Assay of Tocainide in Blood by High-Pressure Liquid Chromatography

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Abstract □ A sensitive, specific, high-pressure liquid chromatographic assay for the determination of tocainide in whole blood is described. The residue from a methylene chloride extract of alkalinized blood was resolvated in a mobile phase of methanol–water (47:53) containing 1% acetic acid and 6.16 m*M* 1-octanesulfonic acid, adjusted to pH 4.0. Chromatography was performed on a reversed-phase column with detection at 254 or 225 nm. The limits of accurate measurement were 2 μg/ml for a 1-ml blood sample monitored at 254 nm and 0.2 μg/ml for a 2-ml sample monitored at 225 nm. The assay was tested on samples from emergency protocol patients and was also found suitable for single-dose pharmacokinetic studies.

Keyphrases □ Tocainide—high-pressure liquid chromatographic analysis in blood □ High-pressure liquid chromatography—analysis, tocainide in blood □ Cardiac depressants—tocainide, high-pressure liquid chromatographic analysis in blood

Tocainide, 2-amino-*N*-(2,6-dimethylphenyl)propanamide (I), is an oral antiarrhythmic agent currently under investigation. Its effectiveness, oral route of administration, long half-life (12 hr), and low order of toxicity indicate that this agent may have advantages over other existing antiarrhythmic agents (1–3).

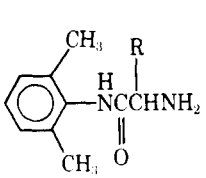
GLC assays for tocainide have used heptafluorobutyrate (4) and trifluoroacetamide (1) derivatives. Tocainide also has been assayed by high-pressure liquid chromatography (HPLC), but the drug required derivatization with dansyl chloride to be detectable with precision (5).

This report describes a new HPLC assay that requires no derivatization and that has a high degree of precision, linearity, and reproducibility in the range of therapeutic and subtherapeutic concentrations.

EXPERIMENTAL

Reagents and Materials—Tocainide and the internal standards, *N*-(2,6-dimethylphenyl)-2-aminobutanamide (II) and glycinexylidide (III), were obtained in purified form as the hydrochloride salts[1]. ^{3}H-Tocainide, of 97.5% radiochemical purity, was obtained with the label in the 4-position on the aromatic ring[2]. 1-Octanesulfonic acid was supplied as the sodium salt and used without further purification[3].

Methanol and methylene chloride were supplied and used as triple-distilled solvents[4]. Water was distilled and deionized by ion-exchange resins, filtered through charcoal, and passed through a 0.45-μm filter prior to use. All other reagents were analytical grade or better.



I: R = CH_3
II: R = CH_2CH_3
III: R = H

Instrumentation—The liquid chromatograph consisted of a high-pressure pump[5], a valve and loop injector[6], a 10-liter water bath with a circulating heater, a fixed wavelength UV detector[7], and a variable wavelength detector[8]. The solvent from the mobile phase reservoir was filtered through a 30-μm inlet filter[9]. A 2-μm filter[10] and 0.5-μm filter[11] in series were placed between the pump outlet and the injection valve inlet to remove any remaining particulate matter from the solvent.

Separations were done on a 30 × 4-mm i.d. column containing 10-μm silica with bonded octadecylsilane[12]. Chromatograms were produced on a strip-chart recorder[13] with 2-, 5-, and 10-mv inputs.

Chromatographic Conditions—The mobile phase was pumped through the column at 2.0 ml/min. The back-pressure of the system was 3500 psig. The column effluent was monitored at either 254 nm with the fixed wavelength detector or at 225 nm with the variable wavelength detector. Columns were flushed each morning with mobile phase for 1 hr at 1.0 ml/min prior to sample analysis. For clinical specimens run at 254 nm, the 0.02 absorbance scale was expanded to 0.01 and 0.004 by recording the 10-mv detector output on the 5- and 2-mv scales of the recorder, respectively. The column water bath was maintained at 25 ± 0.2°.

The mobile phase was prepared by combining 47 parts of a methanolic solution with 53 parts of an aqueous solution. Each solution contained 6.16 m*M* 1-octanesulfonic acid and 1% (v/v) acetic acid. The methanolic solution was adjusted to pH 4.0 with 1.0 *N* KOH in methanol, and the aqueous solution was adjusted to the same pH with 5.0 *N* NaOH in water. Acetonitrile (1%) was added to the aqueous component as a preservative.

Extraction—The extraction was done in 15-ml conical bottom centrifuge tubes capped with polyethylene stoppers.

The extraction mixture consisted of 1.0 or 2.0 ml of sample, 1.0 ml of internal standard, 1.0 ml of 1.0 *N* NaOH, enough water to bring the volume of the aqueous phase to 5.0 ml, and 5.0 ml of methylene chloride. The two phases were rotary mixed[14] for 15 min and separated by centrifugation[15] at 500 rpm for 10 min. The aqueous (upper) phase and coagulated proteins at the interface were removed by vacuum aspiration. The methylene chloride was poured into a 16 × 100-mm glass culture tube and evaporated at approximately 55° in a water bath.

The extracts were stored at room temperature (not more than 6 hr) until reconstituted with 50 μl of HPLC mobile phase. The extracted drug was solvated by vortexing[16] for 30 sec and sonicating[17] for 1 min. Aliquots of 20 μl were chromatographed.

Assay Reproducibility and Linearity—The linearity and precision of the assay in the 5–15-μg/ml range were determined by adding known amounts of tocainide hydrochloride to pooled human plasma, extracting 1.0-ml aliquots, and monitoring the chromatographic eluant at 254 nm. To test the subtherapeutic range (0.5–5.0 μg/ml), microliter volumes of a tocainide stock solution were added to 2-ml aliquots of pooled blood. The samples were extracted and chromatographed with detection at 225 nm. The slope, intercept, and correlation coefficient were determined

[1] Astra Pharmaceutical Products, Framingham, Mass.
[2] New England Nuclear, Boston, Mass.
[3] Eastman Organic Chemical, Rochester, N.Y.
[4] Burdick & Jackson Laboratories, Muskegon, Mich.
[5] Model M6000, Waters Associates, Milford, Mass.
[6] Model CV-6-UHPa-N60, Valco Valve, Houston, Tex.
[7] Model 202, Waters Associates, Milford, Mass.
[8] Model SF 770, Schoeffel Instruments, Westwood, N.J.
[9] Inlet solvent assembly filter 25551, Waters Associates, Milford, Mass.
[10] SS-2-F-2 Swagelok, Crawford Fitting Co., Cleveland, Ohio.
[11] SS-200-6-1-SR12 Swagelok, Crawford Fitting Co., Cleveland, Ohio.
[12] μBondapak C_{18}, Waters Associates, Milford, Mass.
[13] Model DSRG, Sargent Welch Scientific Co., Skokie, Ill.
[14] Rotary mixer, Drummond Scientific, Broomall, Pa.
[15] Model H-NS centrifuge, International Equipment Co., Needham Heights, Mass.
[16] Model K-550-G Vortex Genie, Scientific Instruments, Springfield, Mass.
[17] Model 220, Branson Cleaning Equipment Co., Shelton, Conn.

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Table I—UV Spectrum of Tocainide Hydrochloride in Distilled Water [a]

Wavelength, nm	19.9-μg/ml Absorbance	125-μg/ml Absorbance
200	—	—
205	1.25	—
210	0.960	—
215	0.720	—
220	0.450	—
225	0.280	—
230	0.190	1.37
235	0.120	0.850
240	0.070	0.480
245	0.040	0.370
250	0.035	0.200
255	0.025	0.190
260	0.025	0.215
265	0.025	0.230
270	0.025	0.190
275	0.010	0.050
280	0.000	0.020

[a] Spectrum was taken on a Perkin-Elmer model 202 UV-visible spectrophotometer with the slit at 25 and a 1-cm light path.

by a least-squares regression analysis.

Daily Calibration Curves and Analysis—A four-point calibration curve was run with each day's set of samples. The levels of internal standard were 10 μg/ml for clinical samples and 1.0 μg/ml for pharmacokinetic studies. The slope, intercept, and correlation coefficient were determined by a least-squares regression as already described.

Interferences—Stock solutions of the following pure drug substances were prepared in the mobile phase, and their retention times relative to tocainide were determined: procainamide, *N*-acetylprocainamide, quinidine, dihydroquinidine, lidocaine, theophylline, caffeine, diazepam, and chlordiazepoxide.

Also chromatographed were intravenous solutions of other common drugs containing no preservatives, including isoproterenol, epinephrine, levarterenol, propranolol, phenytoin, penicillin G, and furosemide. Compounds that were isographic with either tocainide or the internal standard were tested for their extractability from alkalinized blood with methylene chloride.

Blood Level Study—Tocainide was administered to patients with life-threatening arrhythmias. To monitor blood levels and to adjust the dose, samples of venous blood were periodically drawn in heparinized containers[18]. Tocainide, 400 mg, also was given to a normal volunteer fasted 12 hr prior to administration. Samples were drawn at appropriate intervals for 48 hr after dosing. Blood and plasma samples were stored at 4° until assayed.

Partition and Recovery—^{3}H-Tocainide was used to determine the partition coefficient and absolute recovery of the extraction procedure. A solution of 5.0 μg of tocainide/ml containing 0.5 μg of labeled species/ml was extracted by the described method. The radioactivity of the organic phase, aqueous phase, precipitated proteins, and reconstituted extract was determined by scintillation counting.

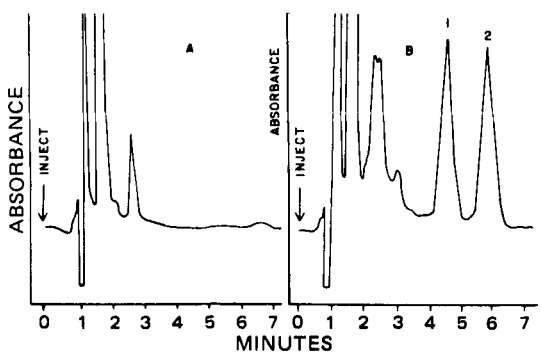

Figure 1—*A: extract of drug-free blood. B: patient sample containing 5 μg of tocainide/ml. Key: peak 1, tocainide; and peak 2, 2-amino-N-(2,6-dimethylphenyl)butanamide. HPLC conditions were: flow, 2.0 ml/min; detection, 254 nm; and 0.01 aufs.*

[18] Vacutainers, Becton-Dickinson, Rutherford, N.J.

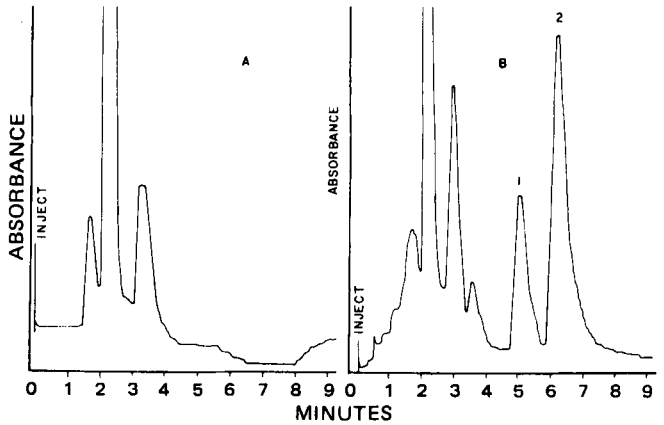

Figure 2—*A: extract of drug-free blood. B: sample from normal volunteer containing 1 μg of tocainide/ml. Key: peak 1, tocainide; and peak 2, 2-amino-N-(2,6-dimethylphenyl)butanamide. HPLC conditions were: flow, 2.4 ml/min; detection, 225 nm; and 0.02 aufs.*

The walls of both the extraction and evaporation tubes were etched with a solution containing 0.5 *M* HNO_3 and 0.5 *M* KF, and the etchant was checked for radioactivity. A quench correction (6) was calculated by adding a known amount of ^{3}H-tocainide to each scintillation vial and recounting the samples.

RESULTS AND DISCUSSION

Tocainide may be detected by UV absorbance at wavelengths below 275 nm (Table I). Figures 1 and 2 show chromatograms of samples monitored at 254 and 225 nm, respectively. Detection at 225 nm yielded a ninefold increase in sensitivity over detection at 254 nm (Fig. 3). Detection at 254 nm had a limit of useful measurement of 2.0 μg/ml for a 1.0-ml sample and was adequate for the monitoring of blood levels in the therapeutic range (4–10 μg/ml) (3). In pharmacokinetic studies, a subtherapeutic dose was administered and blood levels did not exceed 2.0 μg/ml. These levels were measured at 225 nm.

Most clinical data were obtained by monitoring at 254 nm. Use of 225 nm was a compromise between increased sensitivity and ability to null out the background absorbance of the mobile phase. This wavelength is presently recommended for the measurement of both therapeutic and subtherapeutic levels. The 225-nm setting also offered increased specificity. In several patient samples, a large peak at 254 nm interfered with quantitation of the internal standard but did not interfere at 225 nm.

The data from two standard curves are summarized in Table II. The

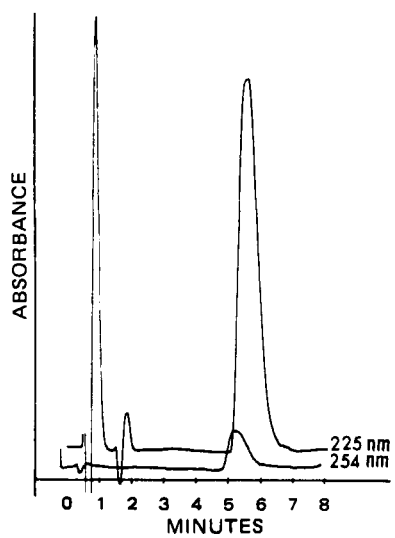

Figure 3—*Comparison of detection response at 254 and 225 nm. Both detectors were operated at 0.04 aufs; 4.0 μg of tocainide was injected. The flow was 2.0 ml/min.*

Table II—Standard Curves: Tocainide to Internal Standard Peak Height Ratios *versus* Micrograms per Milliliter Extracted

Blood Tocainide Concentration, μg/ml	Peak Height Ratio[a], Mean ± *SD* (n = 5)	*CV*, %
15[b]	2.366 ± 0.0928	3.9
10[b]	1.570 ± 0.0507	3.2
7.5[b]	1.101 ± 0.0500	4.5
5.0[b]	0.695 ± 0.025	3.6
5.0[c]	3.22 ± 0.111	3.4
1.5[c]	0.936 ± 0.0361	3.9
1.0[c]	0.642 ± 0.0248	3.9
0.5[c]	0.3470 ± 0.0214	6.2

[a] Internal standard was 2-amino-*N*-(2,6-dimethylphenyl)butanamide. [b] Column effluent was monitored at 254 nm; internal standard = 10 μg/ml. [c] Column effluent was monitored at 225 nm; internal standard = 2 μg/ml.

Table III—Mean Coefficient of Variation Calculated from Duplicate Analysis of Samples Monitored at 254 nm

Number of Samples, N	Concentration Range of Means, μg/ml	Mean *CV*[a], %
3	0.0–1.0	34
11	1.0–2.0	23
4	2.0–3.0	10
18	3.0–4.0	5.7
14	4.0–5.0	5.2
8	5.0–6.0	6.6
11	6.0–7.0	3.9
8	7.0–8.0	5.6
3	8.0–9.0	2.9
11	9.0–13.0	3.3

[a] Calculated from the formula:

$$\overline{CV} = \frac{(100)}{(N)}\sqrt{2}\sum_{i=1}^{N}\frac{|(A_i - B_i)|}{|(A_i + B_i)|}$$

where A_i and B_i are duplicate extractions of the same sample.

coefficients of variation were less than 4.5% in the 1.0–15-μg/ml range and only 6.2% at 0.5 μg/ml. Both curves were linear, with correlation coefficients of 0.99.

Daily four-point calibration curves showed a similar degree of linearity and reproducibility at 254 nm. The average slope for 20 calibration curves in the 5.0–15.0-μg/ml range was 0.1365 ± 0.0148 (mean ± *SD*), and the correlation coefficient ranged from 0.981 to 0.999. At 225 nm, the average slope over the 0.5–5.0-μg/ml range was 0.668 ± 0.078 (mean ± *SD*, n = 11), and the minimum correlation coefficient was 0.978. In Tables III and IV, the mean coefficients of variation as calculated from replicate patient samples provide a measure of the reproducibility that may be expected from actual samples rather than from spiked blood.

The partition coefficient for the organic to alkaline aqueous phases was determined from the extraction of ^{3}H-tocainide. No statistical difference was found between the extraction of blood or plasma. The mean partition coefficient was 0.64 ± 0.01 (mean ± *SD*, n = 4). Negligible (2%) quantities of tocainide adhered to the precipitated proteins and the walls of the extraction tubes. Sixty-three percent of the extracted drug was recovered upon reconstitution by the mobile phase. The balance was adsorbed to the evaporation tube.

Table IV—Mean Coefficient of Variation Calculated from Duplicate Analysis of Samples Monitored at 225 nm

Number of Samples, N	Concentration Range of Mean, μg/ml	Mean *CV*[a], %
8	0.2–0.4	5.9
10	0.4–0.6	6.0
18	0.6–0.8	6.0
43	0.8–1.0	3.0
43	1.0–1.2	8.1
27	1.2–1.4	8.2
34	1.4–1.6	6.8
62	1.6–1.8	9.9
20	1.8–2.0	13.7

[a] Same as footnote *a* in Table III.

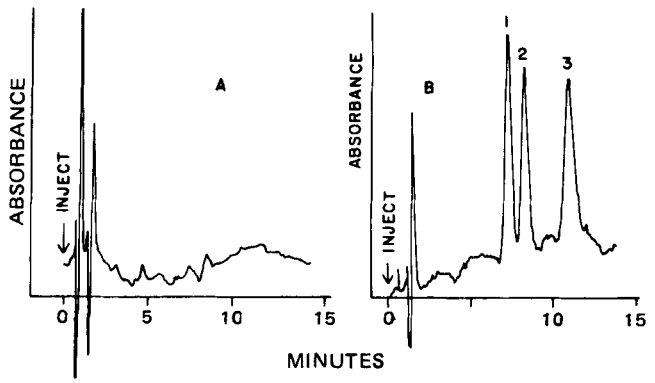

Figure 4—*Use of glycinexylidide as an alternative internal standard for analysis of samples from patients receiving both tocainide and lidocaine. A: extract of drug-free blood. B: extract of samples containing 5 μg/ml each of tocainide (peak 2), lidocaine (peak 3), and glycinexylidide (peak 1). HPLC conditions were: flow, 2.0 ml/min; detector, 225 nm; and 0.04 aufs.*

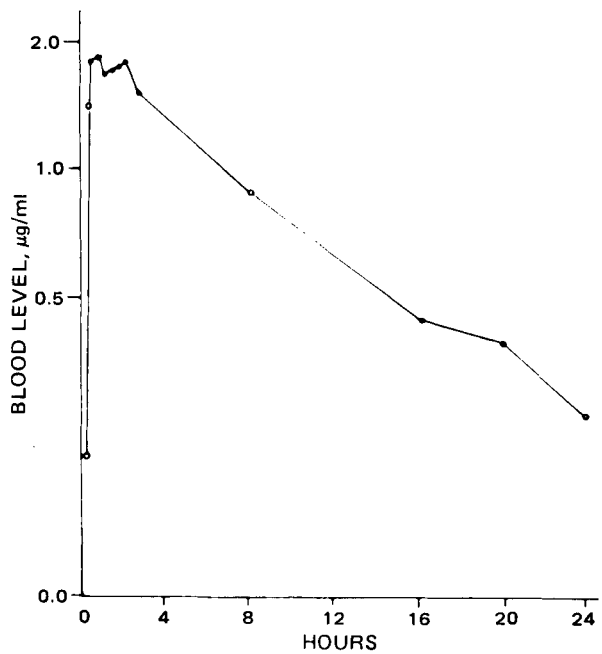

Figure 5—*Blood levels curve for a normal volunteer given 400 mg po of tocainide hydrochloride* ($t_{1/2}$ = ~8 *hr*).

None of the drugs evaluated for interference coeluted with tocainide. Lidocaine and phenytoin, however, were isographic with the internal standard.

Lidocaine, but not phenytoin, was extracted with the described method. For samples containing both lidocaine and tocainide, another internal standard, glycinexylidide (Fig. 4), was used. This compound was resolved from tocainide and other substances in a solvent system of methanol–water (40:60) with the same concentrations of 1-octanesulfonic acid and acetic acid and the same pH.

For samples without lidocaine, 2-amino-*N*-(2,6-dimethylphenyl)-butanamide was the internal standard of choice. Elution times were shorter, and the larger peak height to peak width ratio for a fixed amount injected produced increased assay sensitivity.

A typical blood level curve is shown in Fig. 5. The drug appears to have a rapid phase of distribution and a terminal half-life of approximately 8 hr.

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New Microporous Cholestyramine Analog for Treatment of Hypercholesterolemia

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Abstract □ A new, microporous, uniformly reticulated preparation of cholestyramine is described. The preparation, cholpor, has a higher exchange capacity for chloride than does cholestyramine and swells very little in water. It is 15–20% more potent than cholestyramine in the *in vitro* binding of sodium cholate; moreover, the binding velocity is considerably higher than that of cholestyramine. Colestipol hydrochloride, also used as a reference anion-exchange resin, is about half as potent as the other two resins; its binding velocity is similar to that of cholpor. Cholpor may be prepared in a suspension form of good palatability. Preliminary clinical findings in short-term trials showed a cholesterol-lowering effect similar to that of cholestyramine with lower doses and fewer side effects.

Keyphrases □ Cholestyramine analog—cholpor synthesized, *in vitro* binding of sodium cholate and cholesterol lowering effect in humans evaluated □ Cholpor—cholestyramine analog synthesized, *in vitro* binding of sodium cholate and cholesterol lowering effect in humans evaluated □ Antihypercholesterolemic activity—cholestyramine analog synthesized, *in vitro* binding of sodium cholate and cholesterol lowering effect in humans evaluated

Lowering of plasma cholesterol appears to be a major goal in the prevention of atherosclerosis (1). Anion-exchange resins are the most effective pharmaceutical agents in the clinical management of hypercholesterolemia (2); cholestyramine[1], a styrene-divinylbenzene copolymer with a free quaternary ammonium group, is currently the most widely used agent. Although the clinical efficacy of cholestyramine is not disputed, its unpleasant taste as well as the difficulty encountered by patients in mixing it with water upon each administration makes continued treatments rather difficult (3). The newer anion-exchange resin, colestipol hydrochloride[2] (4), is better tolerated but also requires several daily extemporary preparations.

This report describes a new microporous analog of cholestyramine, cholpor, which may be prepared in suspension form, is of good palatability, and is easily accessible to the patient.

EXPERIMENTAL

Analytical Methods—Swelling of the resins in water was determined by adding 1 g of cholestyramine[1], colestipol hydrochloride[2], or cholpor to enough water (10–30 ml) to provide a suspension. After 24 hr, the suspension was shaken and the volume of resin under water was determined.

Exchange Capacity—Chloride-ion absorption from a 5% NaCl solution, after regeneration of the resin with a 4% NaOH[3] solution, was measured by titrating with 0.1 *N* HCl[3] the sodium hydroxide resulting from the resin and sodium chloride reaction.

Pharmacology—Binding of sodium cholate[4] *in vitro* was estimated essentially according to Whiteside *et al.* (5). Preparations of cholestyramine (100–200 mesh), colestipol hydrochloride (60–150 mesh), and cholpor (60–80 mesh) were added to scintillation vials[5] containing 10 ml of 0.02 *M* phosphate buffer with 2 mg of sodium cholate/ml.

The significance of the differences in the various experiments was determined by the Student *t* test.

RESULTS AND DISCUSSION

Chemistry—Cholpor (6) is obtained by starting with a porous matrix. Porosity is first achieved during preparation of the copolymer. During chloromethylation, porosity is protected by maintaining reaction conditions that do not alter the physical structure of the matrix (low reaction temperature, low acidity of the catalysts, and marked swelling of the polymer chains with appropriate solvents).

Before polymerization, a mixture of compatible organic substances, chemically inert, is added to the monomers. During polymerization, this "porogenic" mixture regulates the molecular weights of the growing monomeric chains by causing precipitation when the chain length exceeds solubility. The copolymer precipitates in the form of microspheres, which, in the presence of the last nonreacted monomers, stably bind to each other. This copolymer is thus composed of numerous microspheres, and the empty space between one microsphere and another constitutes porosity.

Another function of the porogenic mixture is to avoid cross-linking of the macromolecules, allowing a uniform reticulation. Cross-linking inside the porous cavities would alter them, so that the specific properties of the resin, *i.e.*, velocity of diffusion, particularly important in the macroanion exchange, would be lost.

To achieve porosity in cholpor, several methods were tried (6). One method used squalane[3]–1-octanol[3]–paraffin[3] (mp 42–45°) (70:20:10), with the ratio of monomers to porogens being 1.2:1.1. The porogenic mixture, following copolymerization, was removed by prolonged extraction in a soxhlet extractor with a swelling solvent mixture (ether[3]–methylene chloride[3]). Proof of the completed extraction was provided by the final measurement of the extracted porogenic mixture. The general charac-

[1] Cuemid, courtesy of Merck Sharp & Dohme, Rahway, N.J.
[2] Colestid, courtesy of The Upjohn Co., Kalamazoo, Mich.
[3] Carlo Erba, Milan, Italy.
[4] Merck, Darmstadt, West Germany.
[5] Packard Instruments, Downers Grove, Ill.

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