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Purification of capsular polysaccharide from *Neisseria meningitidis* serogroup C by liquid chromatography

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Abstract

Neisseria meningitidis serogroup C capsular polysaccharide (MenCPS) is an important antigen against meningococcal infection. This paper describes a new purification methodology employing liquid chromatography that resulted in a polysaccharide showing the characteristics recommended by the World Health Organization for vaccine purposes. In this method, steps of the traditional procedure that yield low recovery and use toxic materials were modified. The present process consists in the following steps: (1) continuous flow centrifugation of the culture for removal of the cells; (2) supernatant concentration by tangential filtration (100 kDa cutoff); (3) addition of 0.5% DOC, heating to 55 °C during 30 min and tangential filtration (100 kDa cutoff); (4) anion exchange chromatography (Source 15Q) and (5) size exclusion chromatography (Sepharose CL-4B). The polysaccharide C fraction obtained in that way was dialyzed and freeze-dried. The structural identity of the polysaccharide was demonstrated by ^{1}H-NMR spectrometry.

Keywords: *Neisseria meningitidis*; Capsular polysaccharide; Purification by liquid chromatography

1. Introduction

Neisseria meningitidis serogroup C is a Gram-negative, encapsulated bacterium that causes a spectrum of infectious processes. Meningitis, an infection of the central nervous system is the most common complication in meningococcal infection. Its purified capsular polysaccharide (MenCPS) is a linear homopolymer of α 2 → 9 linked *N*-acetylneuraminic acid (NANA), in which 85% of the carbon 7/8–hydroxyl groups are *O*-acetylated [1]. MenCPS has been used as an important vaccine antigen against meningococcal infection since the 1970s, generally associated with the meningococcal polysaccharide serogroups A, Y and W135. However, polysaccharide vaccines are not immunogenic in infants under 18 months showing a limited duration of protection. To solve this problem new generations of polysaccharide-protein conjugated vaccines were developed following the successful introduction of the *Haemophilus influenzae* type b (Hib) PS-protein conjugated vaccines. The polysaccharide (PS), conjugated vaccine induces antibodies against the PS in children probably by switching the MenCPS from a T-independent antigen to a T-dependent antigen [2].

The MenCPS preparation used for the production of the anti-meningococcal vaccine was purified by the first time by Gotschlich [3]. The methodology currently in use in Brazil and based on the Gotschlich procedure, includes the followings steps: (1) the negatively charged polysaccharide is initially precipitated by the use of a cationic detergent, CETAVLON ($C_{19}H_{42}NBr$); (2) the precipitate is recovered by centrifugation and re-suspended in 1 M $CaCl_2$; (3) the cell debris are removed

Abbreviations: WHO, World Health Organization; DOC, deoxycholic acid (sodium salt); MenCPS, capsular polysaccharide from *Neisseria meningitidis* C; NANA, *N*-acetylneuraminic acid; Hib, *Haemophilus influenzae* type b; PS, polysaccharide; LAL, lymulus amebocyte lysate; HPAEC-PAD, high performance anion exchange chromatography with pulsed amperometric detection; LPS, lypopolysaccharide

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by centrifugation; (4) the nucleic acid is removed by centrifugation after precipitation at a final 25% ethanol solution; (5) the polysaccharide C is now precipitated 80% ethanol; (6) after precipitate washings, cold phenol solution in 10% sodium acetate is used for protein removal; (7) the aqueous phase containing the polysaccharide C is dialyzed against water and the contaminating LPS is eliminated by ultracentrifugation [4]. This process has three inconvenient steps for large scale: (1) the manipulation of great amount of ethanol results in a dangerous process, and requires ex-proof room and equipments; (2) phenol is toxic and it can cause serious damage to health by prolonged exposure through inhalation; (3) the ultra-centrifugation step is very expensive. In addition, this is a multi-step process, which incurs in substantial loss of the product (recovery of MenCPS around 37%) [5].

This paper describes a new MenCPS purification methodology, employing suitable liquid chromatography techniques that aim at: (1) reducing the number of purification steps; (2) propitiating a larger product recovery; (3) working with volumes higher than 100 L proceeding from fermentation; (4) eliminating the centrifugation and ultra-centrifugation steps, as well as the use of great amounts of phenol and ethanol; (5) decreasing the overall time of the process. The purified polysaccharide in this work was submitted to several chemical and physical chemical analyses recommended by WHO [6,7]. The results obtained from these analyses suggest the use of the MenCPS, isolated by the described methodology as a vaccine antigen.

2. Experimental

2.1. Microrganism

The organism used was *N. meningitidis* serogroup C 2135 from the Institute Merieux, France.

2.2. Polysaccharide production

N. meningitidis C was grown in Agar Mueller Hinton plates at 37 °C during 16 h with 5% CO_2. The growth was transferred to 250 mL flasks with 50 mL of the Frantz medium. The flasks were incubated in a shaker at 37 °C for 4 h and 200 rpm. The contents of these flasks were transferred to 2 L flasks with 450 mL of the Frantz medium, in the same conditions as used before. The content of the two flasks was used as an inoculum for the fermenter Biostat—B. Braun Biotech International—with 10 L Frantz medium presenting oxygen transfer volumetric coefficient ($K_L a$) value of 36 h^{-1}. After 16 h of fermentation, the culture was inactivated at 55 °C for 10 min.

2.3. Polysaccharide purification

The steps used in the MenCPS purification were: (1) continuous flow centrifugation of the *N. meningitidis* C culture; (2) concentration of the supernatant by tangential filtration—100 kDa cutoff membrane; (3) addition of deoxycholic acid (sodium salt) (DOC)—0.5%, heating to 55 °C/30 min followed by diafiltration (100 kDa cutoff membrane); (4) anionic exchange chromatography (Source 15Q) and (5) size exclusion chromatography (Sepharose CL-4B).

The cultivated cells were removed by continuous flow centrifugation (Sharples, T 1-P); 22,637 × *g*, and flow of 400 mL/min.

The supernatant was concentrated by tangential filtration in a Minisette System (Pall BioPharmaceuticals, USA) using one membrane with a surface of 0.07 m^2 and cutoff of 100 kDa. The conditions were: a pressure differential of 21 psi and pressure transmembrane of 15 psi. The concentrated supernatant was treated with DOC, as mentioned above, diafiltrated and finally equilibrated with 20 mM Tris–HCl buffer, pH 7.5 for the ion exchange chromatography step that ensues.

The workstation used for chromatography was the Äkta Purifier (Amersham Biosciences division of GE Healthcare). Polysaccharide and protein detections were performed in 206 and 280 nm absorption lines, respectively. The Source 15Q anion exchange chromatography employed a saline wise gradient elution formed by the addition of 20 mM Tris–HCl buffer, containing 1 M NaCl, pH 7.5 (buffer B) upon 20 mM Tris–HCl buffer, pH 7.5 (buffer A). The others applied conditions were: linear flow: 179 cm/h; injection volume: 11 mL; column dimensions: 55 mm × 16 mm. Source 15Q (Amersham Biosciences division of GE Healthcare) is a polystyrene/divinylbenzene matrix with quaternary ammonium exchange groups. The resin particle size is 15 μm. The polysaccharide fraction was isolated and equilibrated by dialysis against phosphate buffer for size exclusion chromatography fractionation.

In the Sepharose CL-4B size exclusion chromatography, the elution was performed with 0.05 M phosphate buffer, pH 7.5. The other applied conditions were: linear flow 9.78 cm/h; injection volume 5 mL column dimensions 170 mm × 25 mm. Sepharose CL-4B (Amersham Biosciences division of GE Healthcare) is a cross-linked agarose matrix whose dextran fractionation range is comprised between the molecular weights of 30,000–5,000,000.

The chromatographic polysaccharide fractions were also identified by NANA estimation performed by HPAEC-PAD.

2.4. Analytical procedures

The data obtained by the several analyses performed were estimated based on dry weight.

Protein content was determined by Lowry's method [8]. Nucleic acids were estimated by absorbance at 260 nm and the amount of nucleic acid was calculated, based on the assumption that one absorbance unit is equivalent to 50 μg/mL of nucleic acid solution [7].

The endotoxin content was determined in vitro by the LAL-gel clot method (Endosafe®, Charles River, SC, USA).

The molecular weight of the purified polysaccharide was determined in an analytical TSK-Gel G4000 PWXL-TOSOHAAS column using dextrans, as molecular weight markers (749,000; 410,000; 273,000; 148,000; 48,600) and Blue Dextran (Mw 2,000,000), as a void volume marker. The other conditions applied were: volumetric flow: 0.5 mL/min; injection volume: 0.1 mL; column dimensions: 300 mm × 7.8 mm.

O-Acetyl content was determined by Hestrin's method [9] and the structural identity of the polysaccharide was verified in the Variant Unit 500 MHz ^{1}H-NMR spectrometer. Ten milligrams of dry polysaccharide were dissolved in 0.7 ml of the deuterated water containing 0.01% DMSO and 0.01% DSS. The analysis was performed at 40 °C.

The NANA content was determined by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD). The Chromatographic System DX 500 (Dionex) was used with a CarboPac PA-10 column in combination with the Aminotrap and Boratetrap columns. A waveform with triple potential was used, in the following conditions: E1 = 0.05 V, t1 = 400 ms; E2 = 0.75 V, t2 = 200 ms; E3 = −0.15 V, t3 = 400 ms. The elution used 100 mM NaOH/100 mM sodium acetate and the flow rate was 1 mL/min. The polysaccharide samples were hydrolyzed in 0.1N HCl at 80 °C, for 1 h. Soon after, the hydrolyzates were evaporated in Speed Vac SAVANT, reconstituted in water and applied in the chromatography.

All analyses recommended by WHO aiming at the use of the MenCPS as a vaccine antigen were performed with the last purified polysaccharide fraction.

3. Results

Three identical purification procedures were performed from three successive cultivations of *N. meningitidis* C. The results described below refer to an average of the values obtained from the analysis of related fractions.

Table 1 shows the values obtained for NANA and protein contents (%, w/w) as well as polysaccharide recovery for the fractions isolated after each purification step. The amount of polysaccharide was estimated as NANA and the mass obtained from the culture supernatant (510 mg), was presumed to be 100%. The other polysaccharide fraction recoveries (%, w/w) were obtained from the estimated NANA quantities for each fraction and related to the mass of the culture supernatant. All quantities showed were normalized for the same volume. Crude preparation I was related to the fraction isolated after

Table 1
Controls of the polysaccharide C (MenCPS) purification steps

Purification step	Total volume (L)	NANA mass (mg)	PS recovery (%, w/w)[a]	NANA content (%, w/w)[b]	Protein content (%, w/w)[b]
Culture supernatant	7.62	510.0	100	12.1 ± 2.3	19.1 ± 0.1
Crude preparation I	2.58	505.6 ± 13.0	99.1 ± 2.2	20.0 ± 3.8	21.4 ± 0.1
Crude preparation II	0.50	459.0 ± 12.9	90.0 ± 2.3	56.0 ± 2.7	12.1 ± 0.8
MenCPS fraction I	0.64[c]	376.4 ± 13.0	73.8 ± 1.8	69.6 ± 1.5	2.2 ± 0.7
MenCPS fraction II	3.20[c]	342.5 ± 6.1	67.2 ± 1.2	83.0 ± 2.6	0.10 ± 0.03

[a] Recovery estimation: [NANA mass in each fraction or preparation/NANA mass in culture supernatant] × 100.
[b] Protein and NANA contents were estimated in basis of dried weight.
[c] The volumes were normalized.

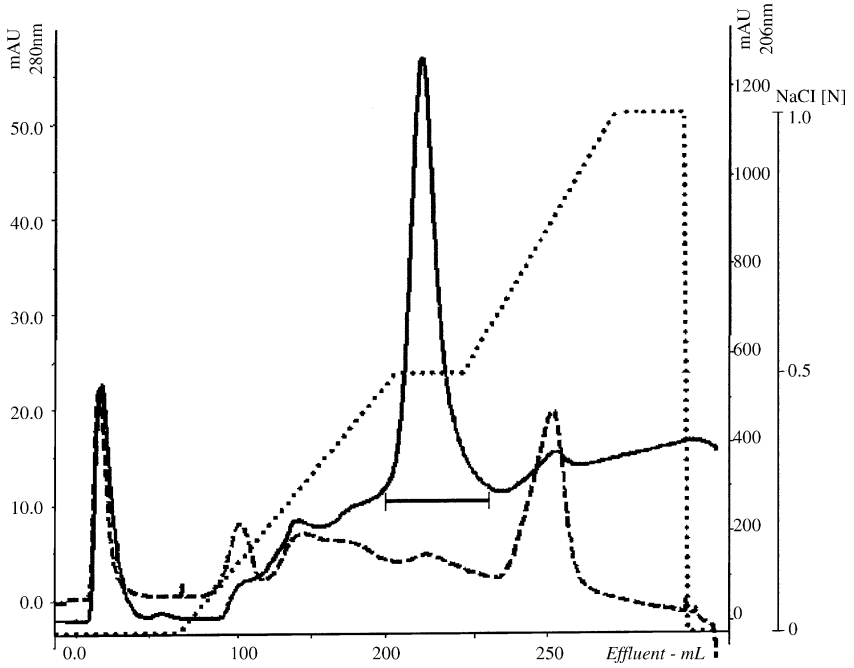

Fig. 1. Chromatographic profiles (206 nm – full line; and 280 nm – dashed line) observed in Source15 Q. The fraction obtained after DOC treatment and ultrafiltration (crude preparation II) was eluted in the anionic exchange column (55 mm × 16 mm) with a stepwise saline gradient performed by addition of 20 mM Tris–HCl buffer containing 1 M NaCl, pH 7.5 upon 20 mM Tris–HCl buffer, pH 7.5. The linear flow was 179 cm/h. The MenCPS fraction I (—) was isolated.

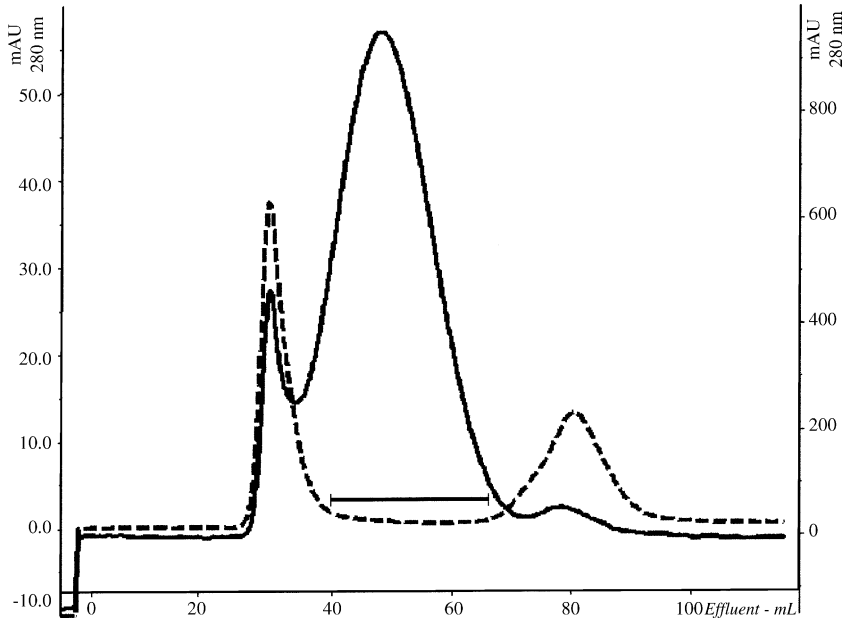

Fig. 2. Chromatographic profiles (206 nm – full line; and 280 nm – dashed line) obtained in Sepharose CL-4B. The polysaccharide fraction isolated after Source 15Q (MenCPS fraction I) was equilibrated in 50 mM phosphate buffer containing 150 mM NaCl, pH 7.5 and eluted with this buffer in the gel filtration column (170 mm × 25 mm). The linear flow was 9.78 cm/h. The MenCPS fraction II (—) was isolated.

continuous flow centrifugation and concentration by tangential filtration. Crude preparation II was obtained after DOC treatment and diafiltration. The MenCPS fraction I was isolated by ion exchange chromatography whereas the MenCPS fraction II was obtained after size exclusion chromatography. The polysaccharide fractions that were identified by 206 nm absorption and NANA estimation (HPAEC-PAD) showed good resolutions, mainly in the anion exchange chromatography column (Fig. 1). In gel filtration experiment was observed a peak detected by a 280 nm absorption slightly overlapping, at the left hand of the polysaccharide fraction (Fig. 2).

The MenCPS fraction I isolated (Fig. 1), after the first polishing step in Source 15Q anion exchange chromatography, showed a protein content of 2.2 ± 0.7 (w/w) while the polysaccharide (PS) content was 69.6 ± 1.5 (w/w). The MenCPS fraction II obtained after Sepharose CL-4B gel filtration chromatography (Fig. 2) yielded a polysaccharide preparation, whose dialyzed preparation showed the specifications showed in Table 2: a protein content of $0.10 \pm 0.03\%$ (w/w), a nucleic acid content of 0.5% (w/w), a NANA content of 83.0 ± 2.6 (w/w), *O*-acetil 2.72 ± 0.9 mmol/mg of PS, endotoxin content <10 EU/μg of PS and molecular mass of 452.750 kDa (Fig. 3). The PS content value was approximately eight times higher than the value observed in the initial PS preparation (see Table 1).

Table 2
Controls of the purified polysaccharide (MenCPS fraction II) and WHO requirements

Characteristics	WHO requirements	Purified polysaccharide
NANA content (%, w/w)	>80	83
Protein content (%, w/w)	<1	0.1
Nucleic acid content (%, w/w)	<1	0.5
Endotoxin content (EU/(g of PS)	<100	<10
O-Acetil (mmol/mg of PS)	>1.50	2.72
Molecular mass (kDa)	>100	452.750

The values (%, w/w) are corresponding to mg/100 mg of the purified polysaccharide.

Table 2 also presents a comparison between the contents determined from MenCPS isolated in this work and required by WHO.

The structural identity obtained for polysaccharide C purified by chromatography was confirmed by NMR analysis. The NMR spectra presented in Fig. 4, shows peaks in the range of 5.0–5.2 ppm corresponding to H-7 and H-8 of *O*-acetylated *N*-acetyl neuraminic acid (NANA) residues. The chemical shifts observed in the range of 3.5–4.2 ppm are related to H-4, H-5, H-6, H-9, H-9′, H-7 and H-8 of deacetylated NANA residues. The peaks related to equatorial H-3 and DMSO are observed in the range comprised between 2.5 and 2.8 ppm. Peaks corresponding to *N*-acetyl are seen in the range 1.95–2.00 while in the 2.17–2.20 ppm range, the observed peak is related to O-acetyl. The chemical shifts observed in the range 1.5–1.7 are due to axial H-3.

4. Discussion

The methods described in scientific reports concerning MenCPS purification are modifications of the traditional Gotschlich's process, that usually employ large volumes of toxic organic solvents such as ethanol and phenol. They are onerous due to cost-dispend centrifugations and ultracentrifugation and large time processing. In this respect, our work is relevant due to the use of a new purification approach mainly based on suitable chromatographic processes. The ion exchange and size

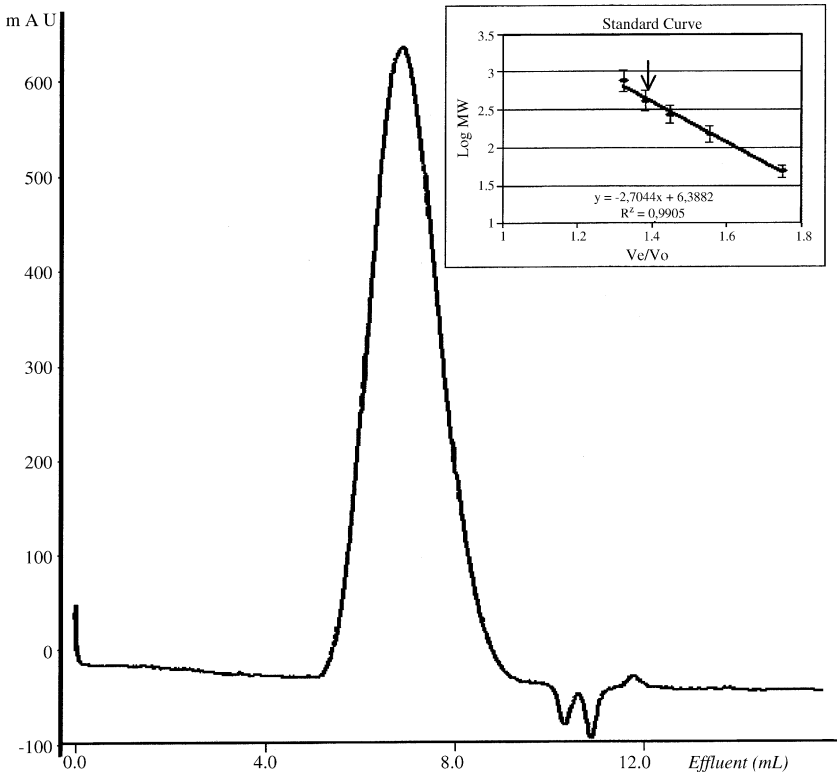

Fig. 3. MenCPS fraction II analytical chromatographic profile (206 nm) obtained in the gel filtration column TSK-Gel G4000 PWXL–TOSOHAAS. The elution was performed by 0.85% NaCl and the volumetric flow was 0.5 mL/min (lower part). The molecular weight standard curve, log PM (kDa) × Ve/Vo is show in superior part to the right. The Ve/Vo mean value obtained for the polysaccharide is indicated as an arrow.

exclusion liquid chromatography here employed as MenCPS polishing steps, accompany the current trends undertaken by pharmaceutical and biotechnology industries such as our Institution.

Liquid chromatography has been used with great success in the industrial purification of several relevant biological molecules. New stationary chromatographic phases aiming at fractionating considerable sample amounts were manufactured, such as Source Q, and used in this work. This anion exchange chromatographic support is comprised by a polystyrene/divinylbenzene inert matrix operating in perfusion mode, which allows for high flow column velocities without compromising resolution. On the other hand, Sepharose CL-4B, also used in this work, is a large pore gel formed by agarose cross-linked by epycloridrin presenting low adsorptive effects. In our work the chromatographic conditions were optimized as recommended by manufacturer in respect to critical parameters such as volume load, mass load, saline gradient, flow rate among others.

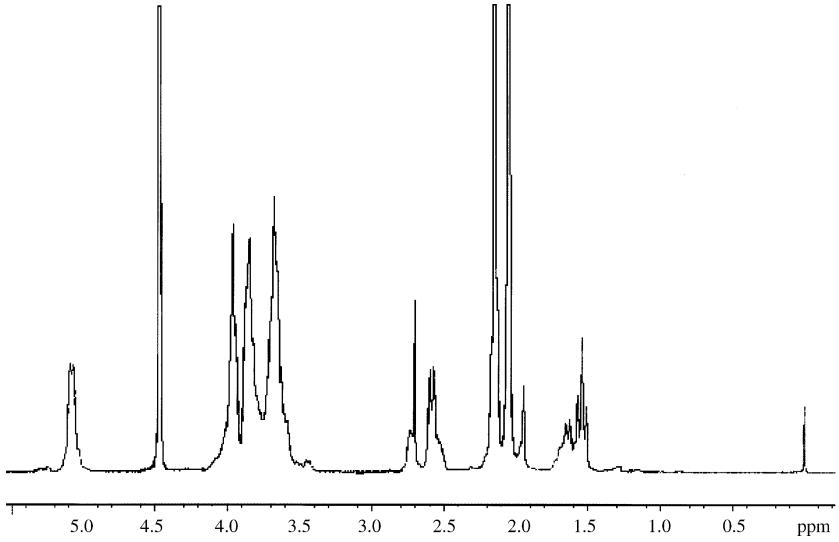

Fig. 4. Structural identity of the purified polysaccharide (MenCPS fraction II) obtained by 500 MHz ^{1}H NMR analysis. Ten milligrams of the polysaccharide dried were dissolved in 0.7 ml of the deuterated water contain 0.01% DMSO and 0.01% DSS. The analysis was performed at 40 °C.

Probably, for large-scale experiments, the use of the Sepharose CL-4B connected columns would determine a better resolution and consequently a higher recovery than that observed for the final polysaccharide preparation here isolated (Fig. 2). In this same context the 0.5 M NaCl step in anion exchange chromatography would be prolonged, in a way that could assure the good resolution observed in Fig. 1.

An attempt to improve the Gotschlich's pioneer work was presented in 1976 by Limjuco and Carlo [10] that introduced a chromatographic step based on hidroxyapatite adsorption for LPS removal, but they maintained the Gotschlich phenol step for protein elimination. Later Tanizaki et al. [11] also proposed modifications in the cited classical methodology for the purification of meningococcal group C polysaccharide. According to Tanizaki's work, the phenol extraction used for the elimination of proteins, was substituted for digestions with proteinases. Here, an extensive diafiltration, using a 100 kDa cutoff membrane, performed in 20 mM Tris–HCl buffer containing 0.5% of deoxycholate, was used to eliminate low molecular weight proteins and lypopolysaccharides (LPS). Despite the introducing these modifications, the isolated polysaccharide C preparation still contained protein and nucleic acid values 2% (w/w) and 1.5% (w/w), respectively, higher than those recommended by WHO for vaccine purposes. Furthermore, the presence of digestive enzymes rendered the process onerous.

In our Institution the Gotschlich's methodology described by Frasch [4] has been traditionally used to obtain the polysaccharide (MenCPS) from *Neisseria meningiditis.* This process shows a MenCPS recovery very low (37%, w/w). The present work, based on suitable chromatographic steps, besides showing high polysaccharide recovery (67%, w/w), also presents several advantages, such as toxic materials elimination (ethanol and phenol), low number of purification steps, centrifuges and ultracentrifuges elimination, among others, when compared to the other methods aiming at the same purpose.

The tangential ultrafiltration used in this experiment for sample concentration also contributed to large sample recoveries. The anionic detergent used was able to disaggregate the polymeric LPS to a lower molecular weight substance that was eliminated by ultrafiltration (100 kDa cutoff membrane). Here a very large amount of protein—estimated in 12.1 ± 0.8 (w/w)—was also eliminated. The chromatographic steps, ion exchange chromatography (Fig. 1) and size exclusion chromatography (Fig. 2), mainly the former, also contributed to protein elimination.

The final, dialyzed polysaccharide preparation obtained by size exclusion chromatography (Sepharose CL-4B) (Fig. 2) showed a consistent relative molecular mass of 452,750 kDa (Fig. 3) within the range suggested by WHO. The significant recovery of the MenCPS (near 70%), the conservation of *O*-acetyl groups observed by NMR analysis (Fig. 4), which is important for immune response, as well as the minimal presence of proteins (0.1%, w/w), nucleic acids (0.5%, w/w) and the low pyrogenicity value (<10 EU/μg of PS), recommend this polysaccharide preparation for vaccine purposes. Furthermore, the chromatographic media, Source 15Q and Sepharose CL-4B, here employed are suitable for either preparative or large-scale purposes. Both gels are also recommended for large-scale use, due to their maintenance (easy hygienization), stability, reproducibility, resolution and recovery.

Thus, we can suggest the current purification approach based on appropriated chromatographic procedures as an alternative to the traditional MenCPS isolation method, considering the significant levels of polysaccharide recovery, the overall quality of the polysaccharide vaccine, the simplicity of the methodology that ensue from the application of this new approach.

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