Table V—Urinary I Excretion by Four Human Volunteers Given 130 mg/60 kg po for 5 Days

Time Interval	I Concentration, mg
2000–0800	13.13 ± 2.30
0800–1200	20.03 ± 1.68
1200–1600	37.44 ± 7.31
1600–2000	29.06 ± 9.31
Excretion, 24 hr	89.03 ± 15.01
Percent of administered dose	80.35 ± 14.08

tography was carried out by focusing the instrument on the molecular ions of methylated I and trideuteromethylated I occurring at m/z 197 and 200, respectively, and on the fragment ions at m/z 133 and 136, which arise from the loss of sulfurous anhydride from the molecular ions. Figure 4 shows a typical mass fragmentogram obtained from a human plasma extract after I intake.

During both GLC with flame-ionization detection and mass fragmentography, no interference due to endogenous substrates was observed. Good linearity in detector response also was found over a concentration range of 50 ng/ml–10 μg/ml with multiple-ion detection mass fragmentography and from 2 μg/ml up to milligram levels for GLC with flame-ionization detection. The precision (percentage coefficients of variation) was calculated from the standard deviation/mean × 100. Assays were made in quadruplicate at six concentrations of I, thus covering the range expected in *in vivo* experiments. The results summarized in Table I refer to determinations from human plasma and urine.

The validity of this method for *in vivo* determinations was tested by studying the plasma kinetics and urinary elimination of I in healthy volunteers after single (50 mg/60 kg of body weight) or repeated (130 mg/60 kg of body weight/day for 5 days divided over the three main meals) oral doses.

Figure 5 shows plasma I kinetics in human volunteers after acute administration. The compound was absorbed rapidly through the GI tract, reaching peak plasma values between 30 and 60 min. Elimination of I followed a monoexponential decay pattern with a calculated half-life of 121 ± 16 min at the times considered (Table II).

Table III reports the plasma I levels determined in the volunteers given repeated oral doses. Concentrations were higher close to the time of I intake, *i.e.*, breakfast, lunch, and dinner. The *AUC* was about half that after a single dose of 50 mg/60 kg of body weight, although the daily dose was almost 2.5 times larger. However, these differences probably arose from differences in absorption since I was taken with food and not in a fasting condition and since the total daily dose was divided *ad libitum*, an experimental condition that may be considered closer to the real situation.

Urinary I excretion by human volunteers given a single oral dose is reported in Table IV. Compound I was eliminated within a few hours of administration, with ~60% of the dose being excreted unchanged by 6 hr, and reached 79.9% at 24 hr. Excretion of I after repeated administration is shown in Table V. Following this schedule of treatment, the 24-hr excretion again amounted to ~80% of the daily dose. These data are in good agreement with previous reports indicating that I is excreted in the urine rapidly and almost completely in humans and laboratory animals (6, 7). This observation is consistent with the relatively short plasma half-life of I, which is poorly metabolized.

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Size Exclusion Chromatography of Liposomes on Different Gel Media

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Received July 24, 1980, from the *Pharmaceutical Research Department, Pharmacia AB, Box 181, S-751 25 Uppsala 1, Sweden.* Accepted for publication January 7, 1981.

Abstract □ Uncharged and negatively and positively charged liposomes of egg lecithin were prepared by sonication and chromatographed on three different gel media. The column effluent was investigated by turbidimetric measurements. The operational parameters were selected to obtain baseline separation of the liposomes. Liposome peaks were fractionated and identified by their K_{av} (distribution coefficient) values. Baseline separation into two fractions was obtained with cross-linked dextran gel, and three fractions were obtained with cross-linked agarose gel.

Keyphrases □ Liposomes—synthesized and fractionated using size exclusion chromatography on different gel media □ Size exclusion chromatography—of liposomes on different gel media □ Fractionation—size exclusion chromatography of liposomes on different gel media □ Chromatography, size exclusion—liposomes, synthesized and fractionated on cross-linked agarose and cross-linked dextran gels

Parenteral and oral dosage forms with increased drug selectivity are achieved by linking drugs to carriers. Such forms are of pharmaceutical importance, and several concepts using liposomes have been suggested (1, 2). Liposomes are formed when phospholipids are allowed to swell in aqueous media. When suitably dispersed, the liposomes consist of a series of concentric lipid bilayers, which alternate with aqueous compartments. The bilayers and aqueous compartments can entrap lipid-soluble and water-soluble substances, respectively.

Size exclusion chromatography[1] (3) commonly is used to separate liposome-entrapped substances from nonentrapped material (4–6), but there has been little evaluation of different gel media for their ability to separate liposomes of different sizes. Huang (7) described the separation of vesicles of homogeneous size by using size exclusion chromatography on a gel with 4% agarose. Chen and

[1] Gel filtration traditionally is used to describe separation according to size on soft permeation media in aqueous solution (3). However, the term size exclusion chromatography is now recommended by the International Union of Pure and Applied Chemistry.

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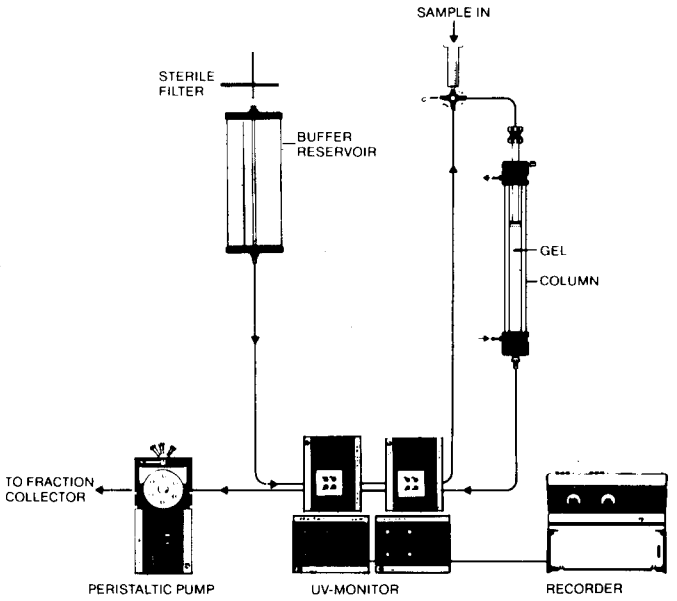

Figure 1—*Equipment for size exclusion chromatography.*

Schullery (8) suggested the separation of multilamellar vesicles from small unilamellar vesicles and sonication degradation products using 2% agarose gel.

This study investigated the ability of three different gels to separate liposomes into different fractions. Uncharged and negatively and positively charged liposomes were studied.

EXPERIMENTAL

Materials—The uncharged liposomes were composed of egg lecithin[2]–cholesterol[3], 41:12 μmoles (33:4.64 mg). The negatively charged liposomes were composed of egg lecithin–cholesterol–phosphatidic acid[4]–dicetyl phosphate[5], 41:12:(mol. wt. unknown):6 μmoles (33:4.46:10:3.24 mg). The positively charged liposomes were composed of egg lecithin–cholesterol–stearylamine[6], 41:12:6 μmoles (33:4.46:1.6 mg).

The buffer was 3 m*M* sodium phosphate[5] (pH 7.3), containing 0.9% NaCl; it was sterilized at 121° for 25 min. Two cross-linked gels of 2[7] and 6%[8] agarose and cross-linked dextran gel[9] were used. To determine the fractionating range ($V_0 \rightarrow V_t$) of the gel bed in one separate run, a test solution was prepared from *Serratia marcescens* (ATCC 14756), which was chemically sterilized with pyrocarbonic acid diethyl ester (V_0), and sodium azide was dissolved in the buffer (V_t). Thirty microorganisms and 1 mg of sodium azide/1 ml of buffer were used.

Preparation of Liposomal Dispersions—Positively and negatively charged and uncharged liposomes were prepared aseptically according to a reported method (9). All glassware in contact with the preparations was sterilized by dry heat. Lipids and amphiphiles were dissolved in 5–8 ml of chloroform[10] in a 50-ml flask. The air in the flask was replaced by nitrogen or argon gas filtered through a 0.2-μm membrane filter. The organic solvent was removed in a rotary evaporator[11] at 25°, and evaporation was continued for 30 min. A thin, uniform film was visible on the wall of the flask.

Nitrogen was flushed into the flask, and 0.1–0.2 ml of the buffer solution was added immediately. Disruption of the lipid film was made by gently shaking the flask manually. A milky dispersion resulted after rotation of the flask with 1.8–1.9 ml of buffer and a few sterile glass beads.

The dispersion was stored at 4° for 12–24 hr. Before further treatment, the dispersions were inspected for microbial contamination. Only sterile dispersions were used for further investigation.

Sonication—The probes and glassware in contact with the product were sterilized by heat at 160° for 2 hr. Samples were sonicated[12] under nitrogen flush at 0–2°. Liposomal dispersions were transferred from a milky dispersion into a colloidal state when the sonication time exceeded 3 min at an amplitude of 8 μm. A homogeneous colloidal state, *i.e.*, a clear solution, was obtained when sonication was continued for 6 min at 8 μm followed by 2 min at 4 μm. The solution was inspected visually in polarized light immediately after preparation and, for some of the dispersions, after a month. Visually, the colloidal state was unchanged.

Centrifugation—All sonicated samples were centrifuged[13] at 2000 rpm for 10 min to remove titanium particles and unidentified fragments. The supernates were collected for evaluation.

Absorption Spectra—The absorption of the liposome components, individually and admixed, and of sonicated liposomes was measured between 600 and 200 nm with a recording double-beam spectrophotometer[14]. The concentration of all samples was identical to the concentration used in the liposomes. For components insoluble in water, chloroform was used as the solvent and reference. For liposome dispersions, the buffer was used as the external phase and as a reference.

Size Exclusion Chromatography—Two cross-linked agarose (2 and 6%) gels and a cross-linked dextran gel were used. These gel media have an effective fractionation range for globular proteins with a molecular weight between 2×10^4 and 3×10^7 daltons, which was of the same order as the liposomes under investigation. All material that would come in contact with the liposomes was sterilized. The gels were autoclaved at 121° for 25 min and then packed into the columns[15] and eluted with the buffer.

Effluent absorption was measured continuously with two UV monitors[16] coupled in series. The first monitor measured UV absorption at 254 or 280 nm. The second monitor was equipped with a high transmittance filter[17], which transmitted the mercury line 546 nm. The 254–280- and 546-nm readings were used to distinguish liposomes of different sizes on an empirical basis. For optimal detector performance, buffer was flushed continuously through the reference cells before entering the column. The effluent was collected in fractions of 0.8–1.2 ml using a fraction collector[18]. The equipment for size exclusion chromatography and the coupling for optimal detector performance are shown in Fig. 1.

The elution characteristics of each gel were determined by eluting 1 ml of the test solution through the column at 20 ± 1°. Flow rates (Table I) were kept constant and were adjusted with a peristaltic pump[19] or hydrostatic pressure. Figure 2 shows the elution profile of the test solution. The void volume (V_0), the total volume (V_t), and the operational volume ($V_t - V_0$) for each gel were determined as indicated in Fig. 2. The column parameters are presented in Table I.

The distribution coefficients[20] (K_{av}) were calculated from the elution

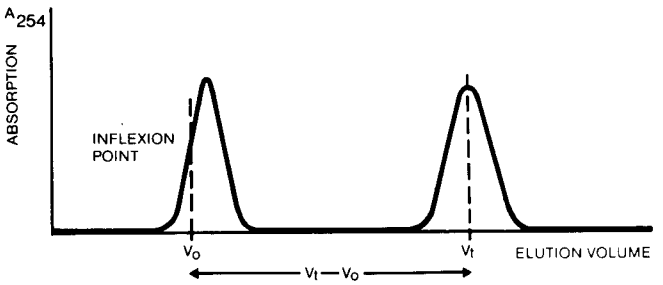

Figure 2—*Absorption profile for the test solution (*Serratia marcescens *and sodium azide).*

[2] Grade 1, Lipid Products, South Nutfield, England.
[3] BDH Biochemicals, Poole, England.
[4] Grade 1, Lipid Products, South Nutfield, England.
[5] Sigma Chemical Co., St. Louis, Mo.
[6] Fluka AG, Buchs, Switzerland.
[7] Sepharose C1-2B, Pharmacia Fine Chemicals, Uppsala, Sweden.
[8] Sepharose C1-6B, Pharmacia Fine Chemicals, Uppsala, Sweden.
[9] Sephacryl S-200, prepared by covalently cross-linking allyldextran with *N,N'*-methylenebis(acrylamide), Pharmacia Fine Chemicals, Uppsala, Sweden.
[10] Merck AG, Darmstadt, West Germany.
[11] Rotavapor-R, Büchi, Switzerland.
[12] Ultrasonic disintegrator, 150 w, amplitude 0–10 μm with 19-mm titanium probe, M.S.E., London, England.
[13] Multex, M.S.E., London, England.
[14] Hitachi Perkin-Elmer model 124.
[15] K16/20 and K16/70, Pharmacia Fine Chemicals, Uppsala, Sweden.
[16] Single-path monitor with recorder REC-2, Pharmacia Fine Chemicals, Uppsala, Sweden.
[17] Wratten No. 16.
[18] Stålprodukter, Uppsala, Sweden.
[19] P-3, Pharmacia Fine Chemicals, Uppsala, Sweden.
[20] The K_{av} value represents the fraction of the total pore volume of the bed that is available for penetration of a given solute species; V_t represents the total liquid volume of the gel bed according to the definition given. In the literature, both K_d and K_{av} are used to signify the fractional pore volume available and, alternatively, the fraction of the gel volume available.

Table I—Operational Parameters Used in Size Exclusion Chromatography

Gel Medium	Column Dimension, Internal Diameter × Height, cm	Gel Bed Height, cm	Bed Volume, ml	Flow Rate, ml/min	Void Volume (V_0), ml	Total Liquid Volume (V_t), ml	Operational Volume ($V_t - V_0$), ml
Cross-linked 2% agarose	1.6 × 70	63.2	127.0	0.12	38.4	124.2	85.8
Cross-linked 6% agarose	1.09 × 25	23.5	21.9	0.34	7.9	23.3	15.4
Cross-linked 6% agarose	1.6 × 20	14.2	28.5	1.22[a]	8.1	27.2	19.0
Cross-linked dextran	1.6 × 20	14.2	28.5	0.99[a]	9.4	26.6	17.2

[a] Flow regulated by hydrostatic pressure measured by volume–time measurements.

volume of the peaks of fractionated liposomes (V_e), the void volume, and the total liquid volume to yield $K_{av} = (V_e - V_0)/(V_t - V_0)$ (10). Newly packed columns or columns cleaned with a buffer solution of high ionic strength were eluted with three samples of liposomes and then washed with a volume of buffer equal to double the operational volume of the respective column before any test sample was applied. Thus, reproducible K_{av} values for the fractions were obtained. The liposomes were stored at 4–8° and kept at 20° for 2 hr before size exclusion chromatography. Size exclusion chromatography was performed within 24 hr after liposome preparation. Sample volumes were 1–2 ml.

RESULTS AND DISCUSSION

The liposomal components, when measured individually or admixed, displayed relatively low absorption in the 600–200-nm range. However, a low maximum or measurable absorbance was observed at 254 nm. The absorbance for lecithin was 0.4. The other components had much less absorbance at this wavelength. When the components were mixed, the absorbance at 254 nm decreased to 0.24. No explanation for this observation can be offered.

The absorption of liposome dispersions gradually increased from 600

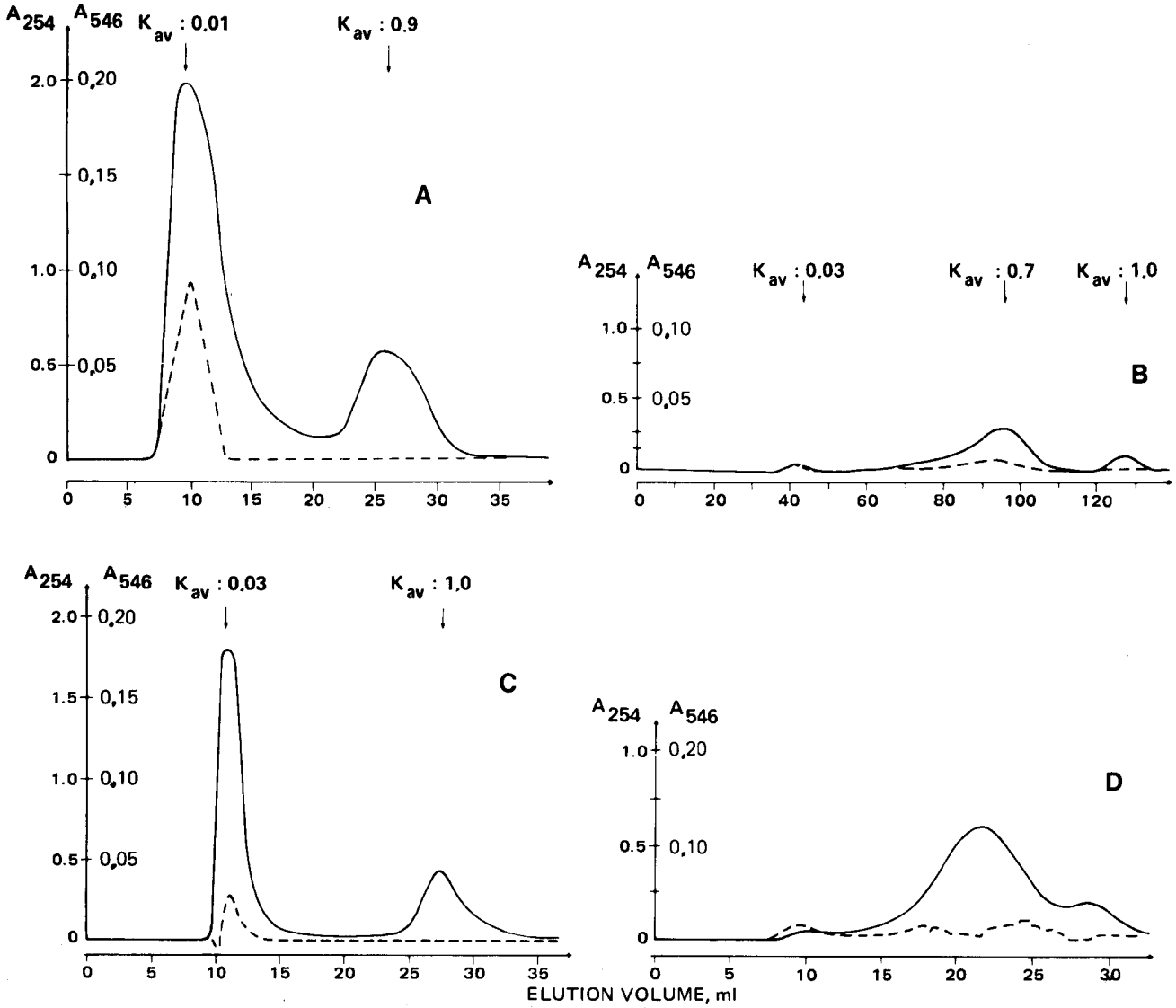

Figure 3—*Size exclusion chromatography of uncharged liposomes on different gel media. The eluent was phosphate buffer–saline (pH 7.3), and the temperature was 20°. Key: —, absorption profile at 254 nm, 0–2.0; and - - -, absorption profile at 546 nm, 0–0.2. A: Cross-linked 6% agarose—operational volume, 19.0 ml; sample volume, 1.5 ml; bed diameter, 1.6 cm; bed volume, 28.5 ml; and flow rate, 1.2 ml/min. B: Cross-linked 2% agarose—operational volume, 85.8 ml; sample volume, 1.5 ml; bed diameter, 1.6 cm; bed volume, 127.0 ml; and flow rate, 0.1 ml/min. C: Cross-linked dextran—operational volume, 17.2 ml; sample volume, 1.0 ml; bed diameter, 1.6 cm; bed volume, 28.5 ml; and flow rate, 1.0 ml/min. D: Cross-linked 2% agarose—operational volume, 26.3 ml; elution profile of uncharged liposomes; sample volume, 1.5 ml; and flow rate, 0.2 ml/min.*

Table II—Size Exclusion Chromatography of Liposomes on Different Gel Media: Population of Separated Liposomes in Percent of Total Peak Area at Different K_{av} Values [a]

Gel Medium and Operational Volume ($V_t - V_0$), ml	Absorption, nm	Uncharged			Negatively Charged			Positively Charged		
		K_{av1}	K_{av2}	K_{av3}	K_{av1}	K_{av2}	K_{av3}	K_{av1}	K_{av2}	K_{av3}
Cross-linked 2% agarose, 85.8		4	83	13	21	51	28	6	81	13
	280	(0.03)	(0.7)	(1.0)	(0.02)	(0.5)	(1.0)	(0.02)	(0.7)	(1.0)
	546	(0.03)	(0.6)	(—)	(0.02)	(0.5)	(—)	(0.02)	(0.6)	(—)
Cross-linked 6% agarose, 15.4		80	20	—	81	19	—	81	19	—
	280	(0.02)	(1.0)	(—)	(0.02)	(1.0)	(—)	(0.04)	(1.0)	(—)
	546	(0.01)	(—)	(—)	(—)	(—)	(—)	(—)	(—)	(—)
Cross-linked 6% agarose, 19.0		71	29	—	71	29	—	78	22	—
	280	(0.01)	(0.9)	(—)	(0.01)	(0.9)	(—)	(0.0)	(0.9)	(—)
	546	(0.05)	(—)	(—)	(0.05)	(—)	(—)	(0.03)	(—)	(—)
Cross-linked dextran, 17.2		72	28	—	75	25	—	72	28	—
	280	(0.03)	(1.0)	(—)	(0.05)	(1.1)	(—)	(0.06)	(1.1)	(—)
	546	(0.07)	(—)	(—)	(0.07)	(—)	(—)	(0.1)	(—)	(—)

[a] Different sizes of liposomes were separated and identified by K_{av} values; K_{av} given in parentheses.

to 200 nm. At 254 nm, the uncharged liposomes showed the lowest absorption of ~1.0. All charged liposomes had absorption of >2.0 at 254 nm. The measured absorption of liposomes was due to the light scattered by the particles and was not influenced by their individual components.

The two UV monitors for measuring the eluted peak profiles (Fig. 1) were used as turbidimeters since a decreased transmission was recorded.

Liposome fractions were separated by size, and these fractions were identified by their K_{av} values. Fraction percentages were obtained by weighing the peaks of the liposome absorption profile at 280 nm (Table II).

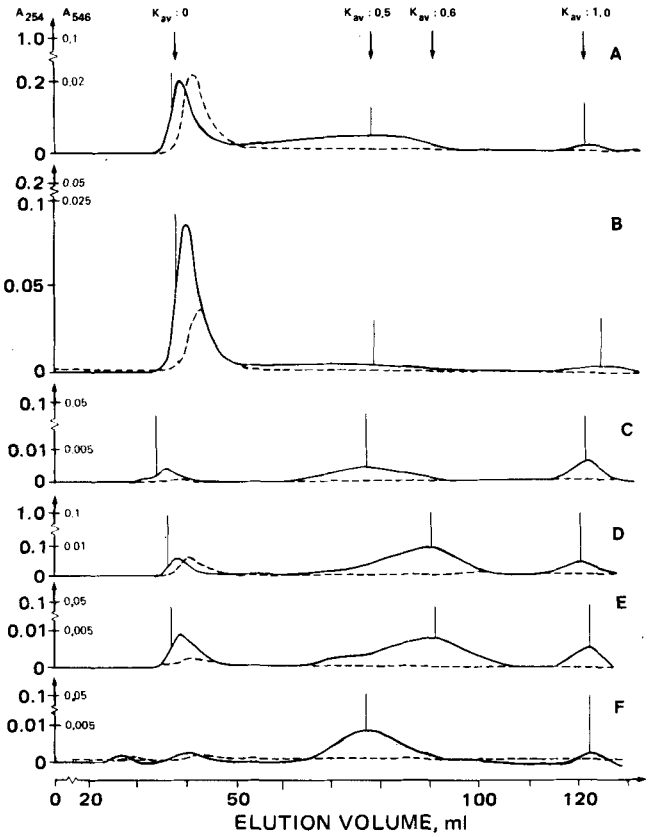

Figure 4—*Rechromatography of peak maxima of liposomes on cross-linked 2% agarose: operational volume, 88 ml; bed diameter, 1.6 cm; bed volume, 127 ml; flow rate, 0.19 ml/min; eluent, phosphate buffer–saline (pH 7.3), and temperature, 20°. Key: —, absorption profile at 254 nm; and - - -, absorption profile at 546 nm. A: Size exclusion chromatography of negatively charged liposomes (sample volume, 1.0 ml). B and C: Rechromatography of Peaks 1 (for B, sample volume was 2.0 ml) and 2 (for C, sample volume was 2.0 ml) from separated top fractions of negatively charged liposomes in A. D: Size exclusion chromatography of positively charged liposomes (sample volume, 1.0 ml). E and F: Rechromatography of Peaks 1 (for E, sample volume was 2.0 ml) and 2 (for F, sample volume was 2.0 ml) from separated top fractions of positively charged liposomes in D.*

Two fractions of uncharged, positively charged, and negatively charged liposomes were separated on cross-linked 6% agarose and cross-linked dextran. The largest fraction was eluted in the void volume (K_{av} 0.05), and the other fraction was eluted in the total volume (K_{av} 1.00). On cross-linked 2% agarose, uncharged and negatively and positively charged liposomes were separated into three fractions. The separation properties of different gel media for uncharged liposomes are shown in Fig. 3. Of cross-linked 6% agarose and cross-linked dextran, baseline separation for all liposomes could be obtained only with cross-linked dextran (Fig. 3C). Baseline separation into three different fractions was obtained for all liposomes with cross-linked 2% agarose with an operational volume of 80 ml (Fig. 3B). As the operational volume of the column was reduced (Fig. 3D), the peaks became incompletely separated.

In additional experiments with negatively and positively charged liposomes, fractions from the maxima of the first and second peaks (Fig. 4) were rechromatographed separately on the same gel (cross-linked 2% agarose). The first peak (K_{av} 0.0) was eluted at the same position, but additional smaller peaks were obtained at K_{av} 0.5–0.6 and 1.0. The second peak (K_{av} 0.5–0.6) also emerged at the same position but with small peaks at K_{av} 0.0 and 1.0. Similar results were observed for uncharged liposomes. The position of the peaks indicates that the separation properties of the gel were unchanged. The appearance of extra peaks may be due to a number of factors such as incomplete separation of liposomes, deformation of the liposomes when penetrating the gel pores, or a natural transfer of liposomes into an equilibrium state of different sizes. This observation indicates that size exclusion chromatography of liposome-entrapped material might lead to a loss of some entrapped material.

Chen and Schullery (8) also found three peaks when egg lecithin vesicles were separated on soft 2% agarose gel. The content of these peaks was found to be nonhomogeneous unless the liposomes were in rapid equilibrium. However, these investigators did not obtain a baseline separation. The assumption from the present study that the fractions obtained are homogeneous is supported by Huang (7), who concluded that a homogeneous fraction of vesicles is obtained on rechromatography on soft 4% agarose gel provided no equilibrium between fractions occurs.

Gregoriadis (11) suggested that the need for liposomes to persist in the blood for some time favors the small unilamellar version, indicating that the separation of liposomes into different sizes may be of great importance.

In conclusion, the experiments showed that macroporous agarose gels can be used to separate liposomes into well-defined fractions. These fractions will be investigated further by small-angle X-ray-scattering techniques.

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High-Performance Liquid Chromatographic Determination of Components of Bleomycin Preparations

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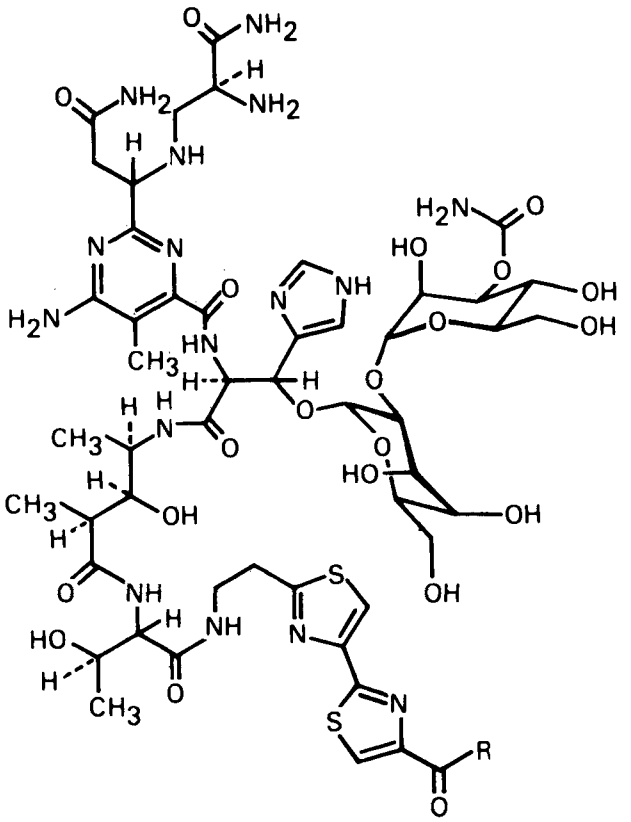
Abstract □ A fast and sensitive method was developed for the quantitative determination of at least 10 components of pharmaceutical bleomycin sulfate preparations. The method is based on the reversed-phase high-performance liquid chromatographic (HPLC) separation of the components on a μBondapak C_{18} column with a mobile phase having a linear gradient of 10–40% methanol in aqueous 0.005 *M* 1-pentanesulfonic acid at pH 4.3. With this assay, the average standard deviations for components A_2 and B_2 are 0.92 and 0.87, respectively, for a 7.5–22.5 × 10^{-3}-mg sample. Regulatory agencies presently use the official Code of Federal Regulations (CFR) method, which is based on CM-Sephadex column chromatography. It was demonstrated that this CFR method does not separate the bleomycin A_2 component from some other minor bleomycin components. After elution from the CM-Sephadex column, the "A_2 component" was separated into five components by the HPLC method. Bleomycin A_2 is stable under these HPLC conditions.

Keyphrases □ High-performance liquid chromatography—reversed phase, bleomycin assay, separation of bleomycin components □ Bleomycin—high-performance liquid chromatographic determination, separation of components □ Antineoplastics—bleomycin, high-performance liquid chromatographic analysis

Bleomycins (I) are a family of glycopeptide antibiotics obtained from the fermentation broth of *Streptomyces verticillus* (1). Bleomycins are effective against various human neoplasms, particularly against squamous cell carcinoma, sarcoma, and malignant lymphoma (2–4). Preparations used in the treatment of these neoplasms contain several bleomycins. The main components are bleomycins A_2 and B_2. Some minor components such as bleomycin acid, demethylbleomycin-A_2, and bleomycin B_4 also are present. Among these minor components, bleomycin B_4 is the most toxic (5).

The Code of Federal Regulations (CFR) (6) requires that bleomycin preparations for therapeutic use meet the following composition specifications: bleomycin A_2, not less than 60% and not more than 70%; bleomycin B_2, not less than 25% and not more than 32%; bleomycin B_4, not more than 1%; and bleomycins A_2 and B_2 combined, not less than 90% of the total bleomycins. The present established method is based on a lengthy CM-Sephadex column chromatographic procedure, essentially that described by Fujii *et al.* (7).

Reported high-performance liquid chromatographic (HPLC) methods were unsuitable for routine quantitative analysis. With one method (8), baseline resolution was incomplete; another method (9) was not designed for quantitative purposes; and a third method (10), which could be used to determine bleomycins A_2 and B_2, did not



bleomycin	R
demethyl-A_2	NH—CH_2—CH_2—CH_2—S—CH_3
A_2	NH—CH_2—CH_2—CH_2—S=$(CH_3)_2$
acid	OH
B_2	NH—$(CH_2)_4$—NH—C(=NH)—NH_2
B_4	NH—$(CH_2)_4$—NH—C(=NH)—NH—$(CH_2)_4$—NH—C(=NH)—NH_2
A_5	NH—$(CH_2)_3$—NH—$(CH_2)_4$—NH_2

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