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STRONG NON-POLAR CATION EXCHANGERS FOR THE SEPARATION OF STEROIDS IN MIXED CHROMATOGRAPHIC SYSTEMS

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SUMMARY

The synthesis of strong cation exchangers with variable polarity is described. The bromohydroxypropyl derivatives of Sephadex LH-20 or Lipidex 1000 are sulphonated with sodium sulphite in aqueous methanol. The polarity of the ion exchangers is decreased by reaction with alkyl olefin oxides in methylene chloride. The materials permit ion exchange in non-aqueous solvents, and the less polar derivatives form reversed-phase systems in aqueous methanol. They are useful in the purification of steroids prior to gas chromatography–mass spectrometry, as they permit the simultaneous removal of organic bases and lipids from the biological extract.

INTRODUCTION

Lipophilic–hydrophobic Sephadex derivatives form excellent stationary phases in organic solvents[1]. Owing to their inertness and high loading capacity they are very suitable for the chromatographic purification of lipid-soluble compounds in biological materials. The polarity of the derivatives can be varied by appropriate choices of the amount and type of non-polar substituents. Ion-exchanging groups can also be attached to yield derivatives suitable for ion-exchange chromatography in organic solvents[2–4]. Such derivatives are useful for the isolation and separation of unconjugated and conjugated steroids in urine and blood prior to gas chromatographic–mass spectrometric analysis[4,5]. While suitable strong and weak anion exchangers with variable polarity have been prepared[3–5], the synthesis of a strong lipophilic cation exchanger with high capacity has not been successful[5]. The latter type of ion exchanger is needed in several methods in which lipid-soluble cations have to be purified or removed from a biological extract. This paper describes a method whereby strong lipophilic cation exchangers with different polarities can be synthesized.

EXPERIMENTAL

Chemicals

Solvents were of analytical-reagent grade and were redistilled when used for chromatography. Methanol was stored over sodium hydroxide prior to redistillation to remove interfering acid.

Nedox 1114 (chain length C_{11}–C_{14}) olefin oxide was obtained from Ashland Chemical Co. (Columbus, Ohio, U.S.A.). Boron trifluoride ethyl etherate (practical grade, Eastman Organic Chemicals, Rochester, N.Y., U.S.A.) and epibromohydrin (purum, Fluka, Buchs, Switzerland) were used as supplied. Sephadex LH-20 (Pharmacia, Uppsala, Sweden) was sieved and the 140–170-mesh fraction was used. Lipidex 1000 and 5000 (Packard, Downers Grove, Ill., U.S.A.) were used as supplied. Cation exchangers when prepared were stored in the Na^+ form in methanol at 4°. Radioactively labelled steroids were purchased from the Radiochemical Centre (Amersham, Great Britain) and NEN Chemicals (Dreieichenhain, G.F.R.). Radioactivity was determined in a Packard Model 3003 liquid scintillation spectrometer using Instagel as the scintillation liquid.

Preparation of sulphohydroxypropyl Sephadex LH-20 (SP-LH-20)

Bromohydroxypropyl Sephadex LH-20 was prepared essentially as described for the chlorohydroxypropyl derivative[2]. Sephadex LH-20 (20 g) was suspended in 80 ml of methylene chloride. The gel was allowed to swell with stirring for 30 min and 5.4 ml of boron trifluoride ethyl etherate were then added. After 15 min, 23 ml of a mixture of epibromohydrin and methylene chloride (7:16, v/v) were added at a rate of 1–2 ml · min^{-1} and the mixture was stirred for a further 30 min. The product (about 29 g, corresponding to 2.3 mequiv. of Br per gram) was collected on a büchner funnel and washed with 1 l each of 85% aqueous ethanol, chloroform and ethanol. It was dried at room temperature overnight.

To prepare SP-LH-20, bromohydroxypropyl Sephadex LH-20 (*ca.* 30 g) was suspended in 150 ml of methanol and the mixture was refluxed for 10 min. Sodium sulphite (30 g in 450 ml of water) was then added, followed by 300 ml of methanol. The mixture was heated under reflux for 4 h. The product (about 29 g, containing 1.6 mequiv. of sulphur per gram) was collected on a büchner funnel and washed with 1 l each of water, 0.5 *M* sodium hydroxide in 50% aqueous ethanol, water, 0.5 *M* hydrochloric acid in 50% ethanol, water, 50% ethanol and ethanol. The gel was then dried at room temperature overnight. Following weighing, the capacity was determined (see below).

Preparation of sulphohydroxypropyl-hydroxyalkyl Sephadex LH-20 (SPHA-LH-20)

SP-LH-20 (about 35 g in the H^+ form) was washed with 0.5–1 l of absolute ethanol on a büchner funnel and dried at 60° for 20 min. About 30 g of the dry material was suspended in 300 ml of methylene chloride and allowed to swell for about 15 min. Boron trifluoride ethyl etherate (3.75 ml; 0.125 ml per gram of gel) was added and the mixture was stirred for about 15 min at room temperature. Nedox 1114 [105 ml (3.5 ml per gram of gel) in 375 ml of methylene chloride] was then added at a rate of 10 ml · min^{-1}. The mixture was stirred for an additional 1 h at room temperature following addition of the olefin oxide. The product was collected on a büchner funnel and washed with 1 l each of chloroform, ethanol, chloroform–methanol (1:1, v/v), 20% aqueous ethanol and absolute ethanol. The material was dried overnight at room temperature. It weighed about 46 g, corresponding to an alkyl group content of about 36% (w/w). The ion-exchange capacity was 0.004 mequiv. · g^{-1}. The esterified sulphonic acid groups were hydrolysed in 500 ml of 0.25 *M* sodium hydroxide in 80% ethanol at 50° for 30 min. The material was then collected on a büchner funnel and

washed with water (until neutral), 0.5 M hydrochloric acid in 50% ethanol, water, 50% ethanol and absolute ethanol. It was dried overnight at room temperature and then had a weight of about 39 g [corresponding to 23% (w/w) alkyl chains per gram] and an ion-exchange capacity of 1.0 mequiv. · g^{-1}.

Preparation of sulphohydroxypropyl Lipidex 1000 (SP-Lipidex)

This ion exchanger was prepared as described for SP-LH-20. Twenty grams of Lipidex 1000 [alkyl group content 10% (w/w)] were used as starting material to yield 29 g of the bromohydroxypropyl derivative (2.3 mequiv. of Br per gram of gel), which yielded 29 g of the sulphohydroxypropyl derivative [ion-exchange capacity 1.4–1.5 mequiv. · g^{-1}, alkyl group content 7% (w/w)].

Determination of capacity, degree of substitution and swelling in solvents

To determine the ion-exchange capacity, 0.5–1 g of the gel in the H^+ form was suspended in 50 ml of a 0.2 *M* solution of sodium chloride in 72% aqueous ethanol and was titrated with 0.1 or 0.01 *M* aqueous sodium hydroxide.

The degree of substitution with bromohydroxypropyl and hydroxyalkyl groups was calculated from the weight increase.

The swelling in different solvents was evaluated by measurement of the bed volume formed by 0.25 g of gel in the H^+ form in a 4-mm I.D. glass column. The material was allowed to settle under gravity flow and a pressure of 200 kPa was applied. The height of the column was then measured.

Column chromatography

Glass columns (150 × 4 mm I.D.) with a 10-ml solvent reservoir were fitted with a Teflon end-piece covered with Teflon gauze to hold the column bed.

The ability of the gels to form a reversed-phase system in 72% aqueous methanol was tested with progesterone and cholesterol. A mixture of trace amounts of radioactively labelled steroids was dissolved in 1 ml of 72% methanol and applied to 100 × 4 mm I.D. column beds (about 0.3–0.5 g gel in the H^+ form) packed in the same solvent. Elution was performed at a rate of about 12 ml · h^{-1} with collection of 1–2 ml fractions for determination of radioactivity.

The ability of the ion exchangers to separate steroid oximes was tested essentially as described previously[6]. Oximes of different radioactively labelled ketosteroids were prepared [7] and extracted with ethyl acetate following evaporation of the pyridine and dilution of the mixture with water. The derivatized steroids were dissolved in 1 ml of methanol and applied to a column of SP-LH-20 (about 100 mg in the H^+ form, 40 × 4 mm I.D.) packed in the same solvent. Elution with methanol was continued at a flow-rate of about 20 ml · h^{-1} with collection of 1–5-ml fractions for determination of radioactivity.

RESULTS AND DISCUSSION

Preparation of cation exchangers

Sulphonation of Sephadex LH-20 and Lipidex 1000 was carried out using the widely applicable and simple reaction between an aliphatic halide and inorganic sulphite in an aqueous solvent (Strecker reaction[8]). Bromine was first introduced into

the gel by an acid-catalysed reaction with epibromohydrin in a non-aqueous solvent[2]. Optimal conditions for the reaction of the bromohydroxypropylated gels with sodium sulphite were investigated. The concentrations of sodium sulphite and methanol in the aqueous reaction mixture were selected to give optimal swelling of the gel in a solution of the salt. Little substitution occurred when the reaction was carried out at 60°. Substitution was obtained when the reaction mixture was boiled under reflux (about 80°). Heating for more than 4 h produced little further increase in the ion-exchange capacity of the products (Fig. 1). The capacity obtained was 70% (1.5–1.7 mequiv. · g^{-1}) of that calculated from the bromine content of the Sephadex LH-20 derivative and 65% (1.4–1.5 mequiv. · g^{-1}) of that calculated from the bromine content of the Lipidex 1000 derivative. Thus, substitution occurred more slowly but with a higher yield than in the corresponding syntheses of anion exchangers. The higher yield of cation-exchanging groups is probably due to a lower degree of hydrolysis of halogen groups during this synthesis.

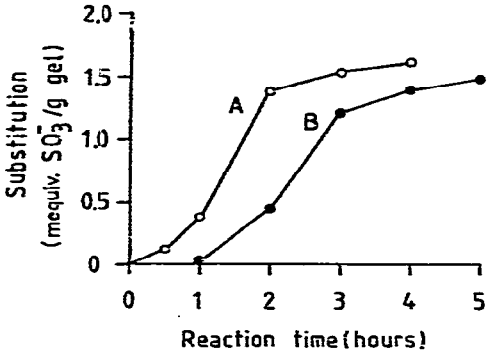

Fig. 1. Rates of sulphonation of the bromohydroxypropyl derivatives of Sephadex LH-20 (A) and Lipidex 1000 (B). Reactions were carried out using 1 g of sodium sulphite and 30 ml of 50% aqueous methanol per gram of gel. The mixture was heated under reflux (about 80°).

The substitution of bromohydroxypropyl Sephadex occurred more slowly than with the derivative of Sephadex LH-20 (Fig. 1). This may be due to steric hindrance by the alkyl chains and to decreased swelling of the less polar Lipidex derivative. Thus, bromohydroxypropylated Lipidex 5000 [containing 50% (w/w) alkyl chains] could not be sulphonated under the conditions used, and reaction times longer than 6–7 h resulted in degradation of the gel. Attempts to use other solvent mixtures containing water, ethylene glycol or isopropanol were unsuccessful. Difficulties in sulphonation of non-polar alkyl halides have been noted previously, and vigorous conditions have to be used[8].

Chlorohydroxypropylated Sephadex derivatives were also studied in the Strecker reaction. These derivatives have been used in the synthesis of, *e.g.*, anion exchangers and sulphydryl derivatives[2–5] and would be convenient as common intermediates in the synthesis of stationary phases carrying different functional groups. However, under the conditions used, products containing only 0.06–0.1 mequiv. of sulphonate groups per gram of gel were obtained. Addition of potassium iodide as a catalyst[9] decreased the substitution (0.025 mequiv. · g^{-1}), while addition of potassium

bromide yielded products with an ion-exchange capacity of 0.65–0.8 mequiv. · g^{-1}. Thus, the yield of ion exchanger was superior when bromohydroxypropyl derivatives were used as intermediates in the syntheses.

As it was not possible to sulphonate bromohydroxypropyl Lipidex 5000 to obtain a non-polar ion exchanger directly, conditions were studied for the hydroxyalkylation of SP-LH-20. Reproducible results required the hygroscopic SP-LH-20 to be dried prior to the reaction. The hydrophobic SPHA-LH-20 could then be prepared by a boron trifluoride catalysed reaction with an alkyl olefin oxide, essentially as described for the hydroxyalkylation of Sephadex LH-20[1]. As expected, the ion exchanger had to be in the H^+ form. Optimal substitution was obtained when the amount of boron trifluoride ethyl etherate was 0.125 ml per gram of gel (Fig. 2), which is less than the optimal amount needed in the reaction with Sephadex LH-20[1]. This may be due to the presence of acidic groups in the ion exchanger. SP-LH-20 could not be hydroxyalkylated to the same extent as Sephadex LH-20 even when a large excess of Nedox 1114 was used. This may be due to steric hindrance or to the presence of charged groups in SP-LH-20. The sulphonic acid groups were also esterified during the reaction (the ion-exchange capacity decreased to 0.004 mequiv. · g^{-1}). Following alkaline hydrolysis, the calculated ion-exchange capacity was reestablished (0.9–1.0 mequiv. · g^{-1}). A suitable final hydroxyalkyl group content [20–30% (w/w), corresponding to a hydroxyalkyl group content of 30–35% in hydroxyalkylated Sephadex LH-20] was obtained when 2.5–5 ml of Nedox 1114 were added per gram of gel (Fig. 2).

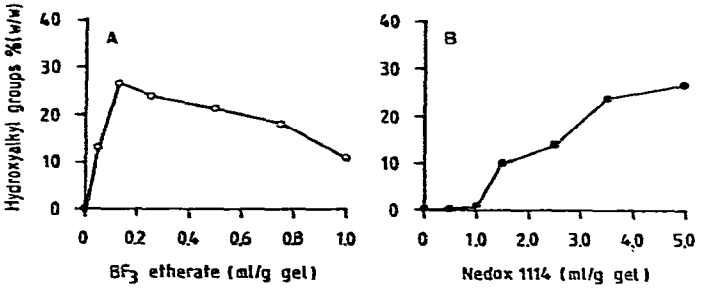

Fig. 2. Effect of the amount of (A) boron trifluoride ethyl etherate and (B) Nedox 1114 on the degree of hydroxyalkylation of SP-LH-20. The weight increase was determined after hydrolysis of the esterified sulphonic acid groups. The optimum amount of boron trifluoride was determined using 5.0 ml of Nedox 1114 per gram of SP-LH-20. When the volume of Nedox was varied, 0.125 ml of boron trifluoride ethyl etherate per gram of SP-LH-20 was used.

Titration curves for the three types of ion exchangers, SP-LH-20, SP-Lipidex and SPHA-LH-20, are shown in Fig. 3. The swelling properties of the gels are compared in Table I. The gels with a high alkyl group content are not wetted by water, while those without long alkyl chains do not swell in *n*-hexane. The gels containing sulphonic acid groups swell more in water than do the corresponding neutral gels.

Chromatographic applications

Reversed-phase chromatography. The analysis of steroids in biological materials by gas chromatography–mass spectrometry usually requires extensive purification of the samples. A method in which lipids and acidic material are removed by filtration

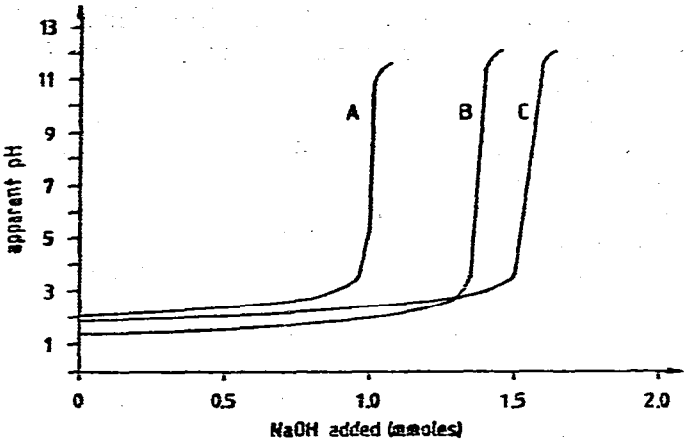

Fig. 3. Titration curves obtained with 1 g of (A) SPHA-LH-20, (B) SP-Lipidex and (C) SP-LH-20 in the H^+ form.

TABLE I

COLUMN BED VOLUMES OF CATION-EXCHANGE GELS IN THE H^+ FORM IN DIFFERENT SOLVENTS

The column bed volumes given by similarly alkylated neutral gels are shown for comparison.

*Gel type**	*Ion-exchange capacity (mequiv.·g^{-1})*	*Alkyl chain content (%, w/w)*	*Column bed volume (ml per gram of gel)*		
			Water	*Ethanol*	*n-Hexane*
SP-LH-20	1.6	—	4.5	3.0	1.4**
Sephadex LH-20	—	—	3.0	3.2	0.7**
SP-Lipidex 1000	1.3	7	4.1	3.6	1.8
Lipidex 1000	—	10	2.3	3.2	1.8
SPHA-LH-20	0.9	25	**	2.4	2.1
Lipidex 5000	—	50	**	2.2	2.6

* For abbreviations, see text.

** Solvent and gel are not compatible and homogeneous column beds cannot be prepared.

through a strong non-polar anion exchanger [triethylaminohydroxypropyl-hydroxyalkyl Sephadex LH-20 (TEAPHA-LH-20)] in a reversed-phase system has been described previously[4,10]. As this anion exchanger is also used for the separation of conjugated steroids, the prior removal of lipids and organic bases by filtration through a non-polar cation exchanger would be simpler and more rapid. The ability of the different cation exchangers to separate progesterone (the least polar steroid hormone tested) and cholesterol (the most common lipid contaminant)was therefore tested using 72% aqueous methanol as the solvent. As can be seen in Fig. 4, no separation is obtained on SP-LH-20, and the retardation of cholesterol by SP-Lipidex does not permit the application of larger volumes of extract. However, the separation factor on SPHA-LH-20 is greater than 20. This material is therefore an effective filter for the removal of cholesterol.

The following sequence of filtrations may be proposed for purification of steroids in a desalted biological extract: biological extract (in 72% methanol) →

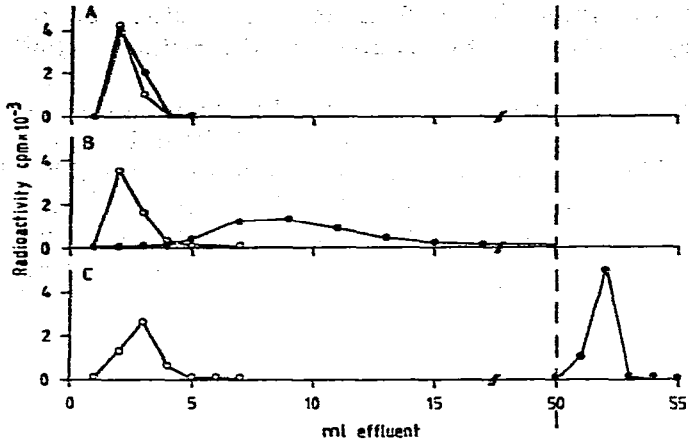

Fig. 4. Chromatography of trace amounts of labelled progesterone (○) and cholesterol (●) on (A) SP-LH-20, (B) SP-Lipidex and (C) SPHA-LH-20 in the H^+ form. Column bed volumes were about 1.25 ml. Elution was performed with 50 ml of 72% aqueous methanol followed by 5 ml of methanol–chloroform (1:1).

SPHA-LH-20 (sorption of organic bases and lipids) → TEAP-LH-20 (sorption of organic acids) → neutral steroids. Estrogens, glucuronides and mono- and disulphates can then be stepwise eluted from the anion exchanger[4,5,11].

Ion-exchange chromatography of steroid oximes. As previously reported, unsubstituted oximes of ketosteroids may be sorbed on strong lipophilic cation exchangers in non-aqueous methanol[6]. This was used for the selective isolation of 3-ketosteroids (as oximes). However, the capacity of the ion exchanger used in these studies was insufficient for the separate isolation of oximes of 17- and 20-ketosteroids. The high capacity of SP-LH-20 permitted the separation of these groups of com-

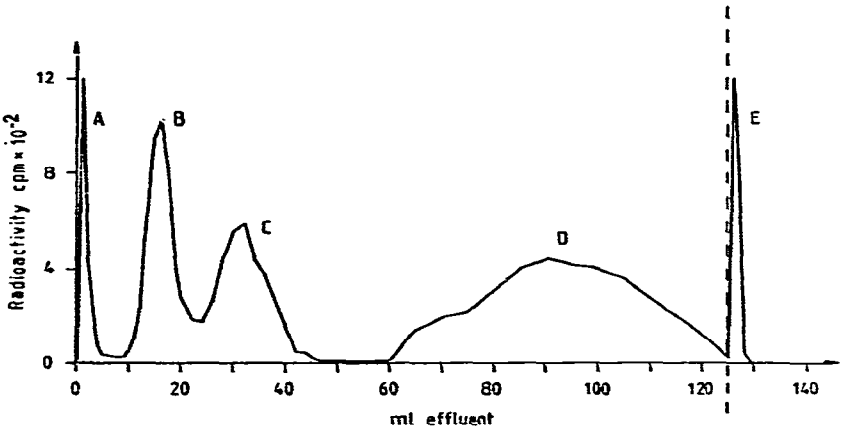

Fig. 5. Separation of trace amounts of radioactively labelled steroid oximes on a column (40 × 4 mm I.D.) of SP-LH-20 (0.1 g) in the H^+ form. (A) Estradiol; (B) 3β-hydroxy-5-androsten-17-one 17-oxime; (C) 3β-hydroxy-5-pregnen-20-one 20-oxime; (D) 17β-hydroxy-5α-androstan-3-one 3-oxime; (E) testosterone 3-oxime. The column was eluted with 125 ml of methanol followed by 15 ml of methanol–pyridine (20:1).

pounds on small columns. This is illustrated in Fig. 5. It should be pointed out that the mobilities of the oximes may be influenced by the presence of substituents close to the oxime groups[6].

The strong non-polar cation exchangers may be useful for the isolation and purification of various positively charged compounds extracted from biological materials with organic solvents. Thus, they could probably be used for the purification of drugs prior to gas chromatography–mass spectrometry. The use of hydrophobic cation exchangers may also be advantageous in ligand exchange chromatography, as the sorption of compounds may be enhanced in non-aqueous solvents. Preliminary experiments indicate that the silver form of SP-LH-20 is useful for the isolation of steroids carrying an ethynyl group.

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