Table I—Predicted Shelf Life of Benactyzine in Multicomponent Formulation

Temperature	90% Shelf Life	75% Shelf Life
5°	4.5 years	12.3 years
15°	1.4 years	3.9 years
25°	5.9 months	1.3 years
40°	1.3 months	3.6 months
60°	0.2 months	0.6 months

cartridges was found only at 80°. No significant differences were found with regard to k or C_0 at other temperatures. The data for plastic cartridges at 80° were excluded in calculating the regression equation in Fig. 5. The line is expressed by

$$\ln k = 18.0100 - 14{,}900/RT \qquad \text{(Eq. 8)}$$

where k is in moles per liter-day. The standard error for the activation energy is 880 calories.

DISCUSSION

Glass cartridges were found to be more suitable than plastic as containers for trimedoxime, atropine, and benactyzine formulations. Benactyzine is the least stable of the active ingredients in the antidote formulation. Results of this study show that benactyzine degrades ~20% after 1 year at 25° in either plastic or glass. The usefulness of the injector could be prolonged by storage at 5°, where the predicted decrease in benactyzine is 10% after 5 years. Figures 6 and 7 illustrate the enhanced shelf life of benactyzine when filled at 105% of label and stored at 25 or 5°. Predicted shelf life at various temperatures is presented in Table I.

The trimedoxime in the injector is very stable; about 1.4% degradation would occur after 1 year at 25°. Solutions of trimedoxime can exist in equilibrium with its hydrolysis products (7, 8). The degradation rate of trimedoxime in plastic cartridges at 80° was faster then expected. The unusual drop in pH at 80° (Fig. 3), greater than expected based on temperature studies, could shift the equilibrium of trimedoxime and consequently account for the increased degradation rate in plastic.

Atropine sulfate in the medication is quite stable. According to kinetic studies of atropine in solution at pH 2.8, its half-life would be about 200 years at 25° and about 2 years at 80° (2, 3).

The pH values in glass were homogeneous at all temperatures. There was some heterogeneity of pH in the plastic cartridge; *i.e.*, 2 of 23 samples stored at 5° had a pH of about 2.95; one sample at 80° had a pH of 3.01, 0.5 units more than expected. A sample at 40° had a pH of 2.51, 0.2 units lower than expected. The decrease in pH with storage time was consistent with temperatures of 65° and below. At 80°, the decrease in pH was slower than expected, suggesting abstraction of hydrogen ion by the rubber in the cartridge. In the plastic cartridge, the pH decreased at a considerably faster rate than expected; this observation suggests a reaction caused by some material in plastic at this temperature. At 25° the drop in pH, probably due to the formation of benzilic acid, is predicted to be less than 0.15 units after 1 year of storage.

Vapor transmission from the cartridges at ambient temperature is low; it is estimated that water loss would be about 1%/year for the glass cartridge and about 2%/year for the plastic cartridge at 25°.

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Factors Influencing the Apparent Protein Binding of Quinidine

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Abstract □ Various factors influencing the apparent protein binding of quinidine were examined. Different binding values in rabbit plasma were obtained by equilibrium dialysis techniques employing three commonly used buffers. Binding values comparable to those found by ultrafiltration were achieved after dialysis against isotonic phosphate buffer for ~4 hr. Dialysis beyond 8 hr gave an increased free fraction with time. The reported effect of *in vitro* added heparin on plasma protein binding could be prevented by reducing the final concentration in blood from 20 to 5 U/ml, a concentration still sufficient to prevent clotting of the blood sample. Daily freezing and thawing of plasma samples over 1 week did not alter the binding of quinidine. The samples were stable for at least 2 months at −20°.

Keyphrases □ Binding, protein—quinidine, factors influencing binding, rabbit plasma □ Quinidine—factors influencing protein binding, rabbit plasma □ Plasma protein binding—quinidine, factors influencing binding, rabbits

A wide range of protein binding values for quinidine have been reported. Mean unbound fractions of 0.10–0.29 were observed in normal volunteers (1–5), while mean values of 0.19–0.42 were found for patients with cirrhosis (2, 3). Although interindividual differences in binding properties may contribute significantly to the variability in these results, differences in the blood collection method and in the binding determination could be important contributing factors. Ultrafiltration and equilibrium dialysis have been used previously for quinidine binding determinations. In equilibrium dialysis, various investigators have used different temperatures, equilibrium

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times, and buffers in their studies (1, 4, 6–9). A significant influence of the ionic composition of the buffer on the extent of apparent protein binding determined by dialysis has been found (10, 11). Woo and Greenblatt (9) demonstrated higher binding at room temperature than at body temperature which could explain differences in results obtained by different investigators. However, no information is available on the time course of equilibrium or whether the dialysis time affects the quinidine binding. Recent studies suggest that the addition of heparin to blood samples may alter the plasma protein binding of quinidine (11).

To determine the influence of the dialysis medium and the equilibrium time on the apparent protein binding of quinidine, the protein binding in rabbit plasma was measured by dialyzing the plasma against various buffers. The influence of heparin *in vitro* at two different concentration levels and *in vivo* was established. Furthermore, the stability of a spiked rabbit plasma sample under conditions of repeated freezing and thawing was tested over 2 months.

EXPERIMENTAL

Assay—Quinidine concentrations in plasma and buffer from equilibrium dialysis were determined using a modification of a previously reported specific high-pressure liquid chromatography (HPLC) method (12). A 50-μl sample of plasma or a 200-μl sample of buffer was mixed with 200 μl (plasma) or 100 μl (buffer) of methanol containing 193 ng of hydroquinine[1]/ml as internal standard. Plasma samples were centrifuged in a microhematocrit centrifuge[2] at ~12,000×*g*. A 20-μl aliquot of the sample was then injected onto a C_{18} μBondapak column[3] of 30-cm length and 3.9-mm bore, connected to an HPLC pump[4]. The mobile phase consisted of methanol[5]–water–phosphoric acid[5] 85% (27.0:72.95:0.05) at a flow rate of 2 ml/min. Quantitation was achieved by means of fluorescence detection[6] with excitation at 245 nm and emission at 440 nm (cutoff filter). Standard curves were prepared daily using peak height ratios of various quinidine[7] concentrations in plasma or buffer prepared as above.

Equilibrium Dialysis—Aliquots (700 μl) of plasma or serum were dialyzed against 700 μl of buffer in a 1-ml dialysis cell[8]. A dialysis membrane[9] with an average pore radius of 24 Å, pretreated by soaking in distilled water (10 min), ethanol (15 min), and buffer (120 min), was used in these studies. The cells were incubated in a shaker bath[10] at 37° during the dialysis. At the end of the planned equilibrium time (6 hr ± 20 min, when not otherwise stated) the plasma or serum and buffer were removed from the half-cells and assayed for quinidine within 24 hr (stored at 4° until assayed). The ratio of quinidine concentrations in the buffer to the plasma or serum was taken as the free fraction of drug in the biological sample.

A blank serum or plasma sample was dialyzed in each binding experiment to assess the presence of endogenous or exogenous compounds that might interfere with the quinidine measurement.

Ultrafiltration—Protein determination using ultrafiltration was carried out with 3 ml of plasma or serum in a stirred cell[11] using an ultrafiltration filter with a molecular weight cutoff of 25,000[12]. The filtration was accomplished using a positive pressure of 3.5 atm with a gas mixture of 1.4% CO_2 in nitrogen at 37° in an incubator. The filtrate was collected in a polyethylene tubing[13]. Volumes of 100-μl aliquots were sampled from the tubing and assayed for quinidine immediately. Filtration was continued until subsequent aliquots of filtrate contained comparable amounts of drug.

Table I—Influence of Storage with Frequent Freezing and Thawing on Plasma Protein Binding of Quinidine

Sample	Free Fraction of Drug, Day 0	Day 2	Day 4	Day 7	Day 60
1	0.20	0.21	0.21	0.17	0.17
2	0.21	0.23	0.22	0.21	0.17
3	0.19	0.21	0.22	0.21	0.20
4	0.18	0.21	0.22	0.18	—
Mean	0.20	0.22	0.22	0.19	0.18
CV[a]	6.6	4.7	2.3	10.7	9.6

[a] Coefficient of variation in percent.

Sample Stability—To determine the influence of storage at −20° with frequent freezing and thawing on the protein binding, a large sample of rabbit plasma was spiked with quinidine[7] at a concentration of 2 μg/ml. A blank sample was kept separate but treated identically to the spiked portion. The samples were stored frozen with daily thawing and freezing for the first week. Protein binding was determined in 700-μl aliquots (quadruplicate) of the spiked sample by equilibrium dialysis [Krebs–Ringer bicarbonate buffer (14)] immediately after drawing the blood from the animal and again after 2, 4, and 7 days. An additional determination was made after 2 months.

Influence of Buffer Composition and Equilibration Time—The dependence of the apparent protein binding value determined by equilibrium dialysis on buffer composition and equilibrium time was evaluated in three experiments. A large sample of freshly drawn pooled rabbit plasma (stabilized with 5 U of heparin/ml) was spiked with 1 μg of quinidine/ml. Aliquots (700 μl) were dialyzed using 12 separate cells for each buffer. The cells were removed after 1, 2 (duplicates), 3, 4, 6 (duplicates), 8, 12, 18, and 24 (duplicates) hr and analyzed by HPLC. The buffers used were Krebs–Ringer bicarbonate and phosphate buffer pH 7.4, both prepared according to Umbreit *et al.* (14) and isotonic phosphate buffer pH 7.4 (0.107 *M* Na_2HPO_4, 0.024 *M* KH_2PO_4 in double-distilled water). The samples containing Krebs–Ringer bicarbonate solutions were dialyzed in a 5% CO_2–95% O_2 atmosphere, the other in a normal atmosphere.

Quinidine binding in the sample was also determined by ultrafiltration ($n = 6$). The first 200 μl of ultrafiltrate was discarded and the unbound concentration was determined in the subsequent aliquots. The concentration in these subsequent aliquots varied less than 2%.

Influence of Heparin—Blank rabbit serum was spiked with quinidine to a concentration of 0.4 μg/ml and with heparin[14] to final concentrations of 0, 5, and 20 U/ml. Protein binding was determined in triplicate by equilibrium dialysis and the values found in the heparinized samples were compared to those found in the nonheparinized samples (Student *t* test).

To test for an indirect effect of heparin *in vivo*, 450 U/kg was injected into a rabbit. Blood samples (3 ml) were collected immediately before injection and after 5, 10, 30, 60, and 180 min. Only the 0- and 180-min samples coagulated within 2 hr and allowed collection of serum; in the other samples the clear supernate after centrifugation was taken for analysis. All samples collected were spiked with 1 μg of quinidine/ml and protein binding was determined using equilibrium dialysis (duplicates) with the Krebs–Ringer bicarbonate buffer.

Concentration Dependence—Rabbit serum was spiked with quinidine at concentration of 250, 750, 1875, and 3000 ng/ml. Protein binding was determined in the samples (triplicates) using equilibrium dialysis with the Krebs–Ringer bicarbonate buffer.

RESULTS AND DISCUSSION

The assay used for quinidine measurements was fast and precise. The number of samples that can be analyzed in 1 day is only limited by the retention time of the compounds on the HPLC column (quinidine, 3.6 min; hydroquinine, 6.0 min). Because no extraction step was involved in the sample preparation there was little variability in repeated determinations of the same sample. For quinidine in plasma or serum the coefficient of variation (*CV*) in duplicated determinations was between 0.18 and 3.9% ($n = 6$) at 0.5 μg/ml and between 0.76 and 1.2% ($n = 2$) at

[1] Prepared by hydrogenation of quinine (Fluka AG, Chemische Fabrik, CH-9470 Buchs, Switzerland).
[2] Model MB, International Equipment Co., Needham Heights, Mass.
[3] Waters Associates, Milford, Mass.
[4] Model 110, Altex Scientific, Berkeley, Calif.
[5] AR grade, Mallinckrodt, St. Louis, Mo.
[6] Schoeffel Instrument Corp., Westwood, N.J.
[7] Fluka AG, Chemische Fabrik, CH-9470 Buchs, Switzerland; hydroquinidine free material prepared by method of Thron and Dirscherl (13).
[8] Technilabs Instruments, Pequannacock, N.J.
[9] VWR Scientific Inc., San Francisco, Calif.
[10] Braun-Melsungen AG, West Germany.
[11] Millipore Co., Bedford, Mass.
[12] Pellicon Type PS, Millipore Co., Bedford, Mass.
[13] Intramedic, 1.57-mm i.d., 2.08-mm o.d., Clay Adams, Parsippany, N.Y.
[14] Heparin sodium injection USP 1000 U/ml, Invenex.

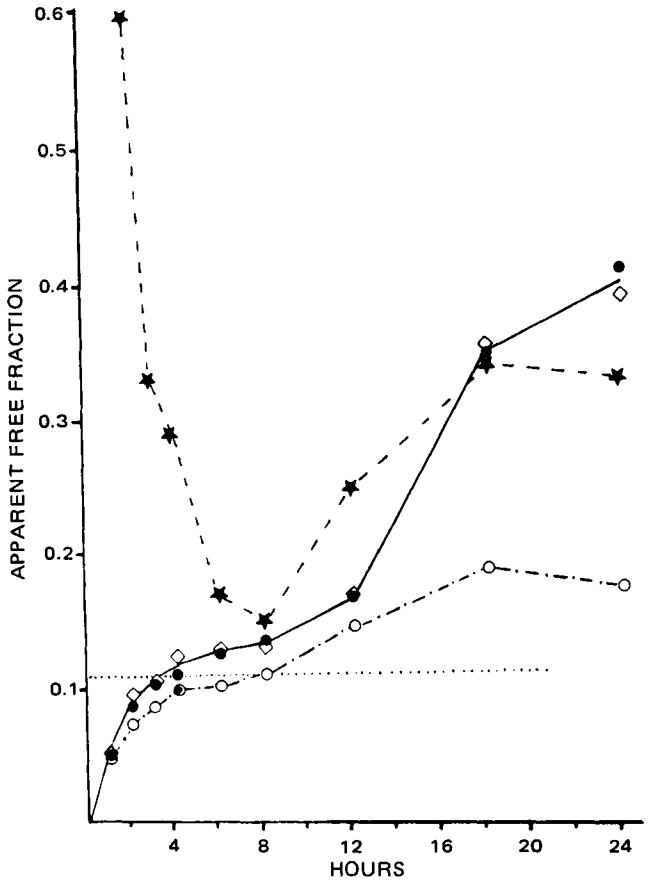

Figure 1—*Influence of buffer composition and equilibration time on apparent free fraction of quinidine in rabbit plasma. Key: ◊, spiked plasma dialyzed against Krebs–Ringer bicarbonate buffer; ●, spiked plasma dialyzed against Krebs–Ringer phosphate buffer; ○, spiked plasma dialyzed against isotonic phosphate buffer; ★ spiked Krebs–Ringer phosphate buffer dialyzed against rabbit plasma; and ·····, ultrafiltration of spiked plasma.*

1 μg/ml. Similar low variability was also observed in duplicate determinations of buffer samples: 0.94–6.3% (n = 4) at 50 ng/ml and 1.7–4.3% (n = 5) at 100 ng/ml. Standard curves were prepared daily by spiking blank plasma or water with various amounts of quinidine. The curves were linear with a CV for concentration-normalized peak height ratios of 4.5–7.5% in plasma and 2.0–5.8% in water.

A great deal of attention has been directed to the stability of biological samples when protein binding is to be measured. Denaturation of a major binding component under the storage conditions could influence the apparent free fraction of drug in the samples. Because freezing of biological fluids collected in pharmacokinetic studies is the most common form of storage, the influence of frequent freezing and thawing on the protein binding of quinidine in rabbit plasma was determined. Table I summarizes the result of a 2-month stability test. There was no significant change or trend in the free fraction of quinidine over a 1-week period with daily freezing and thawing of the sample, and no difference in the extent of binding could be detected after storage of the plasma in the freezer over a 2-month period. These results are in agreement with other findings (9).

The apparent free fraction of quinidine in a test sample was compared using equilibrium dialysis with three different buffers. Because little information exists regarding the time course of the equilibration process of quinidine, studies for each buffer were established. Samples equilibrated for 2, 6, and 24 hr were run in duplicate to allow estimation of the variability in the protein binding determination. Ultrafiltration of the same spiked plasma sample provided an independent estimate of the unbound drug concentration and yielded a value for the free fraction of 0.11.

In the dialysis experiments (Fig. 1), no clear equilibrium was reached for any one of the buffers even after 18–24 hr of dialysis. The values of the free fraction measured at these times exceeded the value of 0.11 determined by ultrafiltration and the samples showed turbidity. However, there appeared to be a temporary plateau discernible in the binding

Table II—Unbound Fraction of Quinidine in Plasma at Various Concentration Levels

Total Quinidine Concentration, ng/ml	Unbound Fraction[a]
250	0.11 (2.8%)
750	0.14 (5.1%)
1875	0.11 (14%)
3000	0.13 (3.2%)

[a] Mean (coefficient of variation).

curves between ~4 and 8 hr with unbound fraction similar to those obtained by ultrafiltration. Therefore, it can be concluded that equilibrium between the free quinidine concentration in plasma and in buffer was established after 4–6 hr, and that a second event causing displacement of drug from the protein or a particularly noticeable reduction of the binding capacity took place when dialysis extended beyond 8 hr. A second, much higher plateau in the equilibration curve was seemingly approached after 18–24 hr.

To further verify the use of the plateau at 4–8 hr as being representative of quinidine binding in plasma, the equilibrium time experiment was repeated using Krebs–Ringer phosphate buffer. However, in this instance the buffer side and not the plasma side was spiked. The result is shown in Fig. 1. After an initial rapid decline in the apparent drug free fraction, a trough value was attained after 8 hr at which time the apparent free fraction increased again, identically to the experiment using spiked plasma samples. The same plateau values were not attained as when the plasma side was spiked and only approached after 8 hr. This is readily explained by the general phenomenon that spiked buffer samples approach equilibrium at a slower rate than spiked plasma samples (15).

These experiments strongly indicate that under the given conditions the equilibrium is reached after 4–6 hr, and beyond 8 hr secondary effects occur that decrease the binding. Special care should be taken when using extended equilibrium times, particularly if the buffer side is spiked when assessing the binding. For example, in a study of demethylchlorimipramine protein binding (16), no apparent equilibrium was found even after 19 hr of dialysis when spiking the buffer side. However, a trough level was observed after 6 hr, after which the apparent free fraction started to increase again. It is possible that the 6-hr trough levels better represent the true unbound levels than the 19-hr dialysis values.

The reason for this phenomenon is not clear. Due to the increasing turbidity of the buffer samples, it is felt that microbiological growth took place, causing either drug displacement or binding protein degradation. This phenomenon is being investigated further.

Krebs–Ringer bicarbonate and Krebs–Ringer phosphate buffers gave higher unbound fraction values than isotonic phosphate buffer after 6 hr. This is consistent with previous findings (10, 11) that chloride displaces quinidine from plasma proteins. The Krebs–Ringer buffers contain chloride (128 mmoles/liter) while isotonic phosphate buffer does not. Because the Krebs–Ringer buffers contain a chloride concentration close to *in vivo* plasma levels, the use of these buffers would be expected to give values close to the *in vivo* unbound levels. However, the ultrafiltration values are closer to the values obtained by use of the isotonic phosphate buffer and therefore do not confirm this assumption.

Figure 1 shows that the increase in the apparent free fraction beyond 8 hr is less pronounced using the isotonic phosphate buffer. For practical reasons it may be more advantageous to use the isotonic phosphate buffer in binding determinations by equilibrium dialysis rather than Krebs–Ringer buffers, although the latter should be the buffer of choice on a theoretical basis.

Heparin is widely used clinically as an anticoagulant, *in vitro* for plasma collection methods, and *in vivo* for blood exchange techniques and for patients undergoing cardiopulmonary bypass or cardiac catheterization. It also has been shown to influence the protein binding or binding determination of various drugs including quinidine (1, 17, 18). The binding of quinidine to serum proteins was unaffected by a heparin concentration of 5 U/ml ($p > 0.10$) but significantly decreased in the presence of 20 U of heparin/ml ($p < 0.05$). Because 5 U of heparin/ml is sufficient to prevent coagulation *in vitro*, the influence of anticoagulant on protein binding could be prevented merely by reducing its concentration.

The effect of injected heparin was immediate with the strongest increase in the free fraction of drug in the first sample (collected at 5 min) and an exponential decline thereafter. Even after 3 hr, the binding of quinidine to proteins had not yet reached the pre-experimental value.

Although the amount of heparin injected may not be large compared to therapeutically administered doses, it is much more than would reach the general circulation by using indwelling heparinized catheters. The effect of lower amounts should be carefully assessed prior to studies using such catheters not only if protein binding determinations are planned, but in kinetic studies in general. This is important because altered protein binding can have a profound effect on kinetic parameters such as clearance (19) and apparent volume of distribution (20) or the interpretation of such data.

An earlier report (12) raised the possibility that protein binding of quinidine, at least in rabbits, might be capacity limited. However, Table II shows that there is no such effect discernible over the concentration range of 250–3000 ng/ml. Similarly, a previous study (9) found no concentration-dependence in the binding of quinidine to human serum over the clinically relevant range.

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Antineoplastic Effects of N^6-(Δ^2-Isopentenyl)adenosine against L-1210 Mouse Lymphocytic Leukemic Cells Using a Polymeric Delivery System

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Abstract □ N^6-(Δ^2-Isopentenyl)adenosine (I), a nucleoside previously shown to be cytotoxic against several types of tumor cells, was impregnated in silicone polymer monolithic disc devices for release *in vitro* against lymphocytic mouse leukemia cells. Plotting the cumulative amount of N^6-(Δ^2-isopentenyl)adenosine released per unit area of the device *versus* the square root of time revealed a linear relationship. However, the higher loading dose tended to rapidly release any drug deposited on the polymer surface. The optimum loading dose of the device for the most effective antileukemic activity in 24 hr was calculated based on a plot of the release rate *versus* the square root of an initial loading dose. The silicone polymer–I delivery system enabled a sustained and controllable release of additional agent. It was thus possible to achieve virtually total inhibition of leukemic cell replication using the polymeric delivery system. Increased concentrations of I, without the use of the polymeric system, resulted in maximum 24 hr inhibition of only ~81%, followed by a decline in overall antileukemic activity. It is possible to achieve a more predictable release rate of N^6-(Δ^2-isopentenyl)adenosine and corresponding antileukemic activity using a polymeric delivery system against L-1210 mouse leukemic cells *in vitro*. The relative data indicate the ED_{50} concentrations to be considerably less using the polymeric delivery system.

Keyphrases □ Delivery systems—impregnated silicone polymer, N^6-(Δ^2-isopentenyl)adenosine □ Release rates—impregnated silicone polymer delivery system, N^6-(Δ^2-isopentenyl)adenosine □ N^6-(Δ^2-Isopentenyl)adenosine—antineoplastic, improved delivery using impregnated silicone polymer

N^6-(Δ^2-Isopentenyl)adenosine (I), a nucleoside previously shown to be both an inhibitor and cytotoxic to human leukemic myeloblast and sarcoma-180 cells (1), was prepared entrapped in the polymeric delivery form of a silicone polymer[1] monolithic disk to evaluate its relative antineoplastic properties.

Earlier studies demonstrated that I, found also in several isoaccepting species of tRNA, can interfere with the transport of unmodified nucleosides through the cytoplasmic membrane of mouse embryo cells at the level of the transmembrane translocation function (2). This membrane transport inhibition is believed to be responsible for its ability to alter RNA synthesis in phytohemagglutinin-stimulated mouse spleen lymphocytes as well as to be immunosuppressive in nature (3). In the latter context, it was possible to prepare an antibody with serologic specificity for I (4). Previous studies (5) demonstrated that L-1210 mouse leukemic cells possess the necessary

[1] Silastic, 382 Medical Grade Elastomer, Dow Corning Corp., Midland, Mich.

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