Radioimmunoassay of Carteolol in Human Plasma

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Received July 7, 1980, from the **Drug Metabolism Department and the ‡Radio-Labeling Service Department, Abbott Laboratories, North Chicago, IL 60064.* Accepted for publication February 12, 1981.

Abstract □ A radioimmunoassay for the direct measurement of carteolol, a new β-adrenoreceptor blocker, in human plasma was developed. Carteolol was acylated to form *O*-glutarylcarteolol, which was conjugated to bovine serum albumin to provide the immunogen. Antibody to carteolol was raised in New Zealand albino rabbits. The tracer was the radioiodinated derivative of *O*-glutarylcarteolol–tyrosine methyl ester conjugate. The method is highly sensitive, with a lower quantifiable concentration of ~0.4 ng of carteolol/ml using 0.1 ml of plasma, and has good specificity, with the major metabolite (8-hydroxycarteolol) showing only 0.2% cross-reactivity. It is reproducible, with relative standard deviations from triplicate standard curves being mostly within ±8%. The method is currently being used to monitor carteolol levels in clinical samples.

Keyphrases □ Radioimmunoassay—determination of carteolol in human plasma □ Carteolol—determination by radioimmunoassay, human plasma □ Antiadrenergics—carteolol, determination by radioimmunoassay, human plasma

Carteolol hydrochloride[1], 5-[3-(*tert*-butylamino)-2-hydroxypropoxy]-3,4-dihydrocarbostyril monohydrochloride, is a β-adrenoreceptor blocker currently under development as a candidate drug. A simple and sensitive fluorometric method was reported (1, 2) for the determination of its concentration in biological fluids. The lower quantifiable concentration was ~20 ng of carteolol (free base)/ml using 2 ml of plasma. This sensitivity is inadequate, especially for pharmacokinetic studies at anticipated therapeutic doses (*e.g.*, <20 mg qid). To provide the needed sensitivity, development of a radioimmunoassay for carteolol was undertaken. Antibody to carteolol was raised in rabbits by immunizing the animals with an immunogen prepared by covalently incorporating carteolol into bovine serum albumin. Radioimmunoassay using this antibody offered great sensitivity and specificity for the measurement of carteolol in plasma.

EXPERIMENTAL

Chemicals and Materials—Commercial reagent grade solvents and chemicals were used. Chloramine-T solution (2.5 mg/ml) was prepared in 0.05 *M* phosphate buffer (pH 7.5). A 3.5-mg/ml solution of sodium metabisulfite also was prepared in the same buffer. The phosphate-buffered saline (saline buffer) was composed of 0.15 *M* NaCl and 0.01 *M* NaH_2PO_4–K_2HPO_4, pH 7.4.

Chromatographic Analysis—TLC was conducted using analytical (250 μm thick) and preparative (1000 μm) silica gel plates. The solvent system was usually ethyl acetate or chloroform–ethanol (2:1 v/v).

High-pressure liquid chromatographic (HPLC) separations were carried out using ion-pair or soap chromatography on reversed-phase columns[2]. The eluent for soap chromatography (soap eluent) was composed of 45% (v/v) acetonitrile, 0.02 *M* phosphoric acid, 0.02 *M* monobasic ammonium phosphate, and 0.15% (w/v) sodium lauryl sulfate. The typical ion-pair eluent was composed of 20% (v/v) acetonitrile, 10% tetrahydrofuran, 10% methanol, and 0.005 *M* *d*-camphorsulfonic acid. Preparative HPLC was conducted using a μBondapak C_{18} column[3] (7.8 mm i.d.)

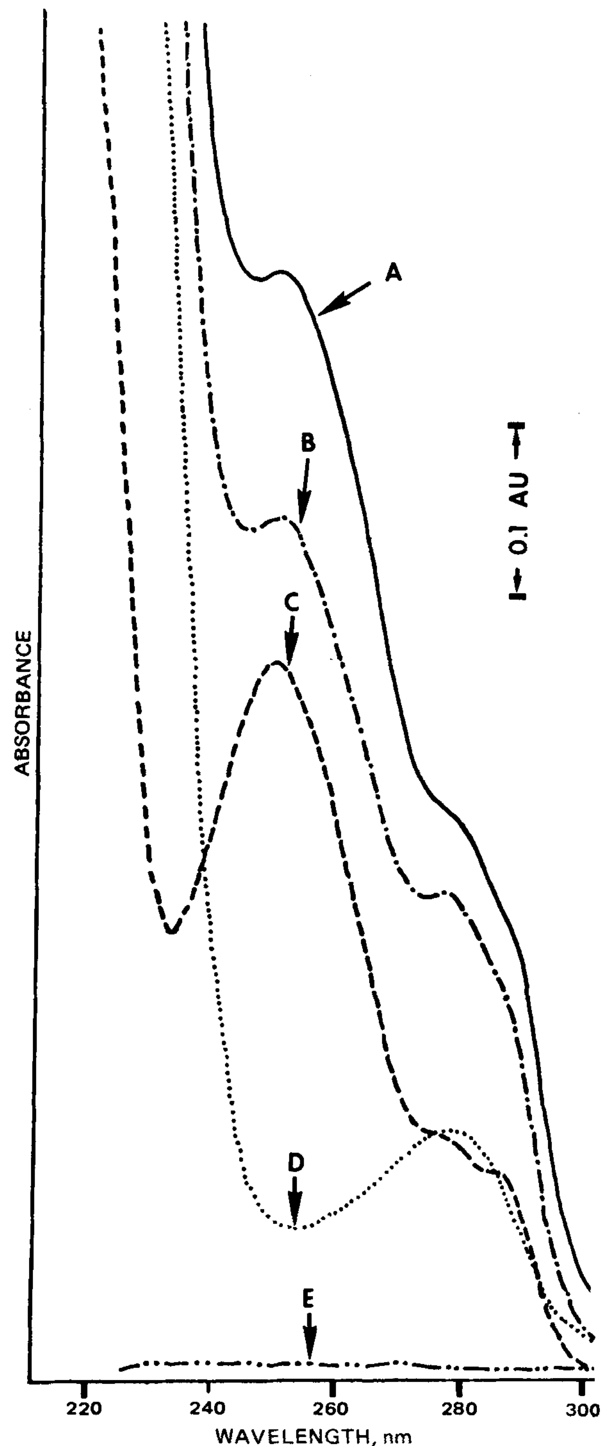

Figure 1—*UV absorption spectra of carteolol immunogen (A), mixture of I (20.6 μg/ml) and bovine serum albumin (242 μg/ml) (B), I (20.6 μg/ml) (C), bovine serum albumin (242 μg/ml) (D), and 0.05* M *tromethamine (pH 8.5) as background (E).*

[1] Abbott Laboratories, North Chicago, Ill.
[2] Zorbax C-8 (DuPont, Wilmington, Del.) or Spherisorb 5-μm ODS (Spectra-Physics, Santa Clara, Calif.).
[3] Waters Associates, Milford, Mass.

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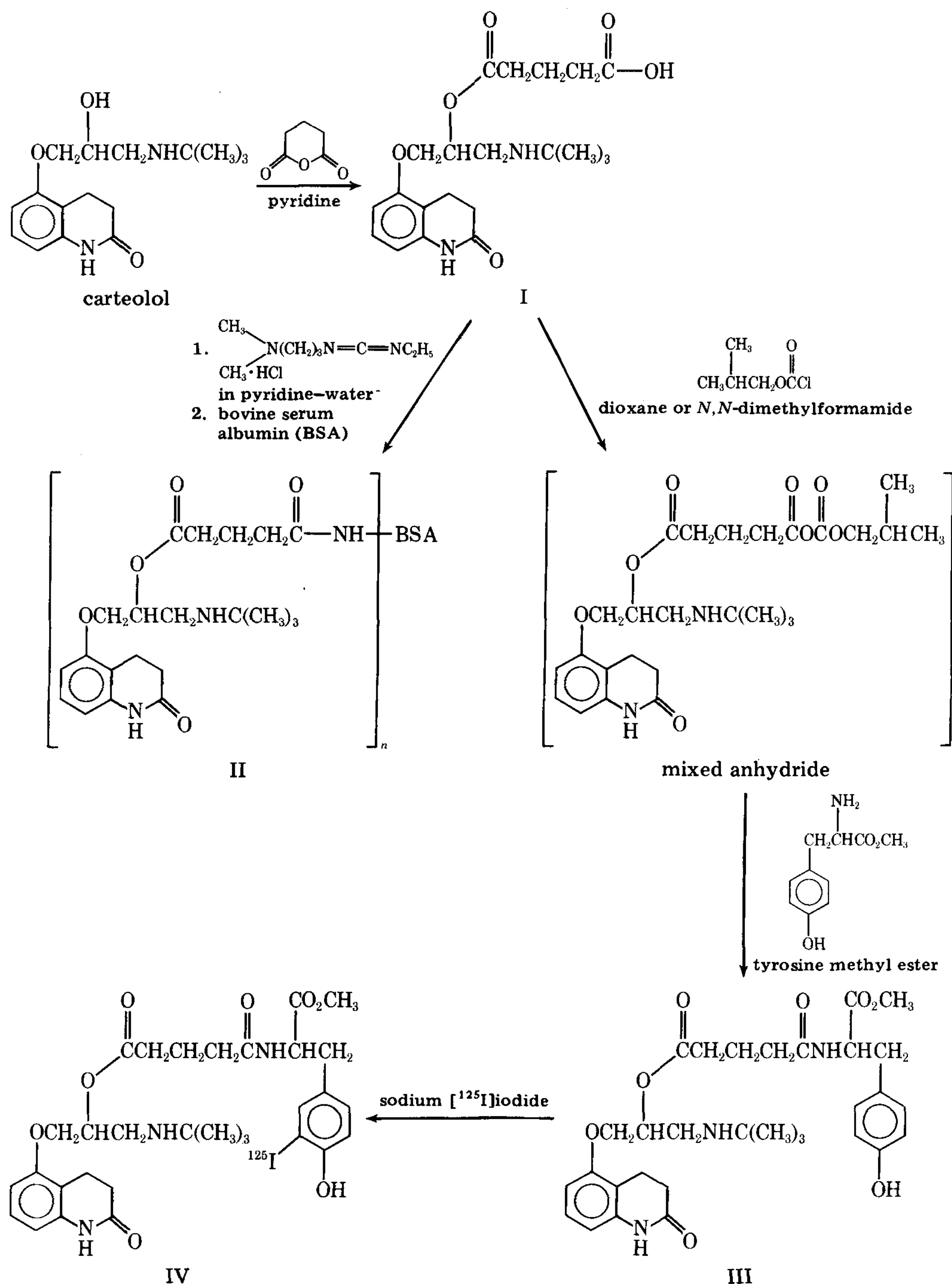

Scheme I—Procedure for the preparation of carteolol immunogen and radioactive tracer.

with an eluent of 18% (v/v) acetonitrile, 8% (v/v) tetrahydrofuran, and 0.005 M d-camphorsulfonic acid. Detection was accomplished by monitoring absorption at 254 nm.

Preparation of Immunogen—Scheme I shows the procedure for preparation of the immunogen and the radioactive tracer.

Synthesis of O-Glutarylcarteolol (I)—Carteolol (450 mg) and 210 mg of glutaric anhydride were dissolved in 7 ml of pyridine. The solution was allowed to stand overnight at room temperature and then was heated at 60° for 3 hr. The solvent was removed under reduced pressure on a rotary evaporator, and the residue was recrystallized from a mixture of ethanol and ethyl acetate. The product, after being air dried, was a colorless solid, mp 184–186°. The yield was 414 mg (66% of theory). The IR spectrum (potassium bromide pellet) showed a strong absorption band at 1740 cm^{-1} (carbonyl absorption for carboxylic acid ester), indicating ester formation. The mass spectral data obtained using a direct-insert probe and electron-bombardment ionization gave the following major ions: m/z (ion, % relative abundance), 406.1 (M^+, 7.5), 391.0 ($M - CH_3$, 25.6), and 86.1 [$CH_2NHC(CH_3)_3$, 100]. PMR spectral data also were consistent with the proposed structure.

Anal.—Calc. for $C_{21}H_{30}N_2O_6$: C, 62.05; H, 7.44; N, 6.89. Found: C, 61.67; H, 7.68; N, 6.69.

Preparation of Carteolol–Bovine Serum Albumin Conjugate (II)—O-Glutarylcarteolol (200 mg) was dissolved in 4.0 ml of pyridine–water (1:1 v/v) with stirring. To the solution was added 240 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, and the mixture was stirred at room temperature for 30 min. A solution of 140 mg of bovine serum albumin in 5 ml of water then was added, and the mixture was stirred overnight.

The product was dialyzed in a cold room (~4°) against 2000 ml of saline buffer containing 5% (v/v) of dioxane and then twice against 2000 ml of water. A 0.50-ml aliquot of the dialyzed solution (~37 ml) was mixed with 1.5 ml of acetonitrile and subjected to HPLC analysis using the described soap eluent. The results showed no free I (< ~0.4 μg/ml) in the dialyzed solution. Another 0.5-ml aliquot was diluted 1:10 with 0.05 M trometh-

Table I—Competition with Radioiodinated Carteolol Antigen for Antibody Binding Sites

Compound	Estimated ID_{50}, pg
Carteolol	370
Dehydrocarteolol	25×10^3
8-Hydroxycarteolol	230×10^3
Isoproterenol	$>500 \times 10^3$
5-Hydroxy-3,4-dihydrocarbostyril	$>500 \times 10^3$
Propranolol	No inhibition at 1×10^6

amine[4] and analyzed by UV spectrophotometry. The conjugated product had a UV spectrum (Fig. 1) nearly identical to that of a mixture of bovine serum albumin and I, showing an absorption maximum at 252 nm (similar to the carteolol derivative) and a shoulder at 278 nm (the absorption maximum for bovine serum albumin). It was estimated to contain an average of ~19 carteolol residues/bovine serum albumin molecule based on UV absorption data.

The remaining product solution was lyophilized to give 152.5 mg of white fluffy product, which was used for the immunization of rabbits.

Preparation of ^{125}I-Labeled Antigen—*Preparation of O-Glutarylcarteolol–Tyrosine Methyl Ester Conjugate (III)*—*O*-Glutarylcarteolol (163 mg) was dissolved in 6 ml of hot *N,N*-dimethylformamide, and the solution was cooled in an ice water bath. To the chilled solution were added 100 μl of triethylamine and 80 μl of isobutyl chlorocarbonate. The mixture was stirred briefly on a vortex mixer and then allowed to stand in an ice water bath for 30 min with occasional stirring. To the reaction mixture was added a solution of 160 mg of tyrosine methyl ester in 4 ml of *N,N*-dimethylformamide, and the mixture was stirred briefly and allowed to stand at room temperature for ~2 hr. The solution then was evaporated to dryness, and the residue was dissolved in 2 ml of the described preparative HPLC eluent. Aliquots (~200 μl each) were chromatographed on a μBondapak C_{18} column, and the fractions corresponding to the major UV-absorbing component were collected and used directly for subsequent radioiodination. The concentration of the product was estimated by comparison of its UV absorption at 252 nm to that of standard carteolol solutions (assuming approximately equal molar absorptivities for both compounds).

Preparation of [^{125}I]-O-Glutarylcarteolol–Tyrosine Methyl Ester (IV)—The procedure was similar to that described previously (3). In a disposable test tube were placed 10 μl of the III solution (~7.1 μg), 25 μl of 0.5 *M* phosphate buffer (pH 7.5), and 17 μl of sodium [^{125}I]iodide solution (4.0 mCi). To the solution was added 20 μl of chloramine-T solution, and the mixture was stirred for 60 sec. The reaction was terminated by the addition of 20 μl of sodium metabisulfite, and the solution was extracted with ethyl acetate (2 × 1.0 ml). The ethyl acetate solution (~95% total radioactivity) then was fractionated[5] with an eluent of 0.1% (v/v) acetic acid in ethanol. The effluent was collected in 1-ml fractions (Fig. 2). The peak fractions of the major radioactive component were pooled (1–2 mCi) and used in the radioimmunoassay. The radiochemical purity of this material was ~87% based on TLC and HPLC analyses. The compound was ~100% bound by the carteolol antiserum at a 1:200 dilution.

Immunization—New Zealand albino rabbits were used. A solution of II was prepared fresh in physiological saline at a concentration of 1 mg/ml and mixed thoroughly with an equal volume of Freund's complete adjuvant. One milliliter of the emulsion containing 0.5 mg of immunogen was injected into four footpads and two thighs of each rabbit. Each rabbit was immunized once a week for 4 weeks and then once every 2–6 weeks. Blood was obtained by cardiac puncture 6–14 days after the booster injections, allowed to clot overnight at 4°, and centrifuged at 2000 rpm for 20 min to yield the antiserum.

Immunoassay Procedures—*Preparation of Reagents*—The gelatin buffer used was saline buffer containing 0.1 mg of thimerosal/ml and 0.1% (w/v) pigskin gelatin. The radioactive tracer (usually with total radioactivity of ~1.5 mCi) was diluted to 100 ml with 0.002 *M* H_3PO_4 to form a stock solution, and the solution was refrigerated until used. A working solution of the tracer was prepared weekly by proper dilution (usually 1:100) of the stock solution with 0.002 *M* H_3PO_4.

A stock solution of carteolol standard was prepared at 1.0 mg/ml in distilled water, which was diluted 1:100 with gelatin buffer to form a working standard at 10 μg/ml. This latter solution was used to spike blank plasma. Typically, the working standard of carteolol was diluted 1:100

[4] J. T. Baker Chemical Co., Phillipsburg, N.J.
[5] A 1 × 17-cm Sephadex LH-20 column.

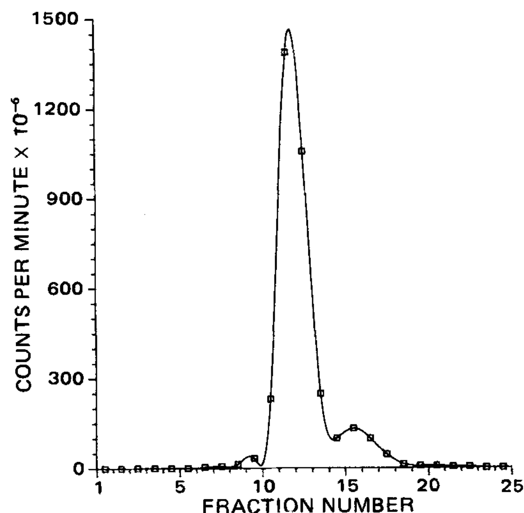

Figure 2—*Elution pattern of the radioiodination products on Sephadex LH-20 column. Fractions 12 and 13 were pooled and used as tracer in the radioimmunoassay.*

with blank plasma to give a plasma standard at 100 ng/ml, and this plasma sample then was diluted serially 1:2 with blank plasma to provide a set of plasma standards.

The antiserum from rabbits was diluted 1:4000 with gelatin buffer. An 18% (w/v) solution of polyethylene glycol 6000 in saline buffer was used to precipitate antibody-bound radioactivity.

Assay—A 100-μl aliquot of the diluted antiserum solution was added to 100 μl of plasma and 200 μl of gelatin buffer, followed by the addition of 100 μl of the tracer solution. The mixture was allowed to stand at ~4° overnight. To each sample was added 1.5 ml of polyethylene glycol 6000 solution, and the mixture was stirred briefly. The samples were centrifuged at 2500×*g* for 30 min at ~5°, and the supernate was decanted. The precipitate was mixed again with 1.5 ml of polyethylene glycol 6000 solution and centrifuged. After the supernate was decanted, the remainder was counted in a γ-counter.

A set of standard plasma samples was prepared in the concentration range to suit the intended analysis. A typical set included standards at 0, 0.17, 0.35, 0.69, 1.39, 2.77, 5.56, 11.11, 22.23, 44.46, and 88.91 ng/ml. These standards were carried through the radioimmunoassay in triplicate. The observed radioactivity counts (B) were divided by the counts from control plasma (B_0). A calibration curve was constructed using the mean ratios of B/B_0 *versus* the logarithms of plasma carteolol concentrations.

RESULTS AND DISCUSSION

Preparation of Immunogen—To prepare the immunogen, carteolol hemiglutarate (I) was conjugated to bovine serum albumin. The hemisuccinate also was examined because hemisuccinate derivatives have been the most commonly used intermediates for the incorporation of haptens to carrier proteins (3–6). The *O*-succinylcarteolol showed only limited solubility in the common solvents such as water, methanol, eth-

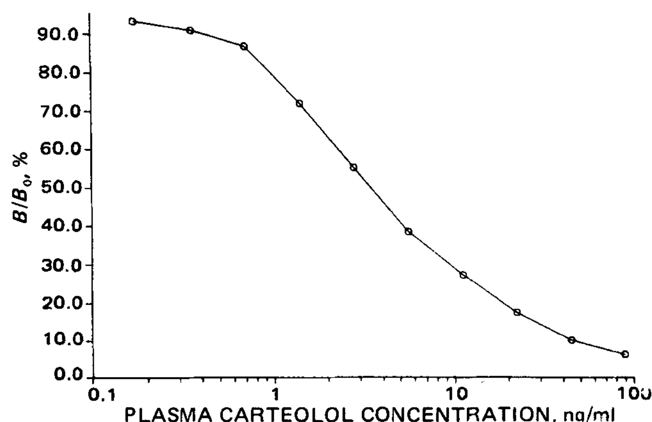

Figure 3—*Typical calibration curve for the radioimmunoassay.*

Table II—Within-Day and Day-to-Day Reproducibilities Using Mean Calculated Carteolol Levels (Nanograms per Milliliter) from the Radioimmunoassay

Item	Theoretical Drug Concentration in Plasma, ng/ml							
	0.35	0.69	1.39	2.77	5.56	11.11	22.23	44.46
				Day 1				
Mean (n = 3)	0.31	0.58	1.44	3.20	6.03	10.9	19.2	41.4
SD	0.02	0.04	0.06	0.12	0.21	0.34	0.24	1.44
RSD, %	6.5	6.1	4.1	3.9	3.6	3.1	1.2	3.5
				Day 2				
Mean (n = 3)	0.38	0.56	1.40	3.17	5.91	10.6	20.8	42.5
SD	0.02	0.05	0.02	0.17	0.22	0.12	0.72	2.03
RSD, %	6.1	8.4	1.4	5.4	3.7	1.1	3.5	4.8
				Day 3				
Mean (n = 3)	0.35	0.72	1.34	2.99	5.48	12.2	20.8	45.6
SD	0.01	0.06	0.11	0.23	0.54	1.43	1.13	0.97
RSD, %	3.2	8.5	8.2	7.8	9.8	11.7	5.4	2.1
				Average of the Nine Standard Curves				
Mean (n = 9)	0.35	0.62	1.39	3.12	5.81	11.2	20.3	43.2
SD	0.03	0.09	0.08	0.19	0.40	1.04	1.06	2.30
RSD, %	9.6	13.8	5.4	5.9	6.9	9.2	5.2	5.3
Percent theory	99	90	100	113	105	101	91	97

anol, dioxane, *N,N*-dimethylformamide, tetrahydrofuran, and acetone. Several attempts to incorporate it to bovine serum albumin using the mixed anhydride method (3, 7), the carbodiimide procedure (8, 9), and the active ester method (*N*-hydroxysuccinimide) (10) were all unsuccessful. In contrast, the *O*-glutaryl derivative was quite soluble in these solvents and could be incorporated readily to bovine serum albumin.

Immunization—The production of carteolol antibody in rabbits was monitored periodically by checking the animal's serum for binding to the radioactive antigen. Antibody to carteolol could be found in the rabbit serum after six injections of the immunogen over 10 weeks. After the rabbits received eight or nine injections over 26–27 weeks, serums of very good antibody titers were obtained.

Assay Procedures—To obtain radioiodinated tracer, tyrosine methyl ester was conjugated to carteolol through the glutaryl group, using the mixed anhydride method. The reaction was complete after ~2 hr, based on HPLC analysis. Several attempts to obtain a solid product by solvent evaporation and crystallization all were unsuccessful. Preparative TLC also was unsuitable for purification of the product, because the isolated products following these treatments were always contaminated with carteolol. Thus, preparative HPLC was used to isolate the product. The fractionated product exhibited one absorption maximum at 252 nm and a shoulder at 278 nm, being consistent with the proposed structure. The product was hydrolyzed quite readily in neutral or alkaline media to give carteolol, providing additional support to the proposed structure.

One important step in radioimmunoassay is the separation of the antibody-bound and the free fractions of the radioactive antigen. Initially, a saturated ammonium sulfate solution was examined for this purpose according to the published radioimmunoassay procedure for propranolol (6). The nonspecific binding was unacceptably high. A 0.25% (w/v) suspension of activated charcoal in gelatin buffer also was unsatisfactory. Polyethylene glycol 6000 in saline buffer was subsequently tested and showed minimum nonspecific binding while providing good precipitation of the antibody–substrate complex. Thus, 1.5 ml of the polyethylene glycol reagent was adequate for 0.5 ml of the incubation mixture.

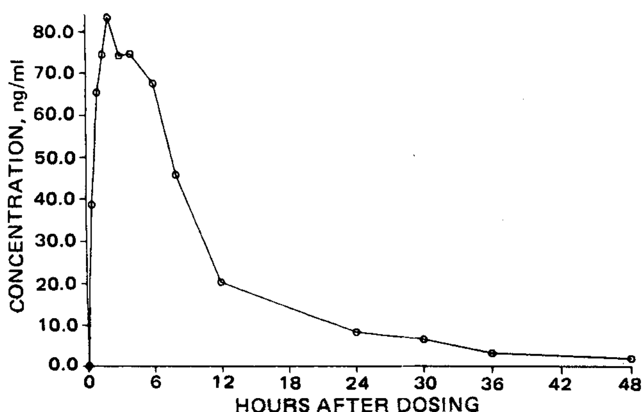

Figure 4—*Human plasma carteolol level–time profile determined by radioimmunoassay.*

Antibody specificity was tested against structurally related compounds by allowing each compound being used as a substitute for carteolol to compete with ^{125}I-labeled antigen for antibody binding sites under the described radioimmunoassay conditions. The amount required to elicit 50% inhibition of initial binding (ID_{50}) was estimated by interpolation of the standard curve at B/B_0 of 50%. The results are summarized in Table I. The cross-reactivity was expressed as the ratio (percent) of the ID_{50} for carteolol to that for the test compound. The only compound that showed significant cross-reactivity was dehydrocarteolol, being ~1.5%. This compound, however, is not a metabolite of carteolol. 8-Hydroxycarteolol, the major human metabolite, exhibited only very low cross-reactivity, <0.2%. Based on the available data, plasma 8-hydroxycarteolol levels within 12 hr after oral dosing are expected to be quite low compared to the corresponding parent drug levels. Thus, this metabolite should not interfere significantly in the radioimmunoassay of clinical samples.

Figure 3 shows a typical calibration curve generated according to the proposed radioimmunoassay procedure. Polypropylene and glass tubes were equally suitable for the assay. To assess day-to-day and within-day reproducibilities, standard plasma samples were prepared and carried through the radioimmunoassay procedure on 3 different days. On each day, three standard curves were prepared; each standard curve was derived from assays of triplicate samples. These B/B_0 ratios *versus* logarithms of concentrations were subjected to a least-squares best fit to a cubic equation using a computer program[1]. The B/B_0 ratios then were reinserted into the equation to calculate plasma concentration (Table II).

The radioimmunoassay procedure appears to be most suitable for the determination of plasma carteolol levels in the range of ~0.35–44.5 ng/ml. The daily replicate curves were quite reproducible, with the relative standard deviations (n = 3) mostly within ±8%. The averages of the assayed carteolol levels from the nine standard curves were in good agreement with the theoretical concentrations, all being within 90–113% of theory. The relative standard deviations for these means were between ±5.2 and 13.8%, showing good day-to-day reproducibilities.

Figure 4 shows a plasma concentration–time curve obtained from the analysis of actual clinical samples from a male subject after receiving a 30-mg oral dose of carteolol hydrochloride. In summary, the proposed radioimmunoassay procedure is quite specific and has good within-day and day-to-day reproducibilities. The workup procedure is simple because it does not require solvent extraction or concentration steps. Most importantly, the method offers sensitivity that is far superior to previously reported methods. The lower quantifiable limit of this procedure is ~0.4 ng of carteolol/ml using 0.1 ml of plasma.

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Interactions of Cephalosporins and Penicillins with Nonpolar Macroporous Styrenedivinylbenzene Copolymers

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Abstract ☐ The conditions of sorption of penicillins and cephalosporins on nonionic macroporous copolymers of styrenedivinylbenzene were evaluated. By increasing the methanol concentration in the eluent, the sorption decreased. Salts exerted little influence on sorption. However, pH exerted a remarkable effect on sorption, and the capacity factor variations according to the pH are quantitatively described. Some typical separations are shown.

Keyphrases ☐ Cephalosporins—interactions with a nonpolar, macroporous styrenedivinylbenzene copolymer stationary phase ☐ Penicillins—interactions with a nonpolar, macroporous styrenedivinylbenzene copolymer stationary phase ☐ Capacity factors—cephalosporins and penicillins, interactions with a nonpolar, macroporous styrenedivinylbenzene copolymer stationary phase ☐ Antibiotics—cephalosporins and penicillins, interactions with a nonpolar, macroporous copolymer stationary phase

Nonionic polymers have come into use recently as nonpolar stationary phases in high-pressure liquid chromatography (HPLC), primarily because of the work of Pietrzyk and coworkers (1–5). The main advantages of these kinds of packing are their low cost, ability to function at any level, compatibility with most solvents, and high adsorbency properties.

Penicillins and cephalosporins are β-lactam antibiotics produced by acylation with different radicals of the amino groups of 7-aminocephalosporanic, 7-aminodeacetoxycephalosporanic, or 6-aminopenicillanic acids, which give cephalosporins, deacetoxycephalosporins, or penicillins, respectively. They are particularly suitable as models to study the effect of protonic equilibria on retention by nonpolar macroporous copolymers because of their structural similarities and, depending on the medium pH, they can be found in undissociated, anionic, cationic, or zwitterionic form.

In this study, a macroporous styrenedivinylbenzene copolymer[1] adsorbent with an average surface area of 780 m^2/g and an average pore diameter of 50 Å (3) was used. The purposes of this work were to study the variables affecting the retention of penicillins and cephalosporins on resin copolymers and to demonstrate that the equations describing the retention of ionic solutes by the nonpolar stationary phases are applicable to the interactions of penicillins and cephalosporins with the copolymer. The results may assist in the development of new analytical methods and may improve β-lactam antibiotic extraction and purification methods; the optimum conditions of adsorption and elution may then be predicted.

[1] Amberlite XAD-4, Rohm & Haas Chemical Co.

EXPERIMENTAL

7-Aminodeacetoxycephalosporanic acid (I), 7-phenylacetamidodeacetoxycephalosporin (II), cephalexin (III), cephradine (IV), 7-aminocephalosporanic acid (V), cephalosporin C (VI), cephalothin (VII), cephaloridine (VIII), cefazolin (IX), 6-aminopenicillanic acid (X), ampicillin (XI), penicillin G (XII), penicillin V (XIII), phenoxyethylpenicillin (XIV), 7-aminodeacetylcephalosporanic acid (XV), and deacetylcephalothin (XVI) were used as supplied[2].

The macroporous styrenedivinylbenzene copolymer[1] was supplied as spheres with an average size of ~500 μm. The resin particles were washed by extraction (soxhlet) with methanol and allowed to dry; they then were ground and sieved, with the 40–70-μm particles being selected (1). The size distribution of these particles was analyzed[3].

The chemicals were the highest commercial grade available and were used without any further purification. The buffer solutions were 0.05 *M* phosphate, and sodium sulfate was used to obtain the desired ionic strength. The mixtures of methanol–buffer solution were expressed in volume percent.

Chromatographic Conditions—A 2.5 × 600-mm steel column, equipped with suitable fittings and a 10-μm filter, was dry packed with the copolymer between 40 and 70 μm.

The liquid chromatograph was fitted with a pump[4], a 20-μl sample valve injector[5], a 250-nm UV detector[6], and a strip-chart recorder[7].

The chromatographed substances were prepared in the mobile phase at a concentration of 0.5 mg/ml, except for the penicillins, which were dissolved at a concentration of 3 mg/ml. In each case, a 20-μl aliquot was injected. The flow was maintained at 0.5 ml/min, and the sensitivity of the UV detector was set between 0.004 and 0.128 aufs, according to the absorptivity of the analyzed product.

The capacity factors (k') were calculated in accordance with:

$$k' = \frac{V_R - V_0}{V_0} \quad \text{(Eq. 1)}$$

[2] Courtesy of Antibióticos, S.A., Madrid, Spain.
[3] Sharples Micromerograph, Franklin Electronics, Bridgeport, Pa.
[4] Constametrik 00, Laboratory Data Control.
[5] Model 7120, Rheodyne.
[6] UV III monitor, Laboratory Data Control.
[7] Model XER, Sargent-Welch Scientific Co., Skokie, Ill.

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