membrane, allowing hydrogen-ion back-diffusion, which results in irritation, bleeding, and possible ulceration. If this mechanism is correct, then the rapid onset of absorption obtained with unbuffered solutions or solutions with less than 16 mEq of antacid buffering may not reduce the incidence of untoward effects as effectively as those containing a greater quantity of antacid buffering.

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Determination of Ethoxzolamide in the Iris/Ciliary Body of the Rabbit Eye by High-Performance Liquid Chromatography: Comparison of Tissue Levels Following Intravenous and Topical Administrations

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Abstract □ A specific high-performance liquid chromatographic method is described for ethoxzolamide following the extraction of the material from iris/ciliary body eye tissue in rabbits. The steps consist of base extraction and protein and enzyme deactivation, followed by acid treatment, extraction into ethyl acetate, evaporation, and solubilization with a 50% aqueous methanol solution. The samples were chromatographed on a reverse-phase phenyl column with a mobile phase consisting of 50% methanol in 1% acetic acid. The recovery was 74.3% over a 10-fold range of tissue concentrations. The sensitivity was 0.03 μg/mL, and the response was linear over the concentration range (0.03–0.5 μg/mL) used in the study. Intravenous (2.0- and 6.0-mg/kg) and topical (1% suspension) doses of ethoxzolamide were administered to rabbits. Iris/ciliary body tissues were excised 45 min after drug administration. The tissue levels after a dose of 6 mg/kg were statistically greater than the levels obtained after a dose of 2 mg/kg. The smaller intravenous dose represented the lowest dose for which a reduction in intraocular pressure could be measured. An initial transitory drop in intraocular pressure was detected for the topical dose. Iris/ciliary body levels in the treated eye could be detected for the 2-mg/kg iv and topical doses.

Keyphrases □ Ethoxzolamide—determination in the iris/ciliary body, rabbits, HPLC, GC □ Dosage forms—intravenous and topical administration of ethoxzolamide, iris/ciliary body of rabbit eyes, HPLC, GC

Ethoxzolamide, a carbonic anhydrase inhibitor used in glaucoma to lower intraocular pressure (IOP), is effective orally but not when administered topically to the eye, presumably due to inadequate drug levels at the active site. In recent studies, the anatomical location of carbonic anhydrase with the anatomical location of aqueous humor formation in the ciliary body have been identified (1, 2). Friedlander and Muther (3) have utilized the high binding affinity of [^{3}H]-acetazolamide for carbonic anhydrase to demonstrate the distribution of carbonic anhydrase in the epithelium of the ciliary body. The greater specificity of ethoxzolamide for the enzyme was shown by displacing [^{3}H]acetazolamide with a high concentration of unlabeled ethoxzolamide.

Although Maren (4) have devised a sensitive method for the determination of carbonic anhydrase and its inhibitors, the assay measures drug activity; therefore, specificity is always in doubt. It is also tedious and time consuming. It was the purpose of this study to develop a high-performance liquid chromatographic (HPLC) procedure for the determination of ethoxzolamide in the iris/ciliary body tissue of rabbit eyes. Data are also presented which compare tissue concentrations after administration by the topical and intravenous routes.

EXPERIMENTAL SECTION

Chemicals—Ethoxzolamide, a gift from a commercial source[1], was used as received. The substances used in the preparation of the various buffers and vehicles were of analytical or USP quality, and included monobasic sodium phosphate[2], dibasic sodium phosphate[3], sodium carbonate[2], hydrochloric acid[2], sodium chloride[4], polysorbate 80[5], and *N*,*N*-dimethylacetamide[2]. Ethyl acetate[6] was used as the organic phase in the extraction procedure. Acetic acid[7], methanol[8], and distilled deionized water were used to prepare the chromotographic mobile phase. The distilled deionized water was also used to make all aqueous dilutions.

Apparatus—The chemicals and tissues were weighed on an electronic[9] or analytical[10] balance. The pH values of the various solutions were measured[11] when necessary.

The HPLC system consisted of a solvent delivery pump[12], a syringe-loading sample injector[13] fitted with a 100-μL loop[14], a reverse-phase phenyl column[15], and a fixed-wavelength UV detector[16] operating at 313 nm. Chromatograms were recorded on a chart recorder[17] operating at 0.5 cm/min. The mobile phase consisting of methanol–1% aqueous acetic acid (50:50, v/v) was deaerated before use; the flow rate was 1.5 mL/min.

[1] The Upjohn Co.
[2] Certified ACS; Fisher Scientific Co., Fair Lawn, N.J.
[3] Baker Analyzed Reagent; J. T. Baker Chemical Co., Phillipsburg, N.J.
[4] USP grade; J. T. Baker Chemical Co.
[5] USP grade; Ruger Chemical Co., Inc., Irvington, N.J.
[6] Analytical Reagent ACS; Mallinckrodt Inc., Paris, Ky.
[7] Certified ACS; Mallinckrodt Inc.
[8] HPLC grade; Burdick & Jackson Laboratories, Inc., Muskegon, Mich.
[9] Model 4100; Cahn Instruments, Paramount, Calif.
[10] Model B6; Mettler Instruments Corp., Hightstown, N.J.
[11] Model 701; Orion Research Corp., Cambridge, Mass.
[12] Model 6000A; Waters Associates, Milford, Mass.
[13] Model 7125; Rheodyne Inc., Cotati, Calif.
[14] Catalog no. 724; Rheodyne Inc.
[15] P/N 27198, S/N μ-Bondapak Phenyl; Waters Associates.
[16] Model 440 absorbance detector; Waters Associates.
[17] Model A5211-1; Omniscribe, Houston Instruments, Austin, Tex.

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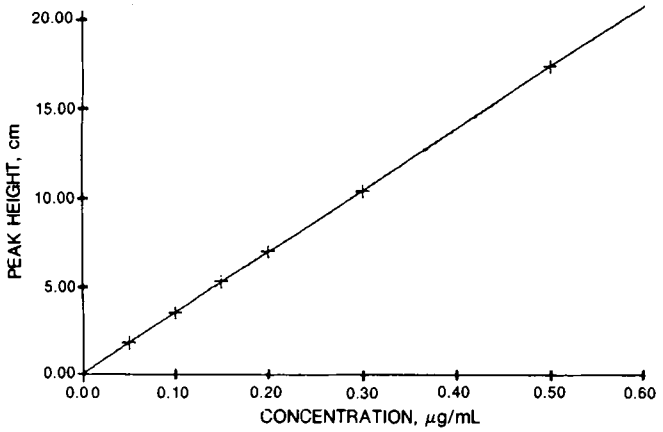

Figure 1—*Typical standard curve for ethoxzolamide over a concentration range of 0.03–0.5 μg/mL (single determinations).*

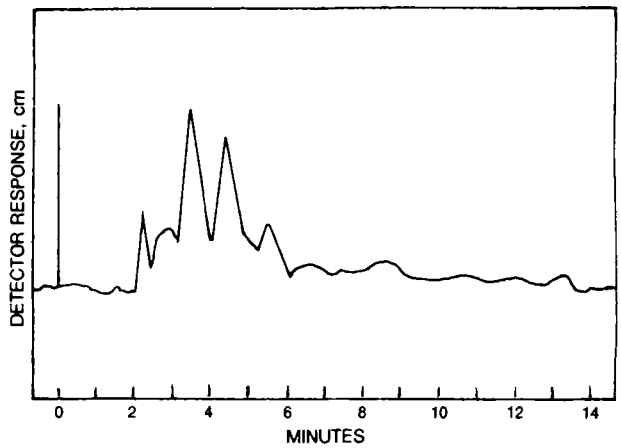

Figure 2—*Chromatograms of 100 μL of tissue extract devoid of drug (AUFS = 0.005).*

Drug Administration—New Zealand White rabbits[18] of either sex (age, 3–4 months; weight 2.0–3.5 kg) were administered ethoxzolamide by intravenous injection into the marginal ear vein or by topical application to the conjunctival sac of the eye. Because of the limited aqueous solubility of ethoxzolamide, a mixed solvent consisting of 60% *N,N*-dimethylacetamide–40% water was used for intravenous administration. With this vehicle, concentrations of up to 20 mg/mL could be injected. The intravenous dose of ethoxzolamide was either 2 or 6 mg/kg. The formulation was prepared immediately before use.

Ethoxzolamide was applied topically as a 1% suspension in Sörensen's modified phosphate buffer (5), to which 0.5% polysorbate 80 was added as a wetting agent. The pH of this vehicle was 7.2. The formulation was prepared ~8 h before use and was stirred continuously on a magnetic stir plate. A 50-μL volume of the suspension was applied every 2 min to one eye for a total of three doses; the blank vehicle was similarly applied to the other eye.

The rabbits were sacrificed 45 min after either intravenous or topical instillation by injecting a bolus of air into the marginal ear vein. The iris/ciliary tissue was immediately excised and weighed. This time interval was chosen to represent nearly the maximal IOP response. Preliminary studies in which ethoxzolamide was administered intravenously showed that a maximal pharmacological response (lowering of intraocular pressure) occurred at 45 min.

Tissue Extraction Procedure—Although the ciliary body has been identified as the active site (2, 3), it is difficult to excise precisely in the rabbit. The iris is easily removed, and the ciliary body remains attached to it when the iris is excised from the rabbit eye. Consequently, tissue levels, as reported here, represent an average level of the combined tissues. The wet tissue was cut into smaller segments and homogenized (30 rpm for 1.5 min) in centrifuge tubes[19] with dry ice; the latter disrupted cellular structure to facilitate extraction. A total of 5 mL of 0.025 M sodium carbonate solution was added to the tubes containing the homogenized tissue. The mixture was vigorously shaken[20] for 1 h at room temperature. The high pH and hypotonicity of the sodium carbonate solution promoted dissociation of the drug from carbonic anhydrase (6) and partitioning into the aqueous phase. The homogenate was then heated to 100°C for 5 min to denature and precipitate the tissue proteins. Identical treatment without iris/ciliary body tissue showed that the drug did not decompose. The mixture was cooled to room temperature, and 1 mL of 0.5 M HCl containing an additional 75 mg of NaCl/mL was added. The acid converted drug to the un-ionized form, and the salt aided in phase separation. Ethyl acetate (3 mL) was added to the mixture, which was shaken on a vortex mixer[21] and centrifuged[22] for 0.5 h. After centrifugation, the top layer, consisting of nearly 3 mL of ethyl acetate and containing drug, was transferred to a 5-mL vial[23]. The extraction was repeated twice with 3 mL of ethyl acetate each time; the ethyl acetate phases were then combined. The ethyl acetate solution was subsequently warmed[24] to 30°C and evaporated to dryness under a stream of nitrogen. To the residue was added 1 mL of 50% methanol to solubilize the drug. The 50% methanol solution containing drug was refrigerated until just before assay by HPLC. To assess the validity of the assay, iris/ciliary tissues were spiked with various known amounts of ethoxzolamide and treated by the extraction and assay procedure described above.

Chromatographic Assay Conditions—One hundred microliters of sample or standard was injected onto the column with the loop injector. Peak heights from five to seven known concentrations of ethoxzolamide, ranging from 0.03 to 0.75 μg/mL, were determined. A standard curve was constructed from these data, relating peak height to concentration. The standard curve was repeated whenever a set of unknowns was assayed. Also, standard concentrations were interspersed after approximately every third unknown sample to assess whether changes in column integrity, mobile phase, or detectors were occurring. The attenuation was 0.005 for all determinations.

Intraocular Pressure—A separate group of New Zealand White rabbits (n = 24) were maintained on 0.3% NaCl solution in place of drinking water for at least 3 weeks before the determination of IOP, because it has been shown that a diet deficient in sodium ion produces a variable IOP response to this class of drugs. Commercial rabbit chow does not provide a sufficient NaCl content compared with the diet available to rabbits in their natural habitat (7). Numerous measurements of IOP were made over 3 weeks to familiarize the rabbits with this procedure before drug administration. Baseline IOP measurements were determined by tonometry[25], followed by either intravenous or topical administration of ethoxzolamide (1% suspension). In each intravenous experiment, 25–50% of the rabbits received vehicle only; the observations were made on a "blind" basis. Whenever ethoxzolamide was administered topically, one eye was dosed as described above, and the opposite eye received vehicle only. Again, observations were made on a "blind" basis. IOP measurements were determined at 20-min intervals for 3 h.

RESULTS AND DISCUSSION

The standard curve (Fig. 1) was found to be linear over the concentration range (0.03–0.5 μg/mL) used in the study. The sensitivity of the assay was determined by analyzing progressively lower concentrations until the coefficient of variation for multiple determinations reached 10%. This occurred at a concentration of 0.03 μg/mL. This value also represented about five times the height of the baseline noise at 0.005 AUFS.

Figures 2 and 3 represent typical chromatograms for the analysis of ethoxzolamide in iris/ciliary body tissue. As shown in the chromatogram (Fig. 3), good separation was achieved from endogenous tissue components. When no drug was present, the baseline was observed (Fig. 2). A metabolite was not apparent in any of the observed chromatograms. The retention time for the drug was 7 min.

To determine the recovery and reproducibility of the assay procedure, iris/ciliary body tissue from the rabbits was spiked with known amounts of ethoxzolamide. The amount of drug was varied from 0.05 to 0.6 μg (Table I); the tissue weight also varied. The known amount of drug was first dissolved in 4 mL of distilled water, to which a freshly extracted tissue sample was added. A 12-mL test tube contained the mixture, which was incubated for ≥3 h at room temperature. Longer incubation periods (up to 72 h) did not increase the percent recovery. After incubation, 1 mL of 0.125 M sodium carbonate solution was added to the mixture to produce a final concentration of 0.025 M. The extraction procedure was continued from this step as described above. Identical extraction experiments were also conducted without the addition

[18] Morrison Rabbitry; West Branch, Iowa.
[19] Tissue grinder (10 mL); Potter-Elvehjem.
[20] Model E-1, Vibromixer; Chemapec Inc., Hoboken, N.J.
[21] Vortex Geni Mixer; American Hospital Supply, McGaw Park, Ill.
[22] Model CS; International Equipment Co., Needham Heights, Mass.
[23] ReactiVials; Pierce Chemical Co., Rockford, Ill.
[24] ReactiTherm; Pierce Chemical Co.

[25] Applanation Pneumatonograph; Alcon Labs, Fort Worth, Tex.

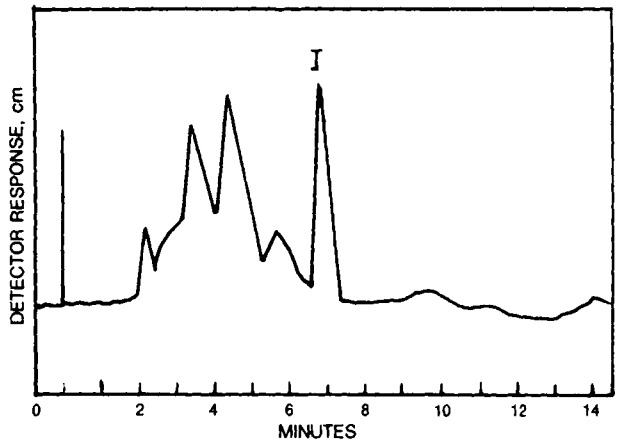

Figure 3—*Chromatograms of 100 μL of tissue extract containing ethoxzolamide (AUFS = 0.005); peak I is ethoxzolamide.*

of iris/ciliary body tissue but with varying amounts of drug (0.05–10 μg). Without added tissue, 85.8 ± 4.97% of the added drug could be extracted. When tissue was added, 74.3 ± 7.83% of the drug could be recovered. The presence of tissue was responsible for a significant increase in the extraction variability.

Without the presence of drug but including tissue, no ethoxzolamide peaks were observed in the chromatograms. A linear increase in the amount of drug recovered was detected by:

$$y = 0.099 + 0.706x \qquad (n = 31, r^2 = 0.995) \qquad \text{(Eq. 1)}$$

where x is the nanograms of drug added per milligram of tissue, and y is the nanograms of drug recovered per milligram of tissue. Although the mean percentage of drug extracted in the recovery experiments could be used to correct for the fraction of drug unavailable for extraction, its use would be somewhat less precise than the use of Eq. 1. This occurs because the calibration equation incorporates the weight of the tissue on a per-unit basis and because the variance can be expressed at every point on the regressed line.

Assay Application—The method described above was developed to compare iris/ciliary body concentrations of ethoxzolamide after intravenous and topical dosing to the eye.

Intraocular Pressure—Before tissue was extracted, a minimum dose was established which produced a significant reduction in IOP. This was accomplished by linear and quadratic regression analysis. If the IOP dropped below baseline values, a negative statistically significant slope from zero indicated a drug effect. Baseline measurements were made by measuring IOP repeatedly over 20 min just before dosing. Two intravenous doses were given. At 2 mg/kg, only 3 of 10 rabbits showed a response over 2 h, whereas at 6 mg/kg, 6 of 10 rabbits yielded a response, which at 45 min was 1.5 to 2 mm Hg lower than from 2 mg/kg. The maximum response occurred at 45 min after intravenous administration and at 40 min after topical administration (Table II).

When a 1% suspension of ethoxzolamide was administered topically to the eyes of 24 rabbits, a small but statistically significant drop in IOP was observed at 20–60 min. The criteria for observing a change in response was based on a statistical difference in IOP between the treated and untreated eyes at each time interval. Equation 2 expresses a change in IOP brought about by corneal penetration only. The responses measured for the two routes of administration are not directly comparable. For the topical route, a difference in response in treated and untreated eyes was measured over time, whereas by the intravenous route, both eyes responded so that changes were made relative to the baseline (*i.e.*, $t = 0$):

$$\text{IOP Changes} = (IOP_{DET} - IOP_{CET}) - (IOP_{DEO} - IOP_{CEO}) \qquad \text{(Eq. 2)}$$

where IOP_{DET} is the IOP measurement (mm Hg) of the dosed or treated eye at time t, IOP_{DEO} is the IOP measurement (mm Hg) of the treated eye at $t = 0$ but before receiving the dose, IOP_{CET} is the IOP measurement (mm Hg) of the control (nontreated) eye at time t, and IOP_{CEO} is the IOP measurement (mm Hg) of the control (nontreated) eye at time $t = 0$. By subtracting the IOP response from the nontreated eye at each time interval, the reduction in IOP occurring from systemic absorption is subtracted.

Interestingly, various manufacturers of carbonic anhydrase inhibitors have repeatedly been unable to detect a lowering of IOP from direct application to the eye[26]. These studies were often conducted with single doses in a small number of rabbits that had not been maintained on a diet sufficient in sodium chloride. This latter factor is most important in attempting to measure a small but detectable IOP response. These results, along with previously published reports (8, 9), have prompted researchers to conclude that intravenous doses of carbonic anhydrase inhibitors, including ethoxzolamide, were acting only centrally. More recently (1, 2, 10, 11), it has been shown that carbonic anhydrase inhibition occurs at the ciliary body. Nevertheless, studies (12, 13) have indicated that systemic acidosis, another effect of carbonic anhydrase inhibitors, contributes to a reduction in IOP. Maren *et al.* (12) have proposed that the acidotic effect of the lowering of the IOP and the direct effect on aqueous humor secretion are additive.

Tissue Levels—Tissue levels were determined in both eyes after either intravenous or topical administration (Tables III and IV, respectively). At each intravenous dose, the right and left eyes showed similar concentrations of ethoxzolamide. At 2 and 6 mg/kg (10 replications were conducted at each level), a statistically significant higher concentration was measured for 6 mg/kg.

[26] Unpublished results.

Table I—Recovery of Ethoxzolamide With the Addition of Known Amounts of Drug to Iris/Ciliary Body Tissue

Ethoxzolamide Added, μg	Tissue Weight, mg	Mean Recovery ± *SD*, %
0.1–10.0[a]	0	85.8 ± 4.97
0.05–0.60[b]	40.9 ± 12.4	74.3 ± 7.83

[a] Increments added are 0.1, 0.2, 0.4, 2.0, 4.0, and 10.0 μg; n = 2–4 for each determination. [b] Increments added are 0.05, 0.1, 0.15, 0.2, 0.3, 0.4, 0.5, and 0.6 μg; n = 2–4 for each determination.

Table II—Intraocular Pressure Measurement Changes after Topical Administration[a] of 1% Ethoxzolamide to Rabbit Eyes

Time Postdose, min	Change, mm[c]	$p^{b,d}$
0	--	--
20	−0.795 (0.63)	0.019
40	−1.085 (0.68)	0.0012
60	−0.955 (0.81)	0.010
80	−0.295 (0.72)	0.21
100	0.085 (0.75)	0.59
120	0.085 (0.76)	0.59
140	0.625 (0.79)	0.94
160	0.045 (0.86)	0.54
180	0.085 (0.71)	0.59

[a] 50 μL q 2 min × 3 (n = 24). [b] $p < 0.02$. [c] See Eq. 2 in text; values in parentheses represent the 95% confidence interval. [d] Probability determined from paired t test.

Table III—Iris/Ciliary Body Levels of Ethoxzolamide after Intravenous Administration to Rabbits

Dose, mg/kg iv	n^b	Ethoxzolamide Level, ng/mg[a] Left Eye	Right Eye	Pooled[c]
2.0	10	2.67 ± 1.91	2.27 ± 1.00	2.48 ± 1.50
6.0	10	10.08 ± 1.84	10.43 ± 1.94	10.26 ± 1.83

[a] Nanograms of drug per milligram of iris/ciliary body tissue; mean ± *SD*. [b] Number of determinations for either the left or the right eye. [c] Pooled data for the left and right eyes (n = 20).

Table IV—Iris/Ciliary Body Levels of Ethoxzolamide after Topical Administration

Rabbit	Ethoxzolamide Level, ng/mg[a] Treated Eye	Untreated Eye
1	5.64	2.08
2	3.50	0
3	1.60	0.30
4	1.87	0.29
5	2.20	0
6	1.83	0
7	0.444	0
8	0.544	0
9	1.94	0
10	1.76	0
Mean ± *SD*	2.13 ± (1.50)	0.27 ± (0.65)

[a] Nanograms of drug per milligram of iris/ciliary body tissue.

The results for the topical dose (Tables II and IV) show that ethoxzolamide could be detected in the untreated eye in 3 of 10 rabbits and in the treated eye in all of the rabbits. An outlier value of 2.08 ng/mg was determined for rabbit 1 in the untreated eye (Table III). The measurement in the untreated eye likely represents systemic absorption. The pooled treated eyes yielded iris/ciliary body levels of 2.13 ± 1.5 ng/mg (n = 10). The mean level is statistically lower ($p < 0.05$) than the mean value obtained for dosing at 6 mg/kg iv but not statistically different from tissue levels obtained at 2 mg/kg iv.

For an average-sized rabbit (2.5 kg), the minimum intravenous dose to produce a response (2 mg/kg) represents a 3.3-fold higher dose than that administered by the topical route. Thus, the nearly equal tissue levels shown in Tables III and IV for the two routes of administration suggest that the tissue levels detected from the smaller topical dose are, at least in part, a consequence of corneal transport and not primarily systemic absorption. Although our results do not differentiate between the direct and systemic effects of the drug, they do add credence to the possibility of a contribution from the corneal pathway.

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Factors Affecting Quinidine Protein Binding in Humans

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Abstract □ The free (unbound) concentration of drug in plasma is often an important determinant of pharmacological and toxicological effects. Unfortunately, studies examining the factors influencing the free fraction of quinidine in plasma have yielded inconsistent results. It is probable that differences in the type of blood collection tubes utilized and the analytical procedure employed biased some of these estimates of quinidine binding. The present study was executed in a manner free of factors now known to introduce artifacts into estimates of the free fraction of quinidine. In healthy volunteers, the free fraction of quinidine (1.0 μg/mL) was 0.129 ± 0.019 (mean ± SD) and was constant throughout the therapeutic range. A high-affinity, low-capacity binding site ($K = 1.17 \times 10^5$ M^{-1}; $nP = 3.49 \times 10^{-5}$ M) and a low-affinity, high-capacity binding site ($K = 1.33 \times 10^3$ M^{-1}; $nP = 3.14 \times 10^{-3}$ M) were identified. The characteristics of quinidine binding in a 4.5-g/dL solution of human serum albumin ($K = 3.05 \times 10^3$ M^{-1}; $nP = 1.36 \times 10^{-3}$ M) suggested that the low-affinity, high-capacity binding site was on this protein. In the presence of tris(butoxyethyl) phosphate (75 μg/mL), the quinidine free fraction increased from 0.114 to 0.231. A lidocaine concentration of 250 μg/mL caused a similar increase. Patients suffering traumatic injury had a significant increase in α_1-acid glycoprotein concentration (197 mg/dL) and a decreased quinidine free fraction (0.075 ± 0.019). Patients with hyperlipidemia had free fractions similar to those observed in healthy individuals (0.118 ± 0.019). These data suggest that the high-affinity, low-capacity binding site is on α_1-acid glycoprotein and that lipoproteins are of little importance in the protein binding of quinidine.

Keyphrases □ Protein binding—quinidine, humans □ Quinidine—protein binding, humans

Despite the recent development of a number of new agents, quinidine continues to be a mainstay of oral antiarrhythmic therapy. Effective concentrations of quinidine in serum range between 1 and 5 μg/mL (1, 2). However, much interindividual variability in the concentration necessary for arrhythmia suppression has been noted (2). Studies with other antiarrhythmic drugs, such as disopyramide (3) and propranolol (4), have demonstrated that the free drug concentration in plasma correlates better with the pharmacological effect than does the total concentration. These observations support the principle that free drug in plasma is in equilibrium with drug at receptor sites (*i.e.*, that which is responsible for the pharmacological effect). Furthermore, alterations in the degree of protein binding can also affect the pharmacokinetic properties of a drug. Fremstad *et al.* (5) have demonstrated a significant positive correlation between the free fraction of quinidine and both volume of distribution and total body clearance. For these reasons, a thorough understanding of the determinants of the protein binding of quinidine is important.

Unfortunately, studies in which the factors influencing quinidine protein binding have been examined have yielded inconsistent results. The mean free fraction in studies with healthy volunteers has been reported to be 0.10 (6), 0.105 (7), 0.141 (8), 0.246 (9), and 0.288 (10). Albumin, α_1-acid glycoprotein, and lipoproteins have all been reported to be important for quinidine binding, but their relative roles have been disputed (10–12). Some of the discrepancies between studies may have been due to methodological differences, as several factors have been found to influence quinidine binding *in vitro*. Quinidine free fraction is two- to three-fold higher when blood is collected in evacuated blood collection tubes[1] rather than glass syringes (6). Other factors influencing estimates of

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