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ANALYSIS OF *CINCHONA* ALKALOIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

APPLICATION TO THE ANALYSIS OF QUINIDINE GLUCONATE AND QUINIDINE SULFATE AND THEIR DOSAGE FORMS

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SUMMARY

The application of high-performance liquid chromatography to resolve the individual alkaloids present in marketed *Cinchona* alkaloids was investigated. Normal-phase and several reversed-phase systems were evaluated. The proposed procedure uses an alkylphenyl column; it adequately resolves quinidine, quinine, dihydroquinidine, dihydroquinine, cinchonine, cinchonidine, dihydrocinchonine and dihydrocinchonidine. Epiquinidine, epiquinine, quininone and quinitoxine are also resolved from quinidine and dihydroquinidine. This high degree of resolution enables the analysis of quinidine and its salts for their usual composition and establishes the absence of any cross-contamination or decomposition. The proposed procedure was applied to currently marketed samples of quinidine salts and their dosage forms. It was also applied to samples that were cross-contaminated or which contained decomposition products.

INTRODUCTION

The *Cinchona* alkaloids that are currently used therapeutically are usually mixtures of two or more of the individual alkaloids. There are significant differences in the therapeutic activity of the individual stereoisomers of the quinine series and of their desmethoxy analogs. Consequently, knowledge of the exact composition of the "drug substance" is important. This composition can vary, for example, depending on whether the quinidine is obtained from *Cinchona* bark or from the epimerization of quinine. The compounds that are most likely to be associated with quinidine are quinine, cinchonine, cinchonidine, epiquinidine, epiquinine, quininone and quinitoxine, together with their dihydro analogs.

With the objective of assessing the quality of the *Cinchona* alkaloids marketed as pharmaceuticals, thin-layer chromatographic (TLC) and gas–liquid chromatographic (GLC) procedures had previously been developed in this laboratory[1]. Since no single TLC or GLC system would separate all of the compounds under consider-

ation, multiple systems were used to characterize the samples. Subsequent to our report[1] on the quality of marketed *Cinchona* pharmaceuticals, the United States Pharmacopeia (USP) adopted the TLC fluorescence procedure as a limits test for the dihydro analog content of the individual quinidine and quinine salts in the drug substances and their dosage forms[2]. Each USP monograph also includes a TLC test to differentiate between quinidine and quinine[2]. A recent review of TLC procedures for the *Cinchona* alkaloids[3] cites the procedures developed in our laboratory and concludes that they are the recommended combination of solvent systems to give the most complete composition of the alkaloids. The review also indicates that there is no single solvent system that will resolve all of the alkaloids under consideration.

The continued variation in the therapeutic effect of different quinidine products has generated considerable interest in the study of the bioavailability of quinidine. With the advent of high-performance liquid chromatography (HPLC), many proposed procedures for the determination of quinidine and its metabolites in biological fluids have been published[4–20]. These procedures include either adsorption or reversed-phase columns but do not adequately resolve all of the alkaloids of interest. Recent methods for the application of HPLC to the analysis of *Cinchona* alkaloids in botanical extracts and dosage forms also lack the desired specificity or sensitivity to detect decomposition products such as quininone and quinitoxine[21–30].

In addition, recent instances encountered in our laboratory and elsewhere of cross-contamination of quinine and quinidine dosage forms make it desirable for regulatory purposes to have suitable procedures that will quantitate isomeric composition, relative vinyl- and dihydro-analog concentrations, and desmethoxy-analog content. Investigation of those HPLC procedures that were available[4–13,21–24] served as the starting point in the development of the proposed procedure. It was soon found that the resolution of the individual vinyl and dihydro pairs could be achieved with several systems, but that complete resolution of all of the alkaloids of interest was not readily obtainable. Therefore, a study of the chromatographic behavior of the various *Cinchona* alkaloids with a number of normal-phase and reversed-phase HPLC procedures was undertaken to establish a suitable method. The results of our investigation are presented in this paper; it was found that an alkylphenyl reversed-phase system achieves the resolution required for regulatory purposes.

EXPERIMENTAL

Apparatus

The chromatography was performed with a Model 3500B liquid chromatograph (Spectra-Physics, Mountain View, CA, U.S.A.) equipped with a 7000-p.s.i. injection valve (Valco Instruments, Houston, TX, U.S.A.), a 10-μl injection loop and a chromatographic column oven. The UV detector was a Model 230 (Spectra-Physics) set at 254 nm. The detector sensitivity was set at 0.08 a.u.f.s. A Model 4000 data integration system (Spectra-Physics) operated at fixed parameter was used. The recorder was a printer-plotter (Spectra-Physics) set at 10 mV full scale.

The HPLC columns were stainless-steel, 25–30 cm × 4–4.6 mm I.D., packed with an alkylphenyl phase bonded to a 5- to 10-μm silica support. A suitable brand of column was μBondapak Phenyl 4 × 30 (Waters Assoc., Milford, MA, U.S.A.).

Reagents

Organic solvents. Methanol, 2-methoxyethanol and acetonitrile were HPLC grade, UV quality and distilled-in-glass (Burdick & Jackson, Muskegon, MI, U.S.A.).

Sodium dihydrogen phosphate solution, 0.05 M. Sodium phosphate monobasic monohydrate, reagent grade (13.80 g), was transferred to a 2-l volumetric flask and dissolved with water which was purified by a Milli-Q water system (Millipore, Bedford, MA, U.S.A.). The solution was diluted to volume. The pH of the final solution (adjusted if necessary with 0.05 *M* phosphoric acid or 0.05 *M* sodium hydroxide) was 4.5.

HPLC mobile phase. Acetonitrile (15 volumes) was mixed with 2-methoxyethanol (15 volumes). A 0.05 *M* sodium dihydrogen phosphate solution (70 volumes) was added and the final solution was mixed well. The prepared solution was filtered through a 5-μm LS Millipore filter with a suitable vacuum system that facilitated deaerating at the same time.

Cinchona alkaloids. These compounds were purified as described in ref. 1. Quinidine sulfate and quinidine gluconate were USP Reference Standards. (All standards were used in their available forms, *viz.*, anhydrous or dihydrate, *without drying.*)

Standard stock solutions. An accurately weighed portion of the alkaloid or alkaloid sulfate (*ca.* 50 mg) was transferred to a 50-ml volumetric flask. (Quinidine gluconate (40 mg) was transferred to a 25-ml volumetric flask.) The standards were dissolved in methanol and diluted to volume.

Internal standard solution. An accurately weighed portion of cinchonidine (*ca.* 25 mg) was transferred to a 25-ml volumetric flask. The internal standard was dissolved in methanol and diluted to volume.

HPLC standard solutions. A 4.0-ml portion of internal standard solution and 5.0 ml of the standard stock solution were transferred to a 10-ml volumetric flask. The solution was diluted to volume with methanol and mixed well.

Sample preparation. An accurately measured portion of injection, tablet or capsule was transferred to a suitable volumetric flask to give a final concentration of *ca.* 1 mg of quinidine sulfate or 1.6 mg of quinidine gluconate per milliliter of solution. (For tablets or capsules, the powdered aliquot was added to the volumetric flask followed by *ca.* 75% of the methanol.) The flask was shaken for 30 min with the aid of a mechanical shaker. The sample solution was diluted to volume with methanol.

HPLC sample solution. A portion of the sample solution was filtered through a Millipore filter with the use of a syringe filter holder (*e.g.*, Millex-HV). A 5.0-ml portion of the filtrate of a quinidine sulfate sample or 4.0 ml of a quinidine gluconate sample was transferred to a 10-ml volumetric flask containing 4.0 ml of the internal standard solution. The HPLC sample solution was diluted to volume with methanol.

High-performance liquid chromatography

The HPLC column was equilibrated with the mobile phase with the column set in a chromatographic oven to maintain ambient temperature. A recorder, integrator and UV detector were used to measure the chromatographic response of the HPLC solutions.

System suitability test. Three 10-μl aliquots of the HPLC standard solution were injected onto the column. The response was measured and the resolution be-

tween the peaks of the two major components, cinchonidine and quinidine, was calculated according to the formula in the USP[31]. A minimum resolution value of 4.5 with the flow-rate set at 1.0 ml/min should be obtained. The reproducibility coefficient of variation (CV) of the relative areas should be not more than 2%. (Once the column suitability has been determined, the subsequent system suitability test is simply the area reproducibility of the HPLC standard solution; the CVs of the replicates should be no greater than 2%.) Samples and standards were analyzed with the flow-rate set at 1.0 ml/min. This flow-rate should be adequate to resolve the usual components found in the samples and standards and should shorten the analysis time. (If the samples are grossly cross-contaminated, a lower flow-rate of 0.6 ml/min is required to establish baseline resolution.)

Determination. Aliquots of HPLC standard solutions and HPLC sample solutions were injected onto the HPLC column with the use of a 10-μl loop injector. The response was recorded and the areas of the observed peaks were measured. The relative areas of the internal standard cinchonidine peak and the combined areas of the quinidine and dihydroquinidine peaks were calculated. The response ratios were used to calculate the total weight of quinidine salts in the sample compared with the total weight of quinidine salts in the standard. The percentage of dihydroquinidine in the sample was calculated from the ratio of the area of the dihydroquinidine peak to the sum of the areas of the quinidine and dihydroquinidine peaks.

RESULTS AND DISCUSSION

Both normal-phase and reversed-phase HPLC procedures were evaluated and are discussed separately.

Normal-phase HPLC procedures

The procedure proposed by Cohen[32] that uses a basic solvent system containing methylene chloride, 2-methoxyethanol and ammonia in conjunction with a silica HPLC column (μPorasil, Waters Assoc.) was investigated. After the ratio of the components of the mobile phase was modified, the procedure adequately resolved quinine, quinidine, dihydroquinidine and dihydroquinine, but not the related desmethoxy alkaloids cinchonine and cinchonidine, which interfered with the quantitation of quinine and quinidine. The use of other modifiers together with methylene chloride or ethylene chloride as the primary solvent was investigated next. It was found that the base could be modified from ammonia to ethanolamine but not to diethylamine, which caused decomposition. The order of elution could be changed with the use of methanol as the modifier. The desmethoxy analogs eluted after quinidine and quinine instead of between those two components as they did with the 2-methoxyethanol solvent system. However, full resolution was still unattainable with this column.

Investigation of other silica HPLC columns was initiated. Better resolution was observed with a Partisil-10 (Whatman, Clifton, NJ, U.S.A.) column. However, a shorter Zorbax-Sil (DuPont, Wilmington, DE, U.S.A.) column afforded the best resolution. The solvent system described above gives adequate resolution of quinidine, quinine, dihydroquinidine, cinchonine and dihydrocinchonine. However, the desired resolution of dihydroquinidine and dihydroquinine and that of cinchonine

and cinchonidine were not obtained. Nevertheless, the procedure is adequate for many purposes since the cinchonine–cinchonidine or the dihydroquinidine–dihydroquinine pair is not ordinarily present, except in the case of cross-contamination, which should be readily discernible by the presence of the quinine peak in the chromatograms of the quinidine samples or *vice versa.*

With the availability of a 5-μm silica column (Partisil-5, Whatman) and the modification of the ratio of the components of the proposed mobile phase, the resolution of quinidine, quinine, dihydroquinidine, dihydroquinine, cinchonine and dihydrocinchonine was obtained (Fig. 1). In addition to the resolution of these alkaloids, epiquinidine, epiquinine, quininone and quinitoxine were resolved from the usual components of quinidine by this normal-phase procedure.

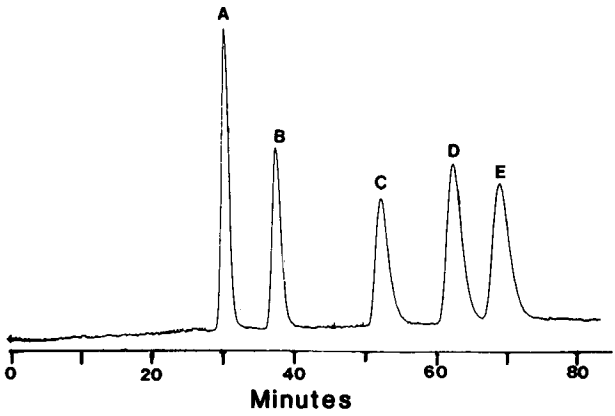

Fig. 1. Separation of *Cinchona* alkaloids on a Partisil-5 column, 25 cm × 4.6 mm I.D. Mobile phase, ethylene chloride–methanol–ammonium hydroxide (96:4:0.25). Flow-rate, 2 ml/min. Peaks: A = quinidine; B = quinine; C = cinchonine; D = dihydroquinidine; E = dihydroquinine.

A complication of the normal-phase procedure is that the UV absorption spectra of the individual alkaloids are pH-dependent; the quinine series spectra differ significantly from those of the desmethoxy series[33]. Because ready sources of pure individual alkaloids were not available, it was thought that the proposed procedure could be simplified by eliminating standard plots of each alkaloid. The spectra of the alkaloids obtained under the conditions of the proposed mobile phase were examined. The UV absorption spectra of quinidine and cinchonine (its desmethoxy analog) in the proposed mobile phase are illustrated in Fig. 2. It appeared that selection of the wavelength at which the two series of alkaloids have the same absorption at the same concentration would simplify the quantitation; this would enable quantitation from measurement of the relative responses of the individual alkaloids within the chromatogram. However, inadequate detector capabilities made this approach unsuccessful. The desired isobestic point was not reproducible with our variable-wavelength detector. Thus suitable internal standard and reference standard curves for each component in the sample would have been required to make this procedure acceptable.

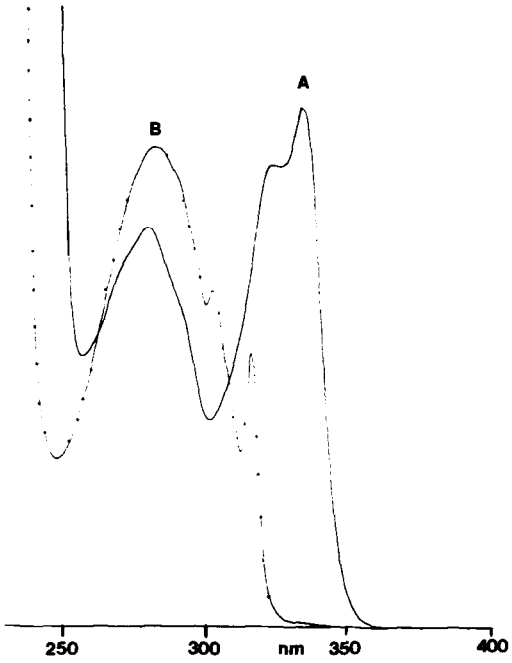

Fig. 2. UV absorption spectra of quinidine (A) and cinchonine (B) in HPLC mobile phase, ethylene chloride–methanol–ammonium hydroxide (96:4:0.25), at a concentration of 4.2 mg/100 ml.

Reversed-phase HPLC procedures

With the use of various bonded reversed-phase columns, the individual pairs of vinyl and dihydro analogs were readily resolved, but the simultaneous resolution of the optical isomers was not obtained. For example, with one microparticulate ODS column (μBondapak, Waters Assoc.) and an acidic mobile phase, the individual pairs were resolved but the quinine and dihydroquinidine peaks overlapped. Further efforts to resolve these two alkaloids were unsuccessful. There are considerable differences in the chromatographic behavior of different brands of C-18 columns. Except for an ODS-3 column (Whatman), the resolution of vinyl and dihydro analogs was not obtained with the acidic solvent system. Investigation of other reversed-phase packings gave similar results. These included supports that were "fully capped" and those that still had available silanols from the silica support. The primary mechanism of chromatographic separation appears to be the interaction of the support backbone rather than the partitioning with the bonded phase. The use of a basic solvent system did reduce the extensive tailing but the desired resolution was not observed. The use of an "ion-pairing reagent" with the acidic solvent system reported by Simon[34] produced resolution of quinine and dihydroquinidine that was no better than that observed in our laboratory with a μBondapak ODS column and the acid solvent system used without the ion-pairing reagent. Because it appeared that this approach would not be fruitful, another reversed-phase procedure[35] was examined. This procedure was adequate for the determination of quinidine and dihydroquinidine content only in the absence of their stereoisomers. Quininone was not detectable with this method,

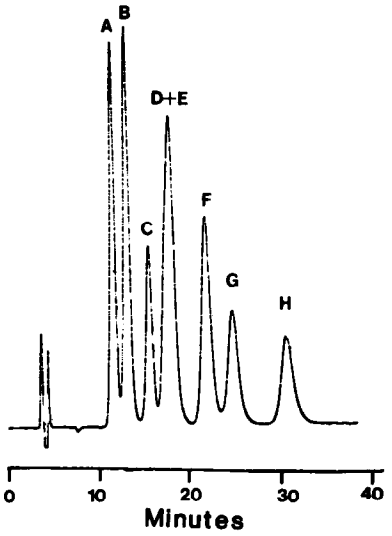

Fig. 3. Separation of *Cinchona* alkaloids with ion-pair HPLC procedure[37] on a μBondapak C_{18} column, 30 cm × 4 mm I.D. Mobile phase, methanesulfonic acid (1.0 *M*)–diethylamine solution (1 *M*)–water–acetonitrile (20:20:860:100). Flow-rate, 1.0 ml/min. Peaks: A = cinchonine; B = cinchonidine; C = dihydrocinchonine; D = dihydrocinchonidine; E = quinidine; F = quinine; G = dihydroquinidine; H = dihydroquinine.

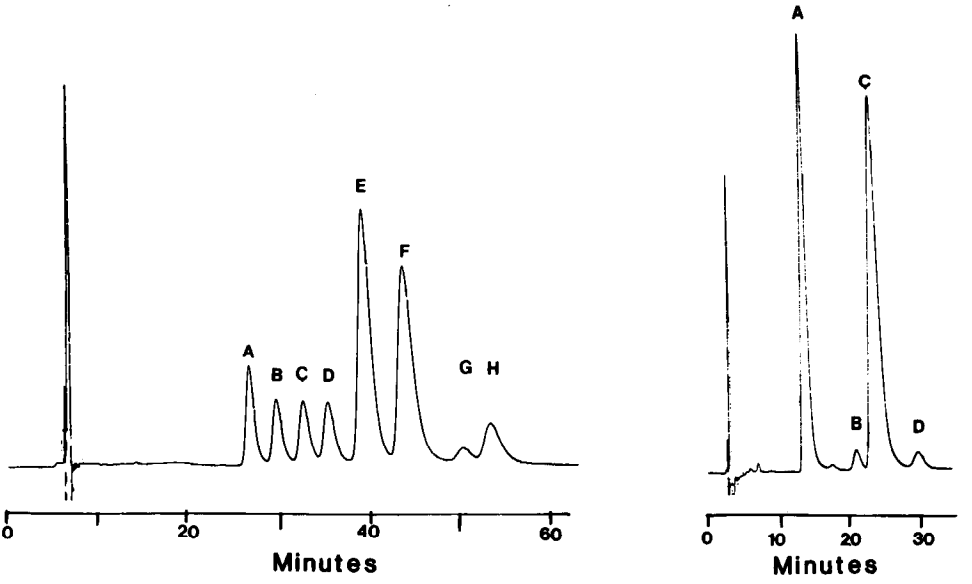

Fig. 4. Separation of *Cinchona* alkaloids on a μBondapak Phenyl column, 30 cm × 4.0 mm I.D. Mobile phase, 0.05 *M* sodium dihydrogen phosphate solution–acetonitrile–2-methoxyethanol (70:15:15). Flow-rate, 0.06 ml/min. Peaks: A = cinchonidine; B = cinchonine; C = dihydrocinchonidine; D = dihydrocinchonine; E = quinine; F = quinidine; G = dihydroquinine; H = dihydroquinidine.

Fig. 5. Chromatogram of quinidine gluconate injection sample on μBondapak Phenyl column, 30 cm × 4.0 mm I.D. Mobile phase, 0.05 *M* sodium dihydrogen phosphate solution–acetonitrile–2-methoxyethanol (70:15:15). Flow-rate, 1.0 ml/min. Peaks: A = cinchonidine (internal standard); B = quininone; C = quinidine; D = dihydroquinidine.

and sensitivity for the desmethoxy alkaloids, which depends on their spectral properties in the mobile phase, was poor. Therefore, this method was found to be inadequate for the proposed purpose.

An ion-pair reversed-phase procedure proposed for the USP[36,37] for quinine and its dosage forms was also evaluated. It requires the use of a shorter wavelength, *i.e.*, 235 nm, to detect the presence of the desmethoxy analogs and does not resolve all of the alkaloids of interest (Fig. 3). In addition, this procedure does not detect quininone.

The procedure proposed in this paper uses an alkylphenyl column and is based on the work of Guentert *et al.*[17] for the determination of quinidine in biological fluids. Their mobile phase of phosphate buffer–acetonitrile–tetrahydrofuran did not give the desired resolution. The effect of pH and the ratio of organic modifiers was examined. Optimum resolution was obtained with the use of 0.05 *M* sodium dihydrogen phosphate solution (pH 4.5) and a mixture of acetonitrile and 2-methoxyethanol as the organic modifiers. The mobile phase proposed here adequately resolves eight related alkaloids (Fig. 4). Their resolution can be compared to the resolution obtained with the recent ion-pair procedure[36,37] illustrated in Fig. 3 for the same eight alkaloids; only seven peaks appear in Fig. 3, and the decomposition product quininone is not detected. In contrast, quininone is readily detected with the proposed procedure with the alkylphenyl column (Fig. 5).

Two different procedures for the preparation of the samples for HPLC analysis were compared. The first used 50% methanol as the solvent and cinchonidine sulfate as the internal standard. The second used methanol as the solvent and cinchonidine as the internal standard. Both procedures gave the same results for samples of quinidine salts and their dosage forms. The procedure using cinchonidine as the internal standard was selected because the cinchonidine that was available was found to have

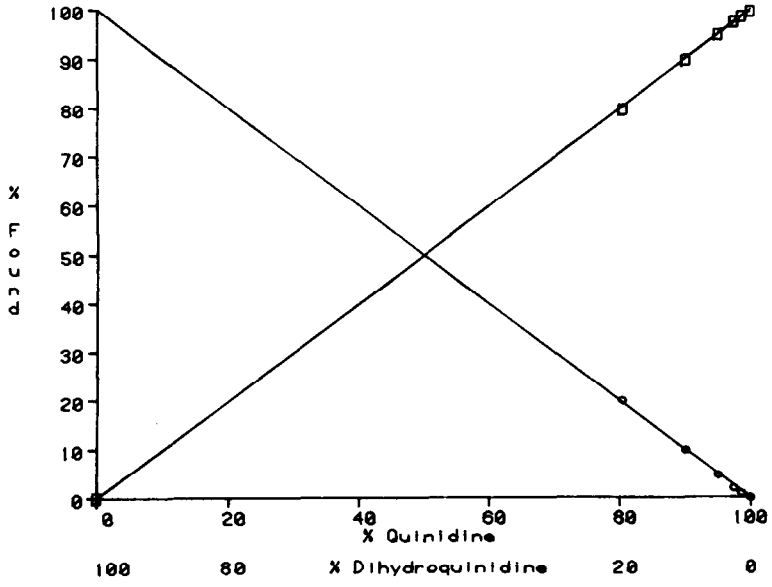

Fig. 6. Linearity data for quinidine–dihydroquinidine mixtures. (□) Quinidine; (○) hydroquinidine.

fewer impurities than the cinchonidine sulfate and because methanol is a better solvent than 50% methanol for most of the *Cinchona* alkaloids and their salts.

The linearity of peak area response was evaluated with respect to the total quinidine salt content (both vinyl and dihydro analogs). Response was linear in the range of 50 to 150% of the usual concentration of total alkaloids found in the HPLC sample preparation step and through the usual range of ratios of dihydro and vinyl alkaloids. An example of a linearity plot is shown in Fig. 6.

The mobile phase originally proposed contained phosphate buffer, acetonitrile and 2-methoxyethanol in a ratio of 80:15:5, which produces a baseline separation of quinine and quinidine as well as their dihydro analogs. With a flow-rate of 2 ml/min, a complete chromatogram is obtained in *ca.* 50 min. The final proposed procedure uses a mobile phase of the same solvents in a ratio of 70:15:15 and a flow-rate of 1.0 ml/min. This was found to adequately resolve those components that are usually

TABLE I

ANALYSIS OF QUINIDINE SULFATE

Product	*Samples**	*Percentage of label***	*Dihydroquinidine (% of total found)*
Tablets, 200 mg	T	101.0	3.1
		102.3	3.0
	L	101.3	6.8
		101.0	6.8
	Z	97.8	10.1
		97.4	10.1
	D	104.5	4.7
		103.4	4.6
	P***	95.8	3.7
		96.7	3.7
	S***	98.3	7.3
		98.4	7.3
Tablets, 300 mg	K	99.1	4.2
		100.0	4.1
	R	98.4	5.5
		101.8	5.0
Capsules, 200 mg	L	101.1	8.5
		102.9	8.3
Capsules, 100 mg	R	96.2	19.9
		95.9	20.1
Injections, 200 mg/ml	M	96.5	6.7
		96.5	6.7
	A_1	101.2	6.6
	A_2	96.3	5.3
Powder	A	100.4	8.0
	B	98.9	5.5
	C	98.8	8.2
	D	99.0	7.9

* Listed by manufacturer. Samples having the same letter and different subscripts are different samples from the same manufacturer.

** Total quinidine and dihydroquinidine sulfate.

*** Analysis of aliquots of tablet composites. All other analyses are of individual tablets or capsules.

TABLE II

ANALYSIS OF QUINIDINE GLUCONATE

Product	*Samples**	*Percentage of label***	*Dihydroquinidine (% of total found)*
Powder	N	99.3	11.9
	P	99.3	3.5
	L	100.0	3.5
Injections, 80 mg/ml	W***	55.0	5.8
		55.0	5.7
	L_1***	95.8	3.0
		97.0	3.8
	L_2	102.4	6.4
	L_3	103.8	7.0

* Listed by manufacturer. Samples having the same letter and different subscripts are different samples from the same manufacturer.

** Total quinidine and dihydroquinidine gluconate.

*** These samples of quinidine gluconate injections were discolored; the presence of quininone was confirmed by TLC. Sample W also contained the thioglycerol adduct of quinidine[38].

present in a quinidine sample as well as any synthesis or decomposition products such as quininone.

The proposed procedure was applied to some aged and discolored samples. The results of the assays were low and the chromatograms showed the presence of a peak that corresponds to quininone (Fig. 5). The presence of quininone was confirmed by TLC[1]. Good precision and accuracy were demonstrated by HPLC analyses of samples of quinidine sulfate and quinidine gluconate and their dosage forms (Tables I and II). Table III lists the results for the assays of cross-contaminated samples in dosage form. Table IV shows the retention times observed for the *Cinchona* alkaloids that were chromatographed with the proposed HPLC method.

TABLE III

ANALYSIS OF CROSS-CONTAMINATED SAMPLES

Product	*Percentage of total alkaloid salt*			
	Quinine	*Quinidine*	*Dihydroquinine*	*Dihydroquinidine*
Quinine sulfate capsules	42.3	49.4	3.6	4.7
Quinidine sulfate tablets*	22.0	73.5	–	4.4
	19.4	76.2	–	4.4

* Two different lots from the same manufacturer.

TABLE IV

HPLC RETENTION TIMES OF *CINCHONA* ALKALOIDS

Mobile phase, acetonitrile–2-methoxyethanol–0.05 *M* sodium dihydrogen phosphate solution (pH 4.5) (15:15:70). Column, alkylphenyl, 30 cm × 4 mm I.D. Flow-rate, 1.0 ml/min.

Alkaloid	*Retention time (min)*
Quinitoxine	3.6
Cinchonidine	14.4
Cinchonine	16.1
Epiquinidine	16.7
Dihydrocinchonidine	17.8
Dihydrocinchonine	19.3
Quininone	21.2
Quinine	21.9
Quinidine	24.4
Dihydroquinine	27.9
Dihydroquinidine	29.7

CONCLUSIONS

HPLC procedures were investigated for applicability to the determination of the individual alkaloids present in quinidine and its salts that are currently being used therapeutically. Both normal-phase and reversed-phase HPLC procedures were evaluated. The *Cinchona* alkaloids examined were quinidine, quinine, cinchonine, cinchonidine, epiquinidine, epiquinine, quininone and quinitoxine, together with their dihydro analogs. The desired resolution of the alkaloids was obtained with an alkylphenyl reversed-phase column and a mobile phase containing phosphate buffer, acetonitrile and 2-methoxyethanol. In addition, the proposed procedure can readily detect the presence of any cross-contamination of stereoisomers (*e.g.*, quinine in quinidine) or the presence of any decomposition or synthesis precursor such as quininone. The proposed procedure was applied to samples of currently marketed quinidine sulfate and quinidine gluconate and their dosage forms.

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