already been demonstrated to provide useful information for the analysis of heparins.

In this paper, we report the results obtained by GPC, PAGE, and tritium labeling in the molecular analysis of C3. In addition, the biochemical profiling of C3 and its components is also reported. GPC demonstrated the MW profile of C3 and allowed the rapid elucidation of MW distribution. To confirm the components in C3, fractionation of C3 was undertaken by PAGE. During electrophoresis, multiple oligosaccharide components were separated. The in vitro activity of the isolated fractions were elucidated by means of different amidolytic and clotting-based assays, including anti-factor Xa activity, activated partial thromboplastin time (APTT), and Heptest in normal human plasma (NHP). The chemical profiles and biological effects of C3 after tritium labeling were analyzed and compared to nonlabeled C3.

2. Materials and methods

2.1. Materials

HDO C3 was obtained from CORCON, Milan, Italy. The compound was provided as a white powder and was stored at room temperature. C3 was manufactured from unfractionated heparin (UFH) by means of a controlled depolymerization induced by γ-irradiation (US patent #4,987,222). UFH in the solid state or in solution is treated with a rectilinear gamma ray beam at doses of 2.5 to 20 Mrad. This irradiation is supplied in successive stages with alternating cooling intervals between the various stages. The degraded material is then fractionated to remove high MW fractions and to obtain C3.

2.2. MW determination of C3

The MW determinations were made using gel permeation chromatography and a high-pressure liquid chromatography (GPC–HPLC) system (Waters 845) equipped with software designed for polymer analysis (Millennium 2000). The HPLC system consisted of a computer (Digital, Pentium III), an LAC/E interface module, two 510 HPLC pumps, a 712 WISP auto-injector, an R401 differential refractometer, and a 484 tunable absorbance detector (Waters). The ultraviolet (UV) and the refractive index (RI) detectors were linked in series, with the outlet of the joint columns (TSK G3000SW and TSK G2000SW) attached to the UV detector. The MW profile of C3 was determined by using a method reported by Ahsan et al. [13]. Analysis of C3 was carried out by injecting 20-μl aliquots of C3 solution (10 mg/ml C3 in 0.3-M sodium sulfate) into the GPC–HPLC system. The flow rate for the mobile phase (0.3 M sodium sulfate) was 0.5 ml/min and the run time was 65 min. UV determination was made at 234 nm at room temperature. Following each run, the elution profile of each sample was analyzed by the narrow range calibration (NRC) method [21]. Calibration of the GPC–HPLC system was performed in a similar manner using narrow range calibrators (10 mg/ml calibrators in 0.3 M sodium sulfate).

2.3. Polyacrylamide gel analysis of C3

A portion (1.25 g in 12.5 ml) of C3 was fractionated into disaccharide through decasaccharide components on a Bio-Gel P-6 column (4.8 × 100 cm) eluted with 100 mM sodium chloride at a flow rate of 1.5 ml/min, with absorbance detection at 232 nm. Fractions consisting of different oligosaccharides were concentrated by rotary evaporation. The fractions were desalted by GPC on a Bio-Gel P-2 column, concentrated again and lyophilized.

The size of C3 oligosaccharide components was analyzed [14]. The dialysed C3 was lyophilized, β-eliminated using mild conditions, and then dialysed against water using a 1000-Da molecular mass cut off (MMCO) membrane. The resulting components were loaded onto a strong anion-exchange resin and the resin was washed sequentially with water, 3% (w/v) NaCl and 16% (w/v) NaCl. The 16% NaCl fraction was dialysed against water using a 1000-Da MMCO membrane. The oligosaccharide components were analyzed on a 12% (w/v) acrylamide gel together with a disaccharide standard, a tetrasaccharide standard, and a mixture of heparin oligosaccharide standards. The gel was visualized with Alcian Yellow staining followed by silver staining.

2.4. Radioactive labeling

Tritium labeling of the C3 was performed in the Heparin Research Laboratory, College of Pharmacy, University of Iowa. C3 (5 mg) was weighed out in a Reacti-vial (Pierce-Rockford, IL) and [^{3}H] $NaBH_4$ (5.5 mCi) was added. The reaction was incubated in a water bath maintained at 40 °C for 24 h. Excess of $NaBH_4$ plus an additional 5.5 mCi of [^{3}H] $NaBH_4$ was then added to the reaction mixture to drive the reaction to completion. The reaction mixture was once again incubated in a water bath (40 °C) for 24 h. The reaction was finally quenched with 10% acetone (500 μl) incorporating excess hydride and tritide into acetone to form isopropanol. The reaction mixture was passed over a

strong anion-exchange column. The column was washed with water, methanol, and 3% NaCl. The bound C3 was released by a 16% NaCl wash and precipitated using methanol (at a final concentration of 80%). The precipitate was centrifuged and washed with ethanol. Ethanol was removed and the precipitate was dissolved in water. This solution was dialyzed against double-distilled H_2O through a 500 MMCO membrane for 2 days with frequent changes of water. The dialysate was added to a preweighed vial and the sample was counted on a Beckman Scintillation Counter. The sample was rotary-evaporated to dryness and the vial was weighed again to determine the approximate dry weight. The specificity of radiolabeled C3 was found to be 0.83 μCi/mg.

2.5. Activated partial thromboplastin time

The anticoagulant properties of C3 components from PAGE analysis were characterized by APTT, Heptest, and amidolytic anti-Xa assays in the NHP.

The APTT is a global clotting assay, which is used to measure inhibition of coagulation factors in the intrinsic pathway and is commonly used to monitor heparin therapy. The assay was performed in the following manner. One hundred microliters (100 μl) of sample was added to 100 μl of APTT reagent (Organon Teknika, Parsippany, NJ) and was incubated for 5 min at 37 °C. Clotting time was measured with a Fibrometer (BBL, Cockeysvile, MD) after the addition of 100 μl of prewarmed 0.025 M $CaCl_2$. Measurement of clotting time was stopped at 300 s as clotting time beyond 300 s was outside the linear range of the instrument.

2.6. Heptest

The Heptest (Haemochem, St. Louis, MO) is an assay used to measure clotting time after the addition of purified bovine factor Xa. The assay was performed in the following manner. One hundred microliters (100 μl) of sample was added to 100 μl of factor Xa and was incubated for 2 min at 37 °C. Clotting time was measured with a Fibrometer (BBL) after the addition of 100 μl of prewarmed Recalmix.

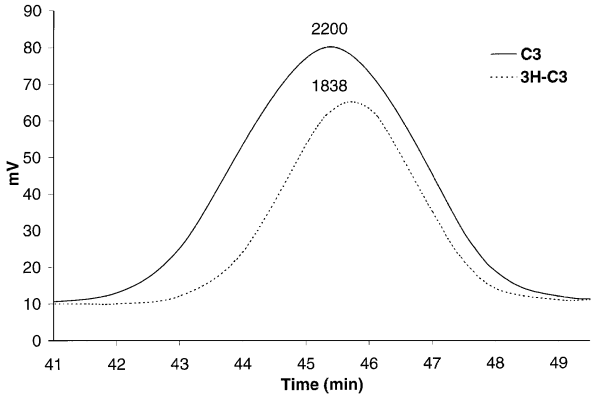

Fig. 1. MW determination of C3 and ^{3}H-C3 using GPC: RI detector. The MW of C3 and ^{3}H-C3 was determined by NRC method.

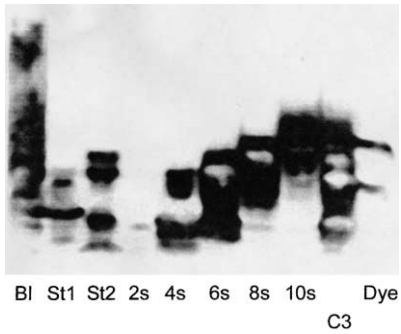

Fig. 2. The separation and purity determination of various oligosaccharide components of C3 by PAGE. The samples were subjected to PAGE (12%, w/v, acrylamide) and stained with Alcian Yellow followed by silver staining. The lanes are as follows: (Bl) MW marker; (St1) disaccharide standard; (St2) tetrasaccharide standard; (2s) disaccharide; (4s) tetrasaccharide; (6s) hexasaccharide; (8s) octasaccharide; (10s) decasaccharide.

2.7. Amidolytic anti-factor Xa assay

Anti-factor Xa activity of the samples was quantitated using a Beckman DU-7 spectrophotometer. Twenty-five microliters (25 μl) of the samples were incubated in 375 μl Tris buffer for 1 min at 37 °C, followed by an addition of 50 μl of bovine factor Xa (Enzyme Research Laboratories, South Bend, IN). Following a 2-min incubation at 37 °C, 50 μl of Spectrozyme factor Xa (2.5 mM, American Diagnostica, Greenwich, CT) was added and the change in optical density at 405 nm was measured for 1 min. Percentage inhibition was determined in relation to baseline using the following formula:

$$\%I = [(\Delta\text{Optical Density}_{\text{baseline}} - \Delta\text{Optical Density}_{\text{sample}}) / \Delta\text{Optical Density}_{\text{baseline}}] \times 100$$

2.8. Statistical analysis

GraphPad Prism version 3.00 for Windows (Graphpad Software, San Diego, CA) was used for statistical analysis.

Table 1
C3 components determined by PAGE analysis

C3 components	Percentage (%)
Disaccharide	≈13.6
Tetrasaccharide	≈16.4
Hexasaccharide	≈34.8
Octasaccharide	≈20.5
Decasaccharide	≈14.1
>Decasaccharide	≈0.2

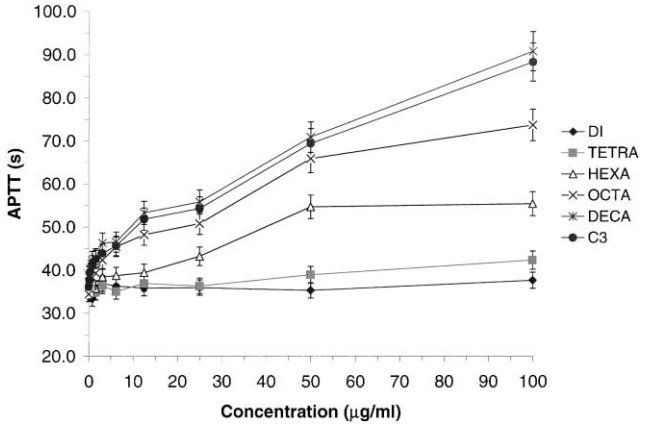

Fig. 3. APTT activity of C3 and its components following supplementation to NHP. The concentrations of C3 and its components represent the final concentration corrected for volume in each assay system. All data are mean observations from three individual experiments, each performed in duplicate, and standard deviation (mean ± S.D.).

All the data are represented as a mean ± standard deviation (S.D.), and appropriate analysis such as one-way ANOVA was performed to determine the statistical significance (declared at a P value of $< .05$).

3. Results

3.1. MW determination of C3

The MW of C3 was determined by GPC using NRC of the columns. The elution profile of C3 is depicted in Fig. 1. The agent was detected in the mobile phase using UV and RI detectors. The results obtained with the UV and RI detectors are 2.23 and 2.29 kDa, respectively. The average MW of C3 was determined to be 2.26 ± 0.02 kDa ($n = 4$) in this analysis. Dispersity, a measure of the homogeneity of C3, was less than 1.5 (1.207 in UV and 1.129 in RI). The slicing data indicated that major components of C3 (55%) had a MW range of 1.30–2.20 kDa, representing hexasaccharide and octasaccharide structures.

3.2. PAGE analysis of C3

Mild β-elimination was used to obtain the intact oligosaccharide components from C3 [14]. The size of each components was analyzed by PAGE (Fig. 2) after β-elimination. The oligosaccharide size was compared with a heparin disaccharide, a heparin tetrasaccharide, and a mixture of heparin oligosaccharide standards. The gel was stained with Alcian Yellow followed by silver staining and scanning. Based on the standards, disaccharide, tetrasaccharide, hexasaccharide, octasaccharide, decasaccharide, and higher saccharide components were identified (Table 1).

3.3. Bioequivalence of radiolabeled C3

Radiolabeling of C3 with tritium did not change the MW profile of the compound significantly, except for a slight decrease of MW to 1.83 ± 0.02 kDa and a minor loss of oligosaccharide components with high MW (Fig. 1). The anti-factor Xa activity between the non-labeled and labeled C3 was measured at 25 μg/ml supplementation in NHP, resulting in 98.8 ± 0.2% and 94.7 ± 0.4% factor Xa inhibition, respectively. No significant difference was observed.

3.4. APTT activity

APTT was a sensitive assay of the anticoagulant effects of C3 and its components as observed in Fig. 3. A statistically significant difference among agents was observed (one-way ANOVA, $P < .05$). Neither disaccharide nor tetrasaccharide exhibited anticoagulant effects in this assay. Using the concentration, which brought a doubled baseline clotting time as an index of potency ($Conc_{2B}$), C3, octasaccharide and decasaccharide were more potent than hexasaccharide as presented in Table 2.

3.5. Heptest assay

In the Heptest assay, only hexasaccharide, octasaccharide, and decasaccharide exhibited concentration-dependent anticoagulant effects. Concentration–response curves are shown in Fig. 4. As with the APTT, no anticoagulant effects were observed for disaccharide and tetrasaccharide. C3 and decasaccharide were sixfold more potent than hexasaccharide (0.8 ± 0.2 vs. 4.5 ± 1.2 mcg/ml, Table 2).

3.6. Anti-factor Xa activity

Factor Xa inhibition by C3 and its components was assessed following supplementation of each agent to NHP. An amidolytic assay using a specific substrate was utilized

Table 2
Comparative potency of C3 and its components in APTT and Heptest assays

Reagent	Concentration doubling baseline ($Conc_{2B}$ μg/ml)	
	APTT	Heptest
Disaccharide	>100	>100
Tetrasaccharide	>100	>100
Hexasaccharide	>100	4.5 ± 1.2
Octasaccharide	58.9 ± 4.0*	1.2 ± 0.4*
Decasaccharide	55.2 ± 3.4*	0.7 ± 0.2**
C3	54.5 ± 4.2*	0.8 ± 0.2**

$Conc_{2B}$ represents the concentration required for doubling the baseline in APTT and Heptest assays.

* $P < .05$, compared with hexasaccharide.

** $P < .01$ compared with hexasaccharide.

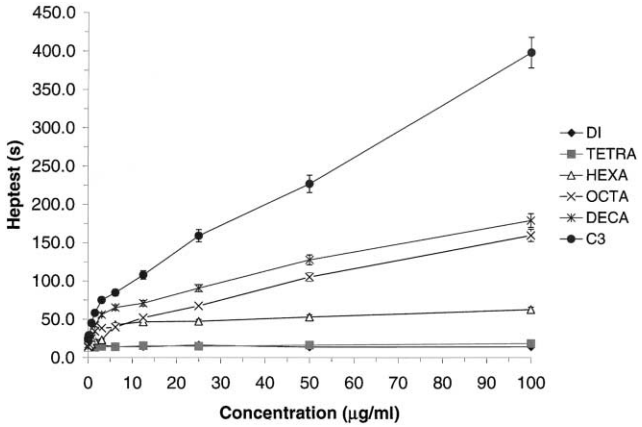

Fig. 4. Anticoagulant effect of C3 and its components in NHP measured by a global clotting test of Heptest. All data are mean observations from three individual experiments, each performed in duplicate, and standard deviation (mean ± S.D.).

such that enzyme inhibition was determined by measuring changes in optical density of the sample. Percentage inhibition was calculated relative to unsupplemented NHP (Fig. 5). IC_{50} values were determined by performing linear regression on the straightline portion of each individual concentration versus percent inhibition curve. A correlation coefficient greater than .92 was obtained for each regression line. Potencies of C3 and its components are compared in Table 3.

C3 and oligosaccharides larger than a hexasaccharide inhibited factor Xa amidolytic activity in a concentration-dependent manner. This is depicted in Fig. 5. However, the inhibition of factor Xa by hexasaccharide was observed to be significantly weaker than that for octasaccharide, decasaccharide, and C3 (one-way ANOVA, $P < .01$). The IC_{50} value for hexasaccharide was twentyfold

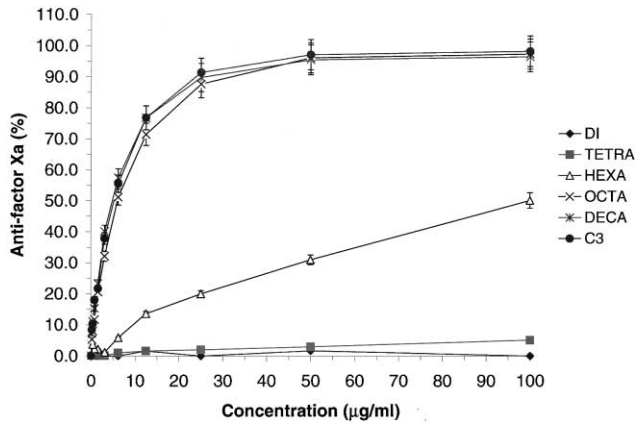

Fig. 5. Anti-factor Xa activity of C3 and its components in NHP measured by an amydolytic assay. All data are mean observations from three individual experiments, each performed in duplicate, and standard deviation (mean ± S.D.).

Table 3
Comparative potency of C3 and its components in anti-factor Xa assay

Reagent	E_{max} (%)	EC_{50} (μg/ml)
Disaccharide	1.7 ± 0.2	>100
Tetrasaccharide	5.1 ± 0.9	>100
Hexasaccharide	50.1 ± 3.9	102.7 ± 1.5
Octasaccharide	97.3 ± 8.9*	6.2 ± 2.4**
Decasaccharide	96.4 ± 7.4*	4.3 ± 1.2**
C3	98.2 ± 8.2*	5.0 ± 2.1**

E_{max} represents the percent inhibition of factor Xa measured by the chromagenic assay at 100 mg/ml of each reagent.
EC_{50} represents the effective concentration at 50% inhibition of factor Xa compared with the saline control.
* $P < .05$, compared with hexasaccharide.
** $P < .01$, compared with hexasaccharide.

higher than that for C3 (102.7 ± 2.5 vs. 5.0 ± 2.1 mcg/ml, Table 3).

4. Discussion

Heparins are polymeric agents consisting of sulfated polysaccharide chains with broad molecular size dispersion [16]. The MW of these chains ranges from 3 to 25 kDa [12]. The measurement of MW of heparin preparations by GPC requires that heparin-like molecules be employed for the calibration of columns. In our previous studies, 19 oligosaccharide fractions isolated from native heparin were used to generate a log MW versus retention time calibration curve [13]. Each of these fractions has a narrow MW distribution with dispersities less than 1.1, making it applicable for the MW determination of heparins [13].

The GPC profile of C3 reveals that this compound is a depolymerized mixture of heparin oligosaccharides with an average MW of 2.26 ± 0.02 kDa ($n = 4$). The oligosaccharides contain four to eight dextrose units, with hexasaccharide and octasaccharide dominating in C3. Since BBB accessibility of a substance is directly related to its MW [17], the relatively small MW of C3 suggests that it may penetrate the BBB and distribute in the brain and cerebral spinal fluid.

Various chemical and biochemical methods have been developed for the characterization of oligosaccharide components in heparins, such as PAGE [14] and mass spectroscopy [18]. The clear description of molecular composition is of great help to elucidate the biological activities of heparins, e.g., the identification of the specific antithrombin III (AT-III) binding sequence in heparin has led to the development of a synthetic anticoagulant, pentasaccharide [19].

The oligosaccharide composition of C3 is revealed by PAGE analysis. Multiple oligosaccharides are included in C3, which may predict the multipharmacological effects of this compound as with UFH. Although the molecular mechanisms of purified heparin oligosaccharides are still not fully clear, their pharmacological effects are mostly

involved in anti-inflammation and more predictable anticoagulation [20].

Compared with UFH (average MW 10,000–15,000 Da), C3 has a much lower MW. Accordingly, the anticoagulant effect of C3 is much weaker than that of UFH (about 1/6–1/7 of UFH). This observation is consistent with the fact that the anticoagulant effects of heparins are closely associated with their MW. Commercially available low-molecular-weight heparins (LMWHs) are less potent than UFH in terms of anticoagulation [20]. Based on the present findings, therefore, the effects of C3 on the coagulation cascade may not be a concern for the further studies and clinical trials.

An apparent MW dependence was noted in the biochemical analysis of C3 components. Since the minimum structure required for the antiprotease effects of heparin is the pentasaccharide sequence [19], it is the hexasaccharide, octasaccharide, and decasaccharide components in C3 that contribute to the anti-factor Xa activity of this compound. Although C3 and its components showed weak anticoagulant effects through the intrinsic pathway according to APTT and Heptest results, it is of interest to study the role of these effects in the treatment of AD. In addition, the functional components of C3 in the central nervous system warrant further elucidation.

GAGs, including UFH, LMWHs, and HDO have been labeled with radioisotopes, such as deuterium and tritium, and fluorescein for molecular profiling and further biological studies, including measurement of their pharmacokinetic behavior [15,21,22]. Since the ideal labeling of GAGs should have high detectable activity without interference of structure and components [23], tritium is usually employed for the radiolabeling.

Radiolabeled heparin oligosaccharides have been widely used to study the human pharmacokinetics and metabolism of exogenously administered GAGs [24,25]. Tritium-labeled C3 exhibits similar MW profiles and biological activity as unlabeled C3, although the labeling distribution is still unknown. Radiolabeling of C3 may provide a useful approach for detection and quantitation of this agent in biologic fluids. In addition, an elucidation of the distribution of radiolabeled oligosaccharide components of C3 may provide more information on their functions in the treatment of animal models of AD and their transport through the BBB.

In conclusion, the chemical and biochemical profiles of C3 indicate that this HDO is a multicomponent oligosaccharide mixture with multiple components but narrow MW distribution and low anticoagulation effects. Since C3 is designed for the treatment of AD and other neurological disorders and has proven efficacy in animal models, the present study suggests that the anticoagulant effects of HDO may not primarily contribute to their functions at neuronal levels. However, various oligosaccharide components of C3 warrant further isolation and investigation to elucidate their functions and underlying mechanisms.

Acknowledgments

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