Journal of Labelled Compounds and Radiopharmaceuticals—Vol. XXVIII, No. 5

[^{14}C] ACETYLATION OF A GLYCOSAMINOGLYCAN SULPHATE: SULODEXIDE

A. BRECCIA, E. GATTAVECCHIA AND A. FINI*,
Istituto di Scienze Chimiche
Via S.Donato 15, 40127 Bologna

E. MARCHI, G.F. TAMAGNONE
Alfa Wasserman S.p.A,
Via Ragazzi del '99, 5 , 40133 Bologna

SUMMARY

The synthesis of [^{14}C] labelled Sulodexide is reported. Sulodexide is a sulphated polysaccharide of the class of glycosaminoglycan, containing a heparin like fraction (70%), dermatan sulphate (20%) and other minor fractions. The heparin-like fraction, suitably isolated from other components, was partially and selectively N-desulphated, thus making few percent unit -NH_2 groups available for the labelling with [^{14}C]-acetic anhydride (specific activity 0.15 μCi/mg). Due to the small extent of modification, the sulphate to carboxylate group ratio remained practically unchanged on the heparin-like fraction. Sulodexide was reconstituted adding to the labelled fraction the suitable amount of the other components; the chemical and biological properties of the final labelled Sulodexide were undistinguishable from those of the starting material.

Key words: N-desulphation, N-[^{14}C] acetylation, heparin-like fraction, sulodexide.

*) to whom correspondence should be addressed.

0362–4803/90/050599–10$05.00

Received October 16, 1989

INTRODUCTION

Sulodexide is a sulphated polysaccharide belonging to the class of glycosaminoglycan (GAG), widely distributed in animal tissues. Sulodexide, mainly extracted from intestinal mucosa, has a constant composition: a heparin-like fraction (HLF), with a mean molecular weight of 6-8000 D and moving as a single spot in electrophoresis [1], is the principal component and accounts for more than 70% by weight; dermatan sulphate (DS) represents about 20%; the remaining parts consist of condroitin sulphates (CS) and other slow moving heparin-like fractions.

Sulodexide is the active constituent of Vessel^{R}, a pharmaceutical compound widely used for the treatment of peripheral vascular diseases in atherosclerosis and diabetes [2, 3].

Since GAG are endogenous, many studies on exogeneous GAG absorption have been facilitated by the use of radiolabelled materials, above all ^{3}H [4] and ^{35}S [5].

However, ^{35}S label does not always appear to be the most suitable one, because of the metabolic instability of sulphate groups, which are easily cleaved by ubiquitous sulphatases [6], while tritiated substrates are subject to isotope exchange.

With these sort of labels, the substrate could remain almost chemically unchanged with respect to the starting material, while with ^{131}I labelling, the substrate undergoes slight structure modification [7,8]. Despite its useful applications, to our knowledge, [^{14}C] labelling has not yet been applied till now in pharmacokinetic studies of GAG.

In this work we report the preparation of [^{14}C]-labelled Sulodexide through [^{14}C] acetylation of its fast moving fraction (HLF) properly isolated from the whole sulodexide

EXPERIMENTAL PART

Materials

Sulodexide (Alfa 8639) was a commercial product of standard composition according to an ALFA-WASSERMANN protocol, whose physical and chemical properties are reported below.

[1-^{14}C] Acetic anhydride was a commercial sample, delivered by Amersham (UK). at 104μCi/mmoles of specific activity. Solvents were of analytical grade; toluene was distilled over metallic sodium just prior to use.

Methods

Electrophoretic analyses were carried out as described [1], using an LKB 2197 power supply. The spots were detected through Alcian blue treatment [9]; when necessary the composition of the mixtures were determined on electropherograms with an ACD 18 Gelman Instrument automatic computing densitometer. The mean charge density was expressed in terms of the ratio between the sulphate and the carboxylate groups after potentiometric titration [10].

The ^{13}C NMR spectra were determined in D_2O solution with a Bruker Spectrophotometer CXP 300 operating at 75 MHz. The ^{13}C NMR resonances are reported in ppm, with TMS as reference.

Chemical properties of Sulodexide ALFA 8639 (I)

The composition of Sulodexide ALFA 8639 (I) was determined by quantitative densitometric evaluation of electropherograms on cellulose acetate (0.1 M barium acetate, adjusted to pH 5.8 with acetic acid) as follows: HLF 77%; DS 18%; slow moving heparin like fractions 4%; CS 1% .

The ^{13}C NMR spectrum of (I) showed peaks of HLF and DS, the relative intensities being consistent with the electrophoretic data: 177.2; 105.7*; 104.6*; 101.6; 99.5; 82.9*; 78.7; 78.2;

77.1; 73.7*; 72.2; 71.8; 71.2; 69.0; 63.5*; 62.5; 60.5; 54.5*; 25.2 in units of d (ppm downfield from TMS).

The signals attributed to DS are indicated by an asterisk; the signals of DS are masked by those of HLF in the 177, 77-79 and 71-72 regions. For HLF and DS resonances see references [11] and [12].

Supplementary data: S 8.5%; SO_4^{2+}/COO^- 1.7; $-NH_2$ absent.

The antithrombotic activity was determined according to the method of Reyers et al. [13, 14] and was expressed in term of ED_{50} value preventing thrombus formation.

Isolation of DS (II) from Sulodexide (I)

A solution of Sulodexide (8.4 g) in water (60 ml) was slowly added, at 10 °C, to a solution of calcium acetate (0.20 moles) in water (640 ml), containing acetic acid (0.32 mol) and ethanol (210 ml). After 15 hours at 10°C the suspension was filtered to give a white solid (1.4 g), a crude calcium salt containing 88% DS and 12% slow moving heparin-like fraction, as determined by electrophoresis analysis. The solid was dissolved in water (25 ml) and filtered through a column of Dowex 50w8 Na^+ (35 ml). Percolate and washings (50 ml) were collected treated with NaCl (1 g) and cooled to 5°C. Ethanol (40 ml) was added and the suspension filtered after 3 h at 5°C. Further ethanol (60 ml) was added to the supernatant and the suspension kept overnight at room temperature. Filtration afforded 1.2 g of DS moving as a single spot in electrophoresis.[8]

^{13}C NMR resonances: 177.7; 176.4; 105.7; 104.6; 82.9; 78.7; 77.1; 73.7; 71.9; 63.5; 54.9; 24.5.

S 6.8%. SO_4^{2-}/COO^- 1.0; $-NH_2$ absent.

Isolation of HLF (III)

The filtrate from DS calcium salt precipitation was

neutralized with NaOH 1N and treated with ethanol (300 ml). After 6 h at 5°C the suspension obtained was filtered to afford a white solid (7.1 g), which was washed with ethanol and dissolved in water (70 ml). The solution was stirred with Dowex 50 w8 Na^+ and the slurry poured onto a column containing 250 ml of the same resin. Percolate and washings (180 ml) were collected, added to sodium acetate trihydrate (4.9 g) and slowly treated with successive 18 ml aliquots of ethanol. After adding 90 ml ethanol an oily material started to precipitate and was separated by centrifugation. Precipitation was continued until the total volume was 270 ml. Bulk separation afforded HLF (6.2 g), moving as a single spot on electrophoresis.

^{13}C NMR resonances: 177.3; 104.4; 101.6; 99.4; 80.0; 78.9; 78.5; 78.0; 72.2; 71.7; 71.2; 69.0; 62.5; 60.5 ppm.

S 8.9%; SO_4^{2-}/COO^- 1.8; $-NH_2$ absent.

Partial and selective N-desulphation of HLF

A solution of HLF (3.6 g) in 0.4N HCl (50 ml) was heated for 75 min at 55°C. The reaction mixture was cooled and treated with 2N NaOH at pH 7.5. Precipitation with ethanol (120 ml) afforded IV, moving as a single spot on electrophoresis. The ^{13}C NMR spectrum was substantially identical to that of the starting material a small additional peak was evident at 57.4 ppm ($-NH_2$).

S 8.9%; ; SO_4^{2-}/COO^- 1.7; $-NH_2$ 6%.

Reaction of IV with [1-^{14}C] Acetic Anhydride

IV (2.5 g) was dissolved under stirring in 0.2 M solution of sodium acetate (4 ml).

[^{14}C] Acetic anhydride was dissolved in toluene (10 ml), at an amount equivalent to 500 μCi.

The two solutions were mixed and allowed to react under

stirring at 4°C. After 4 hours only 4 μCi of radioactivity were recovered in the organic phase. The aqueous layer was washed twice with ethyl acetate and poured dropwise into 100 ml ethanol under stirring. The labelled solid V was filtered and washed with absolute ethanol and ethyl ether and then stored under vacuum.

Yield 2.2g (88%): specific activity 0.15μCi/mg.

Thin layer radiochromatography revealed that radioactivity was associated to only one spot. Column chromatographic analysis (see below) showed that distribution of radioactivity paralleled that of the molecular weight and that the presence of a low molecular weight fraction was less than 10% (in terms of radioactivity).

In the ^{13}C NMR spectrum the weak resonances already present in compound IV at 56.5 ppm (>$\underline{C}$HNHCOCH$_3$) and at 24.8 ppm (>CHNHCO$\underline{C}$H$_3$) were more evident; the peak at 57.4 ppm (>$\underline{C}$HNH$_2$) completely disappeared.

S 8.8%; SO_4^{2-}/COO^- 1.7; $-NH_2$ absent. Antithrombotic activity: ED_{50}: 0.5 mg/Kg (e.v., rat); 24.5 mg/Kg (os)

Molecular weight determination

A chromatography column K16-100 (Pharmacia) packed with extrafine Biogel-P100 (Bio-Rad) was used. The column was eluted with a solution of 0.5 M NaCl containing 4 micrograms/ml of sodium mertiolate. A solution of the product V (5 mg) in the eluent solution (0.5 ml) was percolated onto the column and 2.5 ml fractions were collected at a flow rate of 10 ml/h. Blue dextrane 2000 (Pharmacia) was used for the determination of Vo. The fractionation range (M.W.) of the column was 15000-1300. The concentration of each fraction was determined either with uronic acid assay of Dische, as modified by Bitter and Muir [15] or by ß counting.

Reaction of IV with unlabelled acetic anhydride

Acetylation with Ac_2O in the above conditions afforded compound Va, with chemical and biological properties indistinguishable from the starting compound III.

Labelled Sulodexide VI

Compound V (0.80 g) and compound II (0.20 g) were dissolved in water (10 ml) and the solution freeze-dried. Compound composition as determined by densitometric evaluation of electropherograms was as follows: compound V 79%; compound II 21%; S 8.4%; SO_4^{2-}/COO^- 1.7; $-NH_2$ absent. The NMR spectrum was identical to that of the unlabelled sulodexide I.

DISCUSSION

As a rule, the method of synthesis for pharmacokinetic studies must be based upon mild and easily reproducible reaction conditions so that the physical chemical and biological properties of each batch of labelled compound are constantly identical to those of the unlabelled one. The labelling procedure implies either the use of a labelled reagent at some step of the synthesis or a suitable modification of the starting compound to allow entry of a labelled moiety. Labelling can also be obtained by introducing moieties unrelated to the molecule, thus altering the original structure. This is equally suitable, provided that the molecule is large enough to make the change negligible (e.g. a protein).

When a polymeric compound of natural origin, such as sulodexide is considered, one is faced with the practical problem of selecting a procedure offering the best compromise between the most uniform labelling with the least modifications to the overall chemical structure. In this work the problem was solved by:

a) isolating the principal and the secondary components of sulodexide (HLF and DS, respectively)

b) labelling HLF through a limited and selective reaction of N-desulphation, followed by acetylation of the free amino groups thus formed;

c) reconstituting sulodexide by adding the due amount of isolated DM to the [^{14}C] labelled HLF.

This method circumvented the major drawback arising with acetylation of the desulphated sulodexide, i.e. modified chemical structure of the secondary component (DS) due to extensive depolymerization.

The labelled HLF contained acetyl and sulphate groups still in the ranges typical of heparin-like molecules (1-10%) [16]. Neither O-desulphation, at the 2-iduronic and the 6-glucosamine position nor depolymerization occurred in the mild reaction conditions applied. The position and intensity of NMR peaks were unaltered and the distribution of the molecular weight curve of the [^{14}C] labelled compound, as determined by gel column chromatography, was the same as that of the unlabelled one. The biological activities in the in vitro anticoagulant and in vivo antithrombotic tests were comparable to those of the starting material. Apparently the sequences of the molecule responsible for these activities are not significantly modified by the limited substitution of the N-sulphate by the N-acetyl groups.

The fact that HLF is not the sole component and that the DS fraction plays a significant role in the overall biological mechanism of sulodexide action [17, 18] cannot be neglected. However, in our opinion, the pharmacokinetics of a natural product with a complex structure, like sulodexide can only be elucidated through a stepwise procedure. Hence we aimed to prepare sulodexide containing [^{14}C] labelled HLF and unlabelled

DS in order to carry out the first step of the pharmacokinetic study. This could be followed by the pharmacokinetic study of sulodexide containing unlabelled HLF and labelled DS. Finally, the use of sulodexide containing both [^{14}C] labelled HLF and DS could reveal the effect of the possible interaction between the above components upon the pharmacokinetics of the whole product.

Ackowledgements. This work was in part supported by a Ministry of Education Fund. We thank Dr.A.Minarini for technical assistance.

References

1. Oreste P. and Torri G. - J.Chrom. 195: 398 (1980)

2. Pisano L., Moronesi F., Falco F., Stipa E., Fabbiani N., Dolfi R., Conoscenti E., Gloria R. and Pepe R. - Throm.Res. 41:23 (1986)

3. Arsenio L. and Strata A. - Clin.Trial J. 24:312 (1987)

4. Sbauderge J.N., Gruhl M., Kitani T., Ambegoonkar S., Kambayashi J., Nakagawa M. and Lenter D. - Throm.Res. 13: 767 (1978)

5. De Swart C.A.M., Sixma J.J., Andersson L.O., Holmer E., Verschoor L., Niymeyer A. - Scand.J.Haematol.(Suppl.) 36:50 (1980)

6. Effeney D.J., Case S.A., Graziano C, Blaisdell W.F. - Surg.Forum 30:1980 (1979)

7. Davies J., Pepper D.S. - Thromb.Res. 14:845 (1979)

8. Dol F., Honin G., Dupony D., Cadray Y., Caranabe C., Gabaig A.M., Mardiguian J., Sie' P. and Boneu B. - Thromb.Haemost. 59:255 (1988)

9. Newton D.J., Scott J.E. and Whiterman P. - Anal.Biochem. 62:268 (1974)

10. Mascellani G., Rasconi A., Brugnoli E., Bianchini P. - Il Farmaco (Ed.Pr.) 43:161 (1988)

11. Gatti G., Casu B., Hamer G.K. and Perlin A.S. - Macromolecules 12:1001 (1979)

12. Hamer G.K. and Perlin A.S. - Carb.Chem. 49:37 (1976)

13. Reyers S., Mussoni L., Donati M.R., De Gaetano G. - Thromb. Res. 18:699 (1980)

14. Bitter T. and Muir H.M. - Anal.Biochem. 4:330 (1962)

15. Casu B. - Advances Carb.Chem.Biochem. 43:51 (1985)

16. Merton R.E., Thomas D.P. - Thromb.Haemost. 58:839 (1987)

17. Ofosu F.A., Fernandez F., Gauthier D. and Buchanan M.R. - Seminar Thromb.Haemost. 11:133 (1985)