Sephadex LH-20

Sephadex™ LH-20 is a liquid chromatography medium designed for molecular sizing of natural products such as steroids, terpenoids, lipids, and low molecular weight peptides (up to 35 amino acid residues). Depending on the chosen solvents, this medium can also separate sample components by partition between the stationary and mobile phases.

Sephadex LH-20 is useful for both analytical and industrial scales for the preparation of closely related molecular species. Due to the unique physico-chemical properties of this medium, it can be used either during initial purification prior to polishing by high performance ion exchange or reversed phase chromatography, or as the final polishing step, for example, during the preparation of diastereomers.

Sephadex LH-20 is characterized by:

- Unique chromatographic selectivity due to dual hydrophilic and lipophilic nature of the matrix
- Easily predicted elution behavior based on the chemical structure of the sample (1–3, 14)
- Chemical and physical robustness, see Table 1
- Excellent batch-to-batch reproducibility

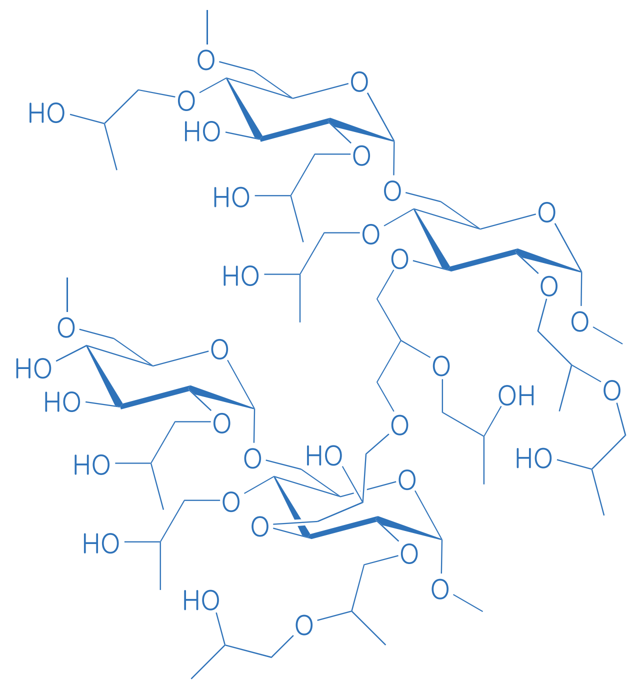


Fig 1. Partial structure of Sephadex LH-20. The medium is based on hydroxypropylated dextran that has been cross-linked to yield a polysaccharide network.

Physical and chemical characteristics

Sephadex LH-20 is beaded, cross-linked dextran that has been hydroxypropylated to yield a chromatography medium with both hydrophilic and lipophilic character. Due to its dual character, Sephadex LH-20 swells in water and a number of organic solvents. The chemical structure of the medium is shown in Figure 1. Both the wet particle size and exclusion limit for the medium vary depending on the solvent used for swelling, see Table 2. Table 1 summarizes the general physico-chemical and chromatographic properties of Sephadex LH-20.

Table 1. General physico-chemical properties and chromatographic performance characteristics of Sephadex LH-20

Matrix	Hydroxypropylated, cross-linked dextran
Bead form	Spherical, porous
Average particle size	
Dry	70 µm
In methanol	103 µm
pH stability	
Working	2–13
Cleaning	2–13
Chemical stability	Stable in most aqueous and organic eluent systems. Not stable below pH 2 nor to strong oxidizing agents
Autoclavable	20 minutes at 121°C
Maximum linear flow rate	700 cm/h
Recommended linear flow rate	60 cm/h
Operating temperature	4°C to 40°C
Sample loading volumes	
Adsorption mode	Depends on resolution required
Molecular sizing	< 2% total medium volume
Partition mode	< 1% total medium volume
Exclusion limit	4000–5000 (depends on solvent)

Isolation of an HIV-1 reverse transcriptase (HIV-1 RT) inhibitor from *Phyllanthus niruri*

Phyllanthus niruri (P. niruri) has been used for many years as a natural medicine against edema and jaundice (13). Early studies showed isolations of a variety of compounds from this plant but none of the isolates were clearly correlated to any antiviral activities. Then in separate studies (5, 6), extracts of *P. niruri* showed inhibitory effects on an endogenous DNA polymerase from hepatitis B virus (HBV pol), on a reverse transcriptase from avian myeloblastosis virus, and on other DNA dependent polymerases. During a recent study (4), an active compound, identified as repandusinic acid A monosodium salt (RA), was isolated from an aqueous extract which showed inhibition of HIV-1 RT.

RA was found to exert significant inhibitory effects on the production of HIV-1 capsid protein in an *in vitro* culture system. These results and others (7) suggest that the tannin-like structure of RA might fit the binding site of template-primer on the enzyme. Table 3 shows the purification results of RA from *P. niruri*. The structure of the free acid is illustrated in Figure 2.

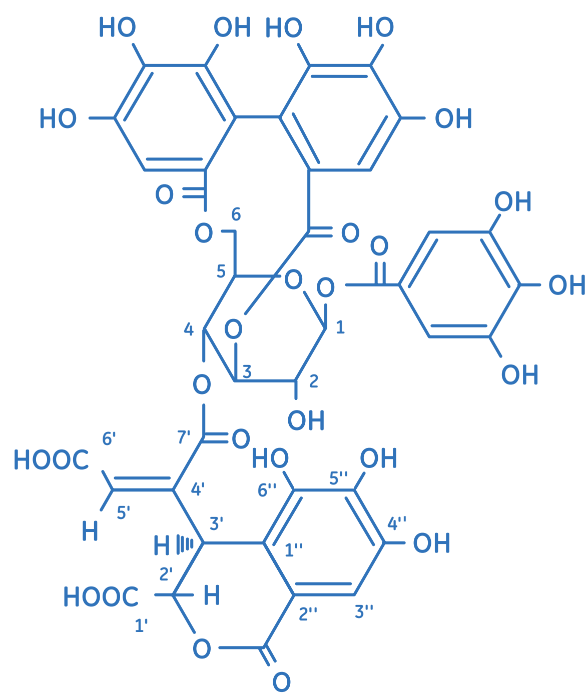


Fig 2. Structure of free acid form of repandusinic acid A.

Table 2. Approximate packed medium volumes of Sephadex LH-20 as swollen in different solvents

Solvent	Approx. medium volume (ml/g dry powder)
Dimethyl sulfoxide	4.4–4.6
Pyridine	4.2–4.4
Water	4.0–4.4
Dimethylformamide	4.0–4.4
Methanol	3.9–4.1
Saline	3.8–4.2
Ethylene dichloride	3.8–4.1
Chloroform[1]	3.8–4.1
Propanol	3.7–4.0
Ethanol[2]	3.6–3.9
Isobutanol	3.6–3.9
Formamide	3.6–3.9
Methylene dichloride	3.6–3.9
Butanol	3.5–3.8
Isopropanol	3.3–3.6
Tetrahydrofuran	3.3–3.6
Dioxane	3.2–3.5
Acetone	2.4–2.6
Acetonitrile[3]	2.2–2.4
Carbon tetrachloride[3]	1.8–2.2
Benzene[3]	1.6–2.0
Ethyl acetate[3]	1.6–1.8
Toluene[3]	1.5–1.6

[1] Containing 1% ethanol.

[2] Containing 1% benzene.

[3] Solvents giving a medium volume of less than about 2.5 ml/g dry powder are generally not useful.

Table 3. Summary of data for the purification of repandusinic acid A from *P. niruri*

Purification step	Yield (mg)	ID50[1] (µg/ml)	Specific activity ($\times 10^2$ IU/mg)[2]	Total activity ($\times 10^3$ IU)[2]
H_2O extract	6600	50	4	2640
Methanol insoluble	2500	20	10	2500
Sephadex LH-20, fr. 4–11[3]	247	3.0–3.6	56–67	1616
Cellulose				
Fr. 1	189	7.8	26	484
Fr. 2	24	5.0	40	96
Fr. 3	18	2.4	83	150
Fr. 4	9	3.4	58	52
Fr. 5	14	1.8	111	156
RA (pure substance)	5.9	0.3	668	394

[1] ID50 indicates the effectiveness of inhibitors expressed as concentrations that cause 50% inhibition of HIV-1 RT. Crude HIV-1 RT was used in this experiment.

[2] IU are arbitrary inhibitory activity units obtained by dividing the total weight of the fraction at each step by the weight of each fraction required to achieve 50% inhibition of [3H]dTTP incorporation into the polymer in the HIV-1 RT assay.

[3] Fractions 4–10 and fraction 11 were combined as both fractions had the inhibitory activity.

Preparative scale separation of 2-acetamidobenzoic acid from 4-acetamidobenzoic acid (1)

The dual character of Sephadex LH-20, hydrophilicity and lipophilicity, provides unique chromatographic selectivity and extremely high resolution of closely related molecular species. Figure 3 shows a preparative scale separation of a mixture containing both 2-acetamidobenzoic acid and 4-acetamidobenzoic acid. The two molecules differ only by the position of the acetamide function on the benzene ring. The separation was possible due to the unique selectivity of the Sephadex LH-20.

Column:	Sephadex LH-20, 2.5 × 200 cm
Sample:	Mixture of 2- and 4-acetamidobenzoic acid
Eluent:	Acetone
Flow rate:	8 ml/min
Detection:	Refractive index (RI)
Yield:	250 mg 2-acetamidobenzoic acid 254 mg 4-acetamidobenzoic acid

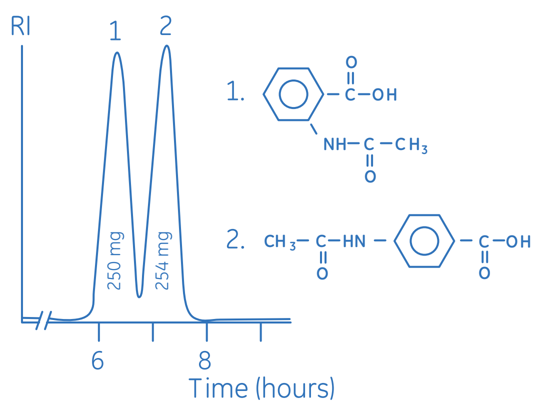


Fig 3. Separation of 2- and 4-acetamidobenzoic acid on Sephadex LH-20.

Isolation of BE-23372M, a novel protein tyrosine kinase inhibitor

BE-23372M was isolated (8) from the culture broth of a fungus and the producing strain, F23372, was identified as *Rhizoctonia solani*. The protein kinase inhibitory activity was purified by a sequence of solvent extractions and then by silica and Sephadex LH-20 column chromatographies.

The particular compound, BE-23372M, was discovered while screening for EGF protein tyrosine kinase inhibitors, which could prove useful as selective drugs for treating cancers. BE-23372M showed strongly specific inhibitory activity to EGF receptor kinase with IC_{50} values of 0.02 and 0.03 μM on two substrates, see Table 4.
The compound also inhibited the growth of A431 human epidermoid carcinoma and MKN-7 human stomach cancer cell lines.

Sephadex LH-20 (2.5 × 50 cm column) was used to prepare 5.7 mg of BE-23372M in the form of a reddish orange solid substance. The physico-chemical properties, structure elucidation, and synthetic studies of this compound are reported in references 9 and 10. The specificity of inhibition of BE-23372M is illustrated through the data in Table 4.

Table 4. The inhibitory effect of BE-23372M on protein kinases

Protein kinase	Substrate	IC_{50} (μM)
EGF receptor kinase	Poly(Glu: Tyr)	0.02
EGF receptor kinase	RR-SRC	0.03
Protein kinase C	Histone (Lys rich)	4.5
cAMP-dependent protein kinase	Histone H2B	> 20

In vitro test to measure physical and chemical changes in doxorubicin (DXR) in liposomal formulations

Two different liposomal doxorubicin (DXR) preparations were solubilized in ethanol and then run chromatographically on a column packed with Sephadex LH-20 in ethanol. The DXR and phospholipid were determined by A_{480} and modified Bartlett assay, respectively (11). Figure 4A shows the elution profile of L-DXR that had been stored at 4°C. All the phospholipids eluted in the void volume probably as micelles. The volume of elution for DXR suggested a molecular weight of 550, which is very similar to its actual molecular weight of 580. Figure 4B shows a comparison of the elution profile of free DXR and L-DXR stored in lyophilized form at 60°C.

The earlier elution of the DXR, which had been stored at elevated temperature (60°C) and in liposomal form, suggests a molecular weight of 1000 to 1100. The appearance of the higher molecular weight of DXR could be the result of either physical aggregation or chemical oligomerization. It is expected that the molecular weight change of DXR might restrict its biological availability. Therefore, this assay might be useful to assess the therapeutic efficacy of liposomal DXR by *in vitro* testing.

The resolving power of Sephadex LH-20 was further demonstrated by its ability to separate DXR and its polar degradation product, which was obtained after 12 hours of incubation in plasma at 37°C (not shown). The elution volume of this compound indicated a molecular weight of approximately 450, which together with spectral data suggested 7-deoxydemethyl DXR or its aglycone.

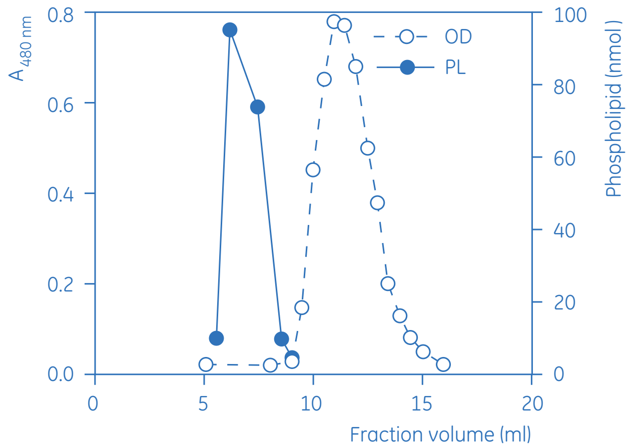


Fig 4A. Sephadex LH-20 (1 × 20 cm) elution profiles of L-DXR clinical batch LT4C stored at 4°C and phospholipid by modified Bartlett assay (•...•...•...•) and A_{480} (o...o...o...o).

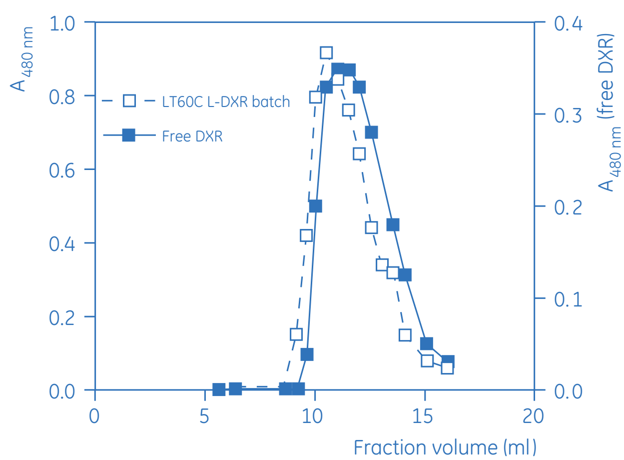


Fig 4B. Sephadex LH-20 (1 × 20 cm) elution profile of L-DXR clinical batch LT60C stored at 60°C (...□...□...) and free DXR (...■...■...). Results of the Bartlett assay are not shown.

Preparative separation of an epimeric mixture of budesonide

Attempts to resolve epimeric mixtures of steroids similar to budesonide by fractional crystallization have met with limited success. The development of a chromatographic separation method on Sephadex LH-20 proved to provide a more effective preparative method (12). As synthesized, the glucocortoid, budesonide, is a mixture of 1:1 of the C-22 epimer, see Figure 5.

During the evaluation of budesonide as an inflammatory, it was desirable to evaluate the chemical and pharmacological properties of the two epimers. Sephadex LH-20 was packed into a preparative column (6.3 × 75 cm) and different loadings of the epimeric mixture of budesonide were run to see the trade-off between resolution and loading, see Figure 6. When restricting the chromatography to a single cycle, sample loading at 1500 mg proved to be a practical preparative load.

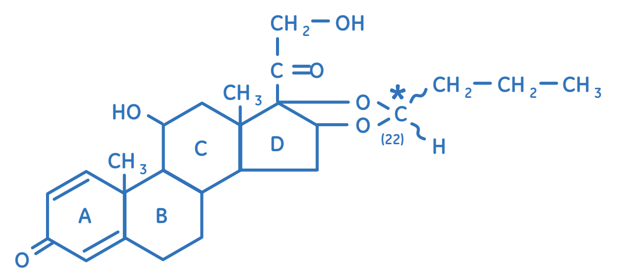


Fig 5. Structure of budesonide C-22 epimer.

Medium:	Sephadex LH-20
Mobile phase:	Heptane-chloroform-ethanol, 20:20:1
Solute:	Budesonide 21-acetate (XVII)
Sample volumes:	A) 500 mg, B) 1500 mg, C) 3000 mg, and D) 5000 mg

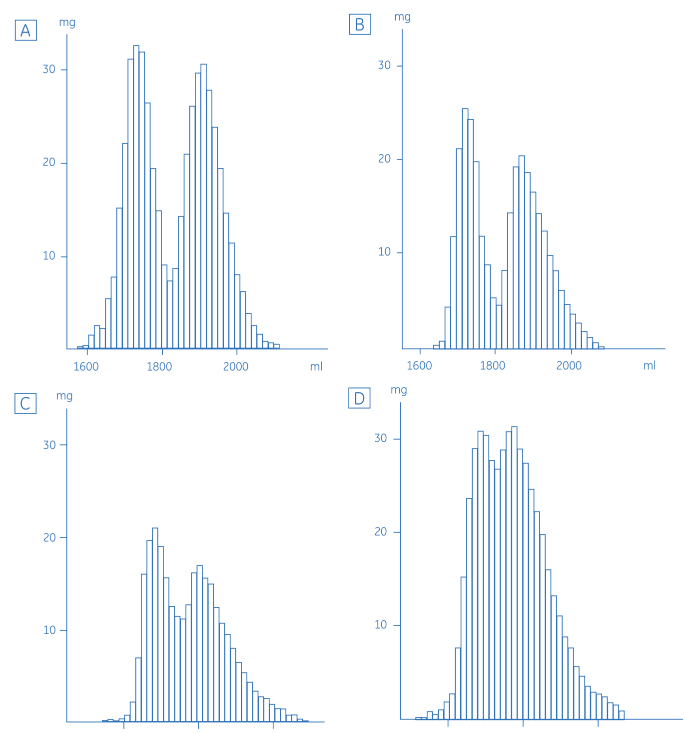
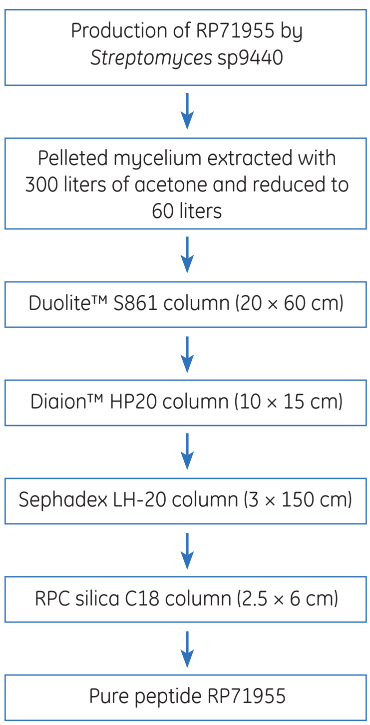


Fig 6. Effect of loading with high sample mass in preparative gel chromatography. Amount of sample/fraction plotted against elution volume.

Isolation of the cyclic peptide, RP71955, an inhibitor of HIV-1 aspartyl protease

RP71955 (15), isolated from *Streptomyces* strain SP9440, has been shown by Helynck *et al.* (1993) to be an effective inhibitor of the HIV-1 protease. After centrifugation of 440 liters from an 800-liter fermentation, the mycelium was extracted with acetone and again centrifuged. The supernatant was then further reduced to 60 liters. Some of the feedstock was then purified through a number of chromatography steps including Sephadex LH-20.

The basic purification scheme is as follows:



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Additional applications of Sephadex LH-20

The following references are a few more publications where Sephadex LH-20 has been employed in the purification strategy. The articles are grouped according to the type of molecule and/or area of research.

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Products	Quantity	Code no.
Sephadex LH-20	25 g	17-0090-10
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