NOTE

Synthesis of tritiated Lomoparan^R (Org 10172)

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Keywords: glycosaminoglycans, acetylation, reductive tritiation.

Introduction

Lomoparan^R (Org 10172) is a highly effective anti-thrombotic drug with low bleeding risks[1]. The product is a mixture of low molecular weight glycosaminoglycuronans obtained from mammalial intestinal mucus by treatment with proteolytic enzymes followed by precipitation[2]. It contains heparan sulphate, dermatan sulphate and chondroitin sulphate with a mean molecular weight of 5000-6000. About 4% (w/w) of the product - the H(igh) A(ffinity)-fraction - has high affinity for the natural inhibitor antihrombin III (AT III); the main fraction - L(ow) A(ffinity)-fraction - does not bind to AT III and consists for the main part of the heparan sulphate (≈75%), dermatan sulphate (~12%) and chondroitin sulphate (<9%).

For pharmacokinetic studies in animals radioactively labelled Lomoparan^R was needed. Recently Breccia et al[3] described their approach for the labelling of sulodexide, which is a mixture of glycosaminoglycuronans, chemically related to Lomoparan^R. They prepared the labelled material by reconstitution of the labelled subfractions, while labelling was achieved through N-desulphation and acetylation with ^{14}C-acetic anhydride.

Because of the rather heterogeneous nature of Org 10172 completely random labelling of all subfractions would be impossible and for this reason we have chosen in agreement with the approach of Breccia et al[3] for separate labelling of the subfractions and reconstitution. However since our method of labelling is on essential points different from that of Breccia et al[3] we present our results in this note.

Because of the biological origin of Org 10172, the product can only be labelled with radioactive isotopes by modification of the natural material. To select a proper method we applied the following criteria:

1. The labelled product should have a reasonable stability both in vitro and in vivo. In view of the reported metabolic in-vivo instability of [^{35}S-sulphate]-labelled glycosaminoglycans[7,8], ^{35}S labelling (through desulphation and resulphation with $^{35}SO_3$) was not tried.

0362-4803/91/020241-06$05.00
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Received September 24, 1990

2. The distribution of molecular weights, chemical composition and biological activity of Org 10172 and its fractions should not be affected by the labelling procedure.
3. Radioactivity in the labelled products should coincide with the mass peak of the unlabelled material in different chromatographic systems. The systems used included HPSEC (TSK-G3000; MW-indication), anion-exchange chromatography (Mono Q; sulphate-content) and in the case of the HA-fraction affinity-chromatography (AT III-silica gel; indication for biological activity).
   This is a very important criterion, because only low specific activities can be obtained (vide infra) and therefore fulfilment of criterion 2 is no guarantee for the quality of the small fraction of labelled material.
4. In the case of tritium-labelling the position of the signals in the ^{3}H-NMR should be in agreement with those of unlabelled Org 10172.

Experimental part

Materials and equipment

Subfractions of Org 10172 were prepared by the Biochemical R&D Labs of Diosynth BV by chromatography on AT III-MAAM silica gel.

AT III-MAAM silica gel was obtained from Diosynth BV. TSK-columns (G3000SW) were purchased from Chrompack, The Netherlands; Mono-Q HR5/5 and Sephadex G25 from Pharmacia, Sweden and Spectrapor 3 dialysis membrane tubing (cut-off 3500) from Hicol.

HPLC-purifications were performed using Waters 510 pumps which were connected in the case of mono Q chromatography with an automated gradient controller or a Model 660 solvent programmer. Mass peaks were detected with a Knauer Differential Refractometer or off-line by using the Azure A method according to Grant[4]; radioactivity was measured on-line with a Ramona-5-detector or a Berthold LB 503, or off-line with a Packard 4430. Electrophoresis was performed in agarose plus (Corning Universal, U.K.) using a LKB 2117 multiphor electrophoresis apparatus in 1 M $Ba(OAc)_2$, pH = 5,0; the gels were stained with toluidin blue.

NMR spectra (^{1}H and ^{3}H) were recorded with a Bruker AM360 spectrometer operating at 360,13 and 384,13 MHz respectively.

Deacetylation

This was carried out according to Shaklee and Conrad[4] in the following way. A mixture of 20 mg Org 10172, 10 mg hydrazine-sulphate and 1 ml hydrazine (100%) was heated at 100°C in a sealed tube for 10 minutes. The mixture was evaporated to dryness under a gentle stream of nitrogen, the residue was dissolved in water and dialysed against water for 16 hours. After freeze-drying the residue was dissolved in water and 0,5 ml of a solution of HIO_3 (44 mg/ml) was added. After thorough mixing for 30 seconds, the mixture was extracted with diethyl ether (3 x 0,5 ml) and the aqueous phase was purified by filtration over Sephadex G25. The high molecular weight fractions were pooled and concentrated by freeze-drying, yielding deacetylated material.

Reacetylation was achieved according to the method of Höök et al[13] by incubation of a solution of about 5 mg of the Org 10172 in 0,2 ml sodium carbonate (pH=10)/10% methanol with

[^{3}H]-acetic anhydride (solution in toluene; 25 µl=0,1 mg) under vigorous stirring. After one hour a second portion of acetic anhydride was added and after another 30 minutes Org 10172 was isolated by gel filtration on Sephadex G25 with water as eluent. Purification was identical to the procedures described for the [NaB^3H_4]-reduced products (see below).

Reductive Labelling

Reactions with NaB^3H_4 were carried out at Amersham International plc Cardiff, U.K. using their tritium labelling services.

Tritiation of HA-fraction:

HA-fraction (25 mg) was incubated for 3 hours at room temperature in 0,1 M aqueous Tris-HCl buffer (pH=8; 2 ml) with 1 Ci (37GBq) of NaB^3H_4 (specific activity 60-80 Ci/mmol (2,5 TBq/mmol). The pH was adjusted to 4 with acetic acid and the mixture was stirred for 30 minutes and the pH was readjusted to 8 with $NaHCO_3$. The resulting solution was lyophilized, dissolved in water and lyophilized again to remove labile tritium. The residues (≈ 30 mCi; 1000 GBq) were, after gel filtration on Sephadex G25 with water, purified by repetitive injections on a TSK-G3000SW column (60 cm x 7,5 mm) with 0,5 M aq. ammonium acetate (pH = 5,0). The high molecular weight fractions were collected, freeze-dried and dissolved in water yielding ≈ 5,0 mCi (185 MBq). This product was chromatographed in portions on AT III-MAAM-silica adsorbent (200 ml bed-volume; capacity 20 µg/ml bed). After washing with 200 ml 0,4 M NaCl/0,05 M NH_4OAc (pH = 7,5) the high affinity fraction was eluted from the column with 300 ml 2,0 M NaCl/0,05 M NH_4OAc (pH = 7,5). The 2M NaCl salt fractions containing radioactivity were pooled and desalted by dialysis in Spectrapor dialysis membrane tubing against water. After freeze-drying, the pure product (yields for two batches were 0,8 mCi (30 MBq) and 1,5 mCi (56 MBq) respectively) was stored in MilliQ water at 4°C. Anti-X_a activities were determined according to the method of Teien and Lie[6].

Tritiation of the LA-fraction.

LA-fraction (25 mg) was incubated at room temperature in 0,1M aq. Tris-HCl buffer (pH = 8,2 ml) with 1 Ci of NaB^3H_4 (specific activity 60 Ci/mmol; 2,2 TBq/mmol). After 3 hours the pH of the reaction mixture was adjusted to 4 with acetic acid and after 30 minutes stirring at room temperature the pH was adjusted to 8 with solid $NaHCO_3$. The resulting solution was lyophilized, dissolved in water and lyophilized again to remove labile tritium. The residues dissolved in distilled water were purified by HPLC by repetitive injections onto TSK G3000SW (60 cm x 7,5 mm) with 0,5M aqueous ammonium acetate (pH = 5,0) after a crude separation on Sephadex G25 in water. The high molecular weight fractions were collected, freeze-dried and dissolved in water yielding 6,3 mCi (233 MBq) and 3,6 mCi (133 MBq) respectively. These crude products were chromatographed in portions on Mono Q type HR5/5 using a gradient of 0 M to 2 M NaCl in 0,05 M aq. Na_2HPO_4, pH = 8,5. The fractions containing tritiated LA-fraction were pooled and desalted by dialysis against water in Spectropor dialysis membrane tubing (MW cut off 3500). After freeze-drying the compound (yield for two batches 2,2 mCi and 2,4 mCi respectively (81 MBq and 89 MBq)) was dissolved in water (Millipore-quality), filtered through cotton-wool and stored in the dark at 4°C.

Results and discussion

Since the modifications introduced by "exchange"-reactions are expected to be small, a number of these methods were applied, however, without success. Reaction with $^3H_2$ in the presence of Pd/C (method of Barlow[9]) resulted in chemical degradation (as checked with Org 10172). Reaction with $^3H_2$ in the presence of RaNi (method of Koch[10]) or reaction with ^3H_2O under alkaline conditions (epimerisation of iduronic acid and glucuronic acid residues) gave no incorporation of tritium while exchange with $^3H_2$ under microwave discharge conditions gave undefined products (as checked by ^{3}H-NMR-spectroscopy).

Since the number of free NH_2 groups in Org 10172 is low (about one per 50 chains) acetylation of the material either with tritiated acetic anhydride or with the N-hydroxysuccinimid ester of 4-OH-propionic acid followed by electrophilic iodination with ^{125}I resulted in products with low specific activity while the bulk of the material was associated with biologically inactive material (see Table I). The number of free NH_2-groups can be increased by N-desulphation or N-deacetylation. Since N-SO_3H groups are involved and essential in the biological activity [11] we selected - in contrast to the approach chosen by Breccia et al. [3] - N-deacetylation. Treatment of the HA-fraction with hydrazine at 100°C for 10 minutes followed by a short treatment with HIO_3 gave no degradation of the material as checked by size exclusion chromatography, measurement of the N-SO_3H, uronic acid content and biological activity (anti-X_a activity). After reaction of this material with excess ^{3}H-acetic anhydride [^{3}H-N-acetyl]- HA-fraction was obtained with between 0,1 and 0,5 labelled N-acetyl groups/chain.

Table I : ATIII-binding fraction of labelled HA-fraction (measured by chromatography on ATIII-MAAM-silica gel).

	before purification	after purification
[N-^{3}H-acetyl]-HA-fraction prepared by direct acetylation	4%	--
[N-^{3}H-acetyl]-HA-fraction prepared after N-deacetylation	42%	96%
[^{3}H]-HA-fraction prepared by reduction	40%	97,5%

As illustrated in Table I this material had to be purified extensively since 60% of the radioactivity was associated with biologically inactive material. Acetylation of this material with 4-hydroxypropionic acid followed by electrophilic iodination was also possible but the material obtained in this way had a slightly altered behaviour on anion exchange chromatography (longer retention time), indicative of unacceptable modification of the material.

A simpler method of labelling was reduction of end groups using tritiated NaB^3H_4 [12]. Using HA-fraction as model compound, labelling conditions were varied and maximum incorporation was observed after 1 hour reaction. A typical ^{3}H NMR spectrum (of the HA-fraction) is shown in

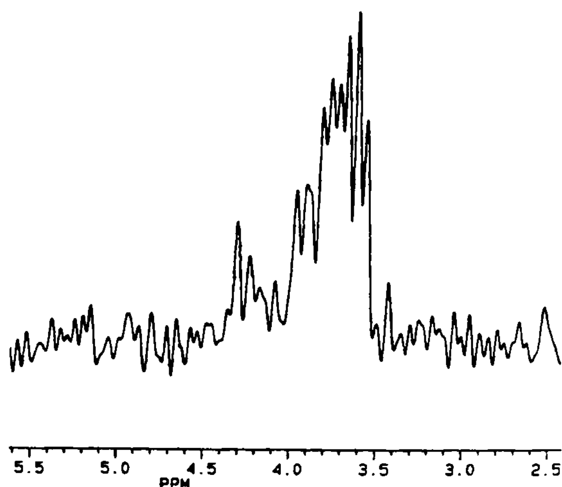
Figure 1. The ^{3}H-signals are in agreement with reduction of end groups (-CH^3HOH-groups) while the complexicity of the signals is indicative of the heterogeneous nature of the HA-fraction. The products obtained had to be purified by size exclusion chromatography (HPSEC) and thereafter either anion exchange chromotography (LA-fraction) or affinity chromatography (HA-fraction) (see Table II).

The amount of tritium incorporated varied for the two subfractions. For the HA-fraction one of every 10-20 chains could be labelled, in agreement with the results described for this method in the literature for other glycosaminoglycuronans[12] while for the LA-fraction a 10 times higher incorporation was observed. As a test for biological activity we included AT III binding of (^{3}H)-HA-fraction while for the LA-fraction an enzymatic degradation indicated that >90% of the labelled material was associated with heparan sulphate.

Table II Characteristics of fractions of Org 10172 prepared by NaB^3H_4 reduction.

	Spec.activity	^{3}H-NMR	Radiochemical purities		
			HPSEC	Anion exchange	ATIII
[^{3}H]-HA-fraction	0,2mCi/mg (2000α-X_AU/mCi)	3,5-4,3ppm	≥ 96%	≥ 98%	≥ 96%
[^{3}H]-LA-fraction	1-2,5mCi/mg	3,5-4,1ppm	≥ 96%	≥ 99%	--

Fig.1 : ^{1}H-decoupled ^{3}H-NMR spectrum of [^{3}H]-HA-fraction; solvent ^2H_2O.



As a conclusion it can be stated that tritiated Org 10172 can be prepared through labelling of its subfractions and reconstitution of the original material. Two methods were applied: partial

N-deacetylation with N_2H_4/HIO_3 followed by reacetylation with tritiated acetic anhydride and, more easily, reduction of end groups with NaB^3H_4. Elaborate purifications were needed to obtain material with properties identical to the unlabelled material.

Acknowledgement

The authors are grateful to Mr. J. Wallaart and Mr. A. van Rooy for their contributions in the initial stage of the study and to Mr. G. Wagenaars for the recording of the NMR-spectra. Dr. J. Dawes (Sidney, Australia) and Dr. L. Roden (Birmingham, U.S.) are acknowledged for their advice and contributions to iodination and deacetylation studies.

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