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Preliminary chemical, biochemical, and pharmacological characterization of a low molecular weight dermatan sulphate

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ABSTRACT

The aim of this study was to set up a depolymerization process which resulted in the formation of a low molecular weight dermatan sulphate (LMWDS), retaining the chemical properties possessed by native dermatan sulphate (DS), fundamental for the expression of its specific biological activity. The depolymerization of DS by a β elimination process led to the production of oligosaccharide chains having a 4,5 unsaturated uronic acid at the nonreducing end. The chemical evaluation has shown that the most important parameters (degree of sulphation, sulphate to carboxyl ratio, and specific rotation) have not undergone any particular modification compared to native DS. The biochemical results demonstrate that the LMWDS obtained retains most, if not all, of the specific biological activity. The reduction in molecular weight significantly enhanced the bioavailability of the product after subcutaneous administration.

INTRODUCTION

Dermatan sulphate (DS) is an endogenous glycosaminoglycan widely distributed in mammalian and other vertebrate tissue[1,2]. It is composed[3] of a linear, repeating sequence of disaccharide units 4-α-L-idopyranosyluronic acid-(1 → 3)-2-acetamido-2-deoxy-β-D-galactopyranosyl 4-sulphate)-(1 → (**1**). DS can inhibit experimental venous thrombosis without manifesting a marked anticoagulant activity [4–6]. It can also inhibit the extension of a pre-existing thrombus when administered by continuous infusion[7]. Its pharmacological profile is further strengthened by the fact that it is less haemorrhagic than either unfractionated heparin (UFH) or low molecular weight heparin (LMWH)[8]. Several clinical studies have been performed to investigate the efficacy of DS in preventing deep vein thrombosis after surgery[9,10], and filter blockage during hemodialysis[11,12]. Pharmacokinetic studies with DS following

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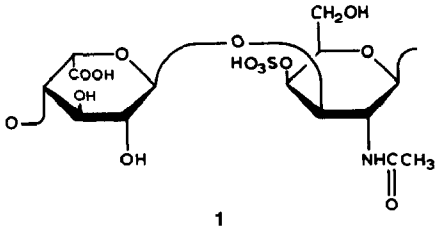
extravascular administration have highlighted its low bioavailability but high absorption; a steady state is achieved only after several days of treatment[9]. This characteristic probably depends on its high molecular mass 20 000–25 000 daltons. In order to improve the pharmacokinetics of DS, we have studied a new original method to depolymerize DS into oligosacharides, which retain the pharmacodynamic profile of native DS[15].

EXPERIMENTAL

Analytical methods.—Optical rotations were determined with a Perkin–Elmer model 241 polarimeter. Organic sulphur and sulphate-to-carboxyl ratios were determined by a potentiometric titration method using a Methrom model 636 Titroprocessor according to the method described by Volpi et al.[16]. The presence of any other glycosaminoglycan (GAG) in the DS preparation (considered as contaminants) was checked using an electrophoretic technique according to the method of Cappelletti et al.[17]. The UV absorption spectrum of the low molecular weight dermatan sulphate at a concentration of 0.02%, in 0.01 M HCl, was registered in the range 210 to 300 nm. Maximum absorption was observed at 230 nm. The value found was expressed as the absorbance of a solution containing 1 g/100 mL contained in a cell having an absorption path of 1 cm ($E^{1\%}_{1\ cm}$).

The molecular weight was determined by high performance size exclusion chromatography, using Waters HPLC equipment (pump 510, injector Rheodyne), a guard column TSK SW, TSK G 4000 SW and TSK G 2000 SW columns, assembled in series. The mobile phase was 150 mM sodium phosphate pH 6.4; flow rate 1 mL/min. The effluent was monitored with a refractive index detector (Waters model R 410), and a UV detector (Waters model 484). Number average (M_n), weight average (M_w), peak molecular mass (M_p) and polydispersity (M_w/M_n), were determined using a specific software program (Maxima 820 GPC). The calibration curve required for the program was prepared using standard dermatan sulphate fractions having known molecular weights, determined by means of dynamic light scattering.

Purification of dermatan sulphate.—A crude glycosaminogycan (GAG) extract from pig intestinal mucosa (a by-product during heparin production) was obtained from Laboratori Derivati Organici (Trino Vercellese, Italy). The crude extract was rich in dermatan, as determined by electrophoresis, with a low quantity of chondroitin and fast moving heparin. Chondroitin sulphate was completely eliminated by preparing a calcium salt of the GAGs contained in the extract, dissolving it in water, and precipitating it with a precise amount of acetone. The precipitate collected was composed of dermatan and traces of heparin. After transforming the calcium salt into the sodium salt the heparin fraction was eliminated with nitrous acid treatment according to the method of Lagunoff et al.[18]. The resulting product, dermatan sulphate (**1**) was submitted to electrophoresis to verify its purity, and to chemical analysis and biological evaluation.



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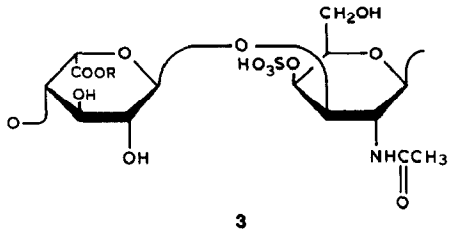

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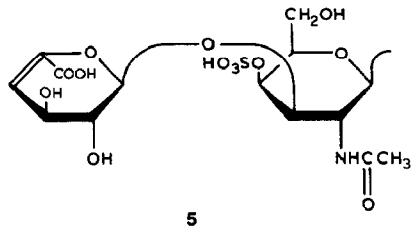
R = H (80–60%), Bn (20–40%)



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2, Benzethonium salt of DS
4, Crude low molecular weight DS

Benzethonium salt of DS.—Compound **1** (50 g) was dissolved in 500 mL of demineralized water and under strong stirring, 500 mL of a 25% solution of benzethonium chloride was added. The stirring was continued for 1 h and then the solution was kept at room temperature for a further 12 h. The resulting precipitate (**2**) was collected and washed several times with demineralized water and dried under vacuum at 40°C (yield, 115 g).

Esterification and depolymerization reactions.—100 g compound **2** (100 g) was dissolved in 500 mL of dimethylformamide (DMF). The temperature of the solution was brought to 35 ± 1°C, 15 mL of benzyl chloride was added under stirring, and the reaction was allowed to proceed for 120 min. Upon termination, 10 mL of solution was removed and treated with 20 mL of MeOH containing 10% sodium acetate, and the resulting precipitate was washed with MeOH and then dried under vacuum to give compound **3**. The degree of esterification of **3** was determined by potentiometric titration[16] of the acidic carboxyl groups compared to those of the starting compound **1**.

To the remaining solution, cooled to 25 ± 1°C, 20 mL of 40% benzyltrimethylammonium hydroxide in MeOH was added under stirring. The reaction was allowed to proceed for 2 h at 30 ± 1°C, then stopped by adding 750 mL of MeOH containing 10% (w/v) sodium acetate, under stirring. The resulting precipitate was collected by filtration, washed several times with MeOH, and then dried under vacuum at 50°C. This gave 36 g of crude low molecular weight dermatan sulphate sodium salt (**4**).

Purification of the low molecular weight dermatan sulphate.—The crude preparation (**4**), was dissolved in a 5% sodium carbonate solution and kept at 20°C for 5 h, then 2.5 vol of MeOH were added. The resulting precipitate was washed several times and dried at 50°C under vacuum. This yielded 27 g of pure low molecular weight dermatan sulphate (**5**).

Biochemical characterization.—The in vitro anticoagulant activity of compounds **1** and **5** was evaluated using a commercial kit, thrombin reagent (TT) (Boehringer Mannheim, Germany). Graded amounts of compounds **1** and **5** were added to the plasmas and the amount needed to triple the control values was determined.

The inhibitory activity of compounds **1** and **5** on thrombin was evaluated using a chromogenic assay. Briefly, 1 vol of plasma containing increasing amounts of low molecular weight dermatan sulphate was mixed with 1 vol of human thrombin (250 nM) and incubated at 37°C for 60 s. The residual thrombin amidolytic activity was determined by adding 1 vol of 1.2 mM of chromogenic peptide Chromozym TH (Boehringer, Mannheim, Germany), diluted in Tris–saline buffer, pH 8.4. The reaction was stopped 3 min later by adding 1 vol of acetic acid. Residual thrombin activity was then measured at 405 nm, and the concentration of low molecular weight dermatan sulphate required to inhibit 50% of the initial thrombin was calculated (IC 50).

A similar test was performed in a buffered system in which thrombin inhibition via heparin cofactor II was investigated. One volume of Tris–saline buffer containing increasing amounts of low molecular weight dermatan sulphate was added to an equal volume of the same buffer containing 0.15 μM of purified human heparin cofactor II (Diagnostica Stago, France) and incubated at 37°C for 60 s. One volume of 3 nM of human thrombin was then added and the resulting mixture incubated at 37°C for 60 s. After adding the chromogenic substrate, the assay and the subsequent calculation of the IC 50 were conducted as described above. The inhibitory activity of native DS and LMWDS on factor Xa, mediated by antithrombin III, was evaluated using a chromogenic assay[19].

Pharmacological activity.—Compounds **1** and **5** were screened pharmacologically in order to assess whether, there was a marked difference in absorption and, if so, whether their antithrombotic activities differed as a consequence. In order to highlight any differences in absorption, two separate doses (20 and 60 mg/kg) of each compound were administered subcutaneously to New Zealand white rabbits (Conelli, Arona, Italy). At fixed time intervals (0, 10, 20, 30, 40, 60, 80, 120, 200, 300, 480, and 1440 min) after administration blood was removed from the

marginal-ear vein. After centrifugation, a commercially available kit (Stachrom DS, Stago, France) was used to determine the amount of DS or LMWDS present in the plasma. For each compound a standard reference curve was constructed in vitro by adding known amounts of either DS or LMWDS to the plasma.

The antithrombotic activities of compounds **1** and **5** were determined in a rat model of venous thrombosis. In this model, thrombus formation is induced in the inferior vena cava by inserting a silk thread and applying a stasis for 15 min. DS and LMWDS (10 and 30 mg/kg) were administered subcutaneously 40, 60, and 90 min before inducing thrombus formation. The thrombus formed was removed, dried, and weighed. The results are expressed as percent inhibition. The levels of circulating DS and LMWDS were measured using a Stachrom DS kit.

RESULTS AND DISCUSSION

The method described to purify dermatan sulphate from chondroitin sulphate resulted in the complete elimination of chondroitin sulphate as evidenced by electrophoresis[17] (Chondroitin sulphate was below the detectable limit of 0.5%). The elimination of heparin was confirmed by determining the anti-Xa activity of DS, which was less than 0.3 U/mg. The pure dermatan sulphate **1** obtained underwent both chemical and biochemical analysis, the results of which are summarized in Tables I and II, respectively.

A novel and original procedure was set up to depolymerize dermatan sulphate in a nonaqueous medium in an attempt to prepare a more active product compared to those obtained using the classical depolymerization reactions conducted in aqueous medium. This process consists of a partial esterification of the carboxyl group of DS, followed by an alkaline cleavage in an organic solvent. The esterification of the carboxyl groups increases the acidity of the α-hydrogen atom, making it vulnerable to β-elimination by bases leading to the formation of oligosaccharides bearing 4,5-unsaturated uronic residues at the nonreducing end (see formula **5**).

TABLE I

Chemical characteristics of native and low molecular weight dermatan sulphates

Parameter	DS (1)	LMWDS (5)
M_w	21500	7500
M_p	22500	6500
M_n	19500	6600
M_w/M_n	1.11	1.13
Organic sulphur (%)	6.87	6.75
Sulphur/carboxyl ratio	1.17	1.20
Specific rotation (deg)	−68.5	−60.5
$E^{1\%}_{1cm}$ at 230 nm	–	8.5

TABLE II

Biochemical characteristics of native and low molecular weight dermatan sulphates

Property	DS (1)	LMWDS (5)
Thrombin time (μg/mL) [a]		
Rabbit plasma	14.2	20.0
Rat plasma	9.8	9.5
Anti IIa activity (μg/mL) [b]		
Rabbit plasma	9.0	10.5
Buffered system	0.68	0.54

[a] Thrombin time μg/mL required to triple (×3) control value. [b] Concentration giving 50% inhibition.

Esterification of the benzethonium salt of dermatan sulphate (**2**) was performed in DMF, using benzyl chloride as the esterifying agent. The experimental conditions for the preparation of the benzyl ester of DS (**3**) were optimized in order to achieve an appropriate balance between the degree of depolymerization and the retention of biological activity. Using the experimental conditions described, the degree of esterification in the dermatan sulphate ester **3** was ~ 30%. Depolymerization by alkaline cleavage was performed in situ using benzyltrimetylammonium hydroxide (40% in methanol), Other organic bases soluble in DMF may also be employed. The reaction was conducted at constant temperature (30 ± 1°C). The depolymerization reaction was stopped by adding methanol containing sodium acetate, in order to precipitate the oligosaccharides and at the same time obtain their sodium salts. The yield of crude low molecular weight dermatan sulphate **4** was 72% with respect to the starting amount of DS.

Due to the possibility that some of the carboxyl groups of the oligosaccharides contained in **4** may still be esterified, it was necessary to perform an alkaline hydrolysis in an aqueous medium with sodium carbonate at 20°C for 5 h. This treatment did not modify the molecular weight and resulted in a 50% yield of pure low molecular weight dermatan sulphate (**5**), with respect to the starting material.

Analytical results.—The results regarding the chemical analysis of the purified LMWDS are shown in Table I. They demonstrate that the depolymerization procedure did not affect the sulphur content of the molecule. The UV spectrum, shows an absorption peak at 230 nm, specific for 4,5-unsaturated uronic acid. The molecular mass (peak) (M_p) was 6500 Da, while the weight average mass (M_w) was 7500 Da. The polydispersion was found to be 1.13, very close to that measured for dermatan sulphate (1.1), this indicates that the method leads to the formation of an oligosaccharide distribution similar to that found for the starting material. Approximately 70% of the total oligosaccharides were contained within the range 5000 to 10000 Da.

Biochemical results.—This depolymerization technique gave rise to a low molecular weight dermatan sulphate which biochemically does not significantly differ from native dermatan sulphate (see Table II). Its specific activity, as determined by measuring thrombin time and anti factor IIa, was maintained. Its anti-thrombin

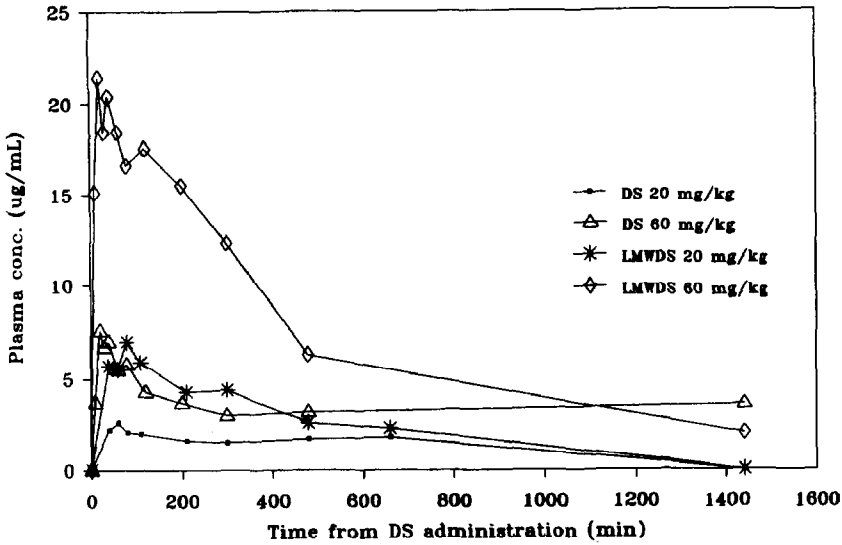

Fig. 1. Circulating plasma levels of DS and LMWDS after subcutaneous administration to rabbits. Eight animals per group.

activity, mediated by the specific cofactor heparin cofactor II (HC II), in a buffered system, was also preserved. This would suggest that the binding site for HC II on the low molecular weight dermatan sulphate chain remains intact.

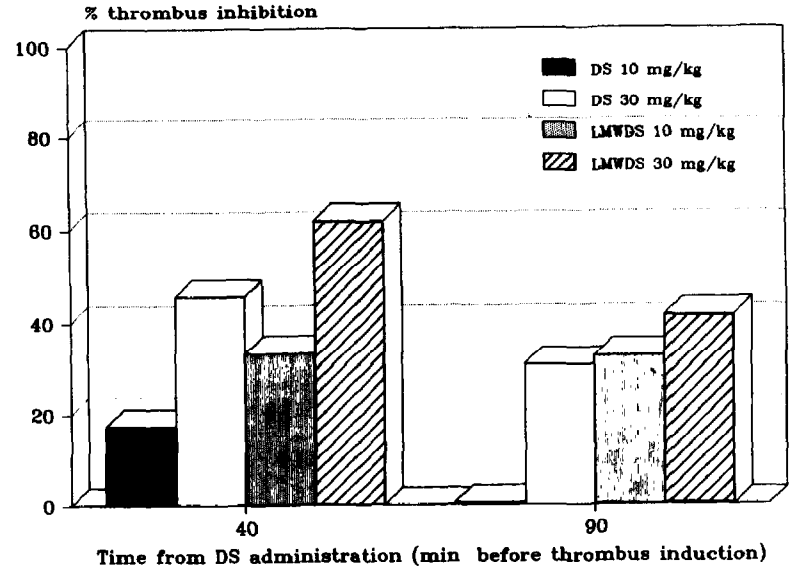

Fig. 2. Antithrombotic activity of DS and LMWDS (10 and 30 mg/kg) in a rat venous thrombosis model, expressed as % inhibition of thrombus formation. Both compounds were administered subcutaneously. Ten animals per group.

Pharmacological results.—From Fig. 1, it can clearly been seen that the LMWDS fraction is able to achieve far higher plasma concentrations in rabbits, as compared to unfractionated DS. In fact, the low dose of LMWDS (20 mg/kg) results in circulating concentrations similar to those found with 60 mg/kg of native DS after a single bolus subcutaneous dose. LMWDS 60 mg/kg $C_{max} = 23.6$ μg/mL, $T_{max} =$ 0.5 h, AUC = 174 μg/mL/h, while DS 60 mg/kg $C_{max} = 8.2$ μg/mL, $T_{max} = 0.6$ h, AUC = 86 μg/mL/h, and LMWDS 20 mg/mL, $C_{max} = 7.1$ μg/mL, $T_{max} = 1.3$ h, AUC = 58.4 μg/mL/h. This would confer a greater bioavailability to the LMWDS. This difference in bioavailability is also expressed by the antithrombotic activity observed (Fig. 2). Forty minutes after a subcutaneous injection into rats of LMWDS (30 mg/kg) the percentage inhibition of thrombus formation was 65%, while a similar dose of native DS, gave only 18% inhibition. After 90 min 42% and 34% inhibition was achieved by LMWDS and DS, respectively.

To summarize, we can conclude that the depolymerization method described can be used to produce low molecular weight dermatan sulphate having a better pharmacokinetic and pharmacodynamic profile than native dermatan sulphate after subcutaneous administration.

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