Journal of Chromatography, 278 (1983) 117—132
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1845

DETERMINATION OF QUINIDINE IN SERUM BY SPECTROFLUOROMETRY, LIQUID CHROMATOGRAPHY AND FLUORESCENCE SCANNING THIN-LAYER CHROMATOGRAPHY

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(First received March 28th, 1983; revised manuscript received July 7th, 1983)

SUMMARY

Quinidine is determined in serum by direct and extraction spectrofluorometry, by reflectance fluorescence scanning thin-layer chromatography (TLC), and by high-performance liquid chromatography (HPLC). Least-squares analyses of patients' sera ($n=62$) analyzed first by direct fluorometry (x) and then HPLC (y) gave a slope of 0.52, an y-intercept of −0.40, a standard error of estimate of 0.65, and a correlation coefficient of 0.83. Comparison of patients' sera ($n=59$) determined by extraction fluorometry (x) and then HPLC (y) gave a slope of 0.998, an y-intercept of −0.175, a standard error of estimate of 0.30, and a correlation coefficient of 0.96. Comparison of patients' sera ($n=36$) by HPLC (x) and then reflectance fluorescence scanning TLC (y) gave a slope of 0.837, an y-intercept of 0.152, and a correlation coefficient of 0.94. Methaqualone and oxazepam interfere with HPLC. Within-run precision is 1.6, 1.0, 5.2 and 3.0% by direct fluorometry, extraction fluorometry, TLC and HPLC while between-run precision is 5, 3.5, 9 and 6.0%, respectively.

INTRODUCTION

Quinidine, a naturally occurring alkaloid, is the dextrorotatory isomer of quinine. It acts as a myocardial depressant by depressing excitability, contractibility, and conduction velocity, and is used to treat cardiac arrhythmias. Quinidine has a narrow therapeutic range [1—7] and knowledge of the quinidine serum concentration is therefore required for effective therapy.

0378-4347/83/$03.00

Analytical methods for quinidine determinations are characterized by a combination of sample-preparation techniques and of instrumentation required for detection and quantitation. Some of the first methods were developed to measure the naturally occurring fluorescence of quinidine and they were very simple and sensitive. An example is the protein precipitation—fluorescence method of Brodie and Udenfriend [8]. Because of its simplicity, this method is non-selective. Quinidine metabolites are not separated or distinguished from quinidine in the sample and other drugs that fluoresce will interfere with this fluorometric analysis. The therapeutic range is 4–8 mg/l [1].

In attempts to improve the analytical selectivity of quinidine methods, various extraction techniques and solvents to preferentially remove the parent drug from the metabolites have been developed. In most of these methods the sample is made alkaline and then extracted with an organic solvent. Generally, these methods employed benzene [2,9,10], ethylene dichloride [9,11], chloroform [8,13], toluene [14] and an amylalcohol—benzene mixture [12,15]. In some studies, the organic extract was acidified with trichloroacetic acid [1,11] whereas in others, the organic extract was re-extracted with acid and subsequently the acidic solution was measured spectrofluorometrically [9,10,14,16]. The most commonly used extraction procedure is the double-extraction method of Cramer and Isaksson [9]. Many interferents and the more polar metabolites of quinidine are eliminated by this double-extraction procedure [9,10]. The therapeutic range is 2—5 mg/l when determined by this method.

Although the selectivity for quinidine analysis is improved by the single- and double-extraction procedures, there are still some disadvantages. Dihydroquinidine and quinidine are not separated and the extraction steps increase the overall length of time for completion of the analysis. Nevertheless, the acquired selectivity outweighs the disadvantage of the increased time for analysis. A double-extraction method is also more applicable to urine samples.

A large group of quinidine methods are based on some type of chromatographic technique. Examples of these types of methods are thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), gas chromatography (GC), and gas chromatography—mass spectrometry (GC—MS). For TLC, the sample may either be directly applied (spotted) on the TLC plate [17,18] or the sample may be extracted and the concentrated extract be applied to the TLC plate [12,19]. Quantitation is done by scraping off the separated compounds of interest from the adsorbent. The concentrated extracts are then measured for quinidine with a fluorometer [3,12,15,20—22]. An alternative to extracting the TLC plate is to directly scan the plate while monitoring either fluorescence [17,18,23] or ultraviolet absorption [19,24].

TLC methods for quinidine are selective and sensitive, with the precision of some of the methods being similar to HPLC. Dihydroquinidine and quinidine metabolites are separated from quinidine by TLC. A major disadvantage to TLC in the past was the lack of an appropriate instrument for detection and quantitation without having to remove the compound from the TLC plate. Our TLC method for quinidine uses a dual-channel reflectance fluorescence scanning spectrodensitometer and circumvents the previous difficulties.

The requirement for more sensitive and selective analytical methods for

quinidine has produced a variety of HPLC methods that use fluorescence [5,25—30] and ultraviolet [31—41] detection. Some of these methods provide the separation and quantitation of quinidine, dihydroquinidine, and metabolites such as 2′-quinidinone, 3-hydroxyquinidine, quinidine-N-oxide and O-desmethylquinidine [5,25,27,28,30,42,43]. A method based on the direct injection of the sample without any type of extraction has been reported [32].

A limited number of GC methods for quinidine have been developed [13,15, 23,44,45,46]. They all require a flame ionization detector except one [15] which used a nitrogen-selective detector. Because of the poor detectability of quinidine by GC the parent drug is usually derivatized either as the trimethylsilyl derivative [13,23] or the methyl derivative [44—46]. Quinidine and dihydroquinidine could not be distinguished by one of the methods [46].

Several attempts have been made to determine quinidine by MS or GC—MS. One method required isobutane chemical ionization [47]. In this method quinidine is extracted with benzene from alkalinized plasma and the extract is evaporated to dryness. The residue is then put into the mass spectrometer by direct insertion probe. Another technique is methane chemical ionization [19]. Although GC—MS methods are sensitive and specific, the instrumentation is very expensive and requires experienced personnel for its operation and maintenance. For the present, these methods can only be used for research studies. The most recently developed analytical method for quinidine is enzyme immunoassay [48,49]. A comparison of EMIT* (enzyme multiplied immunoassay technique) with the fluorescence method of Cramer and Isaksson [9] and HPLC [27,50] indicates that the EMIT method is not specific for quinidine [49], but the method is acceptable for clinical use.

Quinidine therapeutic levels will vary depending on which one of the above analytical methods is used for quantitation. For example, the reported therapeutic range is 4—8 mg/l when determined by the non-specific fluorometric methods [1,6,9,10]. As the methods become more selective, the range narrows from 2.3 to 5 mg/l [2,51]. In a study in which HPLC was used the levels were 50% of that found in the direct fluorometric methods [34]. Those methods that do not separate quinidine from dihydroquinidine may still be useful because both compounds have similar pharmacokinetics and activities [3,52,53]. Regardless of the final method selected for quinidine determinations, the resulting therapeutic range should be a guide to the physician and will have to be interpreted in conjunction with clinical signs and symptoms of the patient.

Quinidine concentrations above 10 mg/l are associated with toxic effects [1,54—56]. About one-third of the treated patients will have an immediate adverse intolerance to quinidine and therapy will have to be altered. Precautions should be taken when patients with congestive heart failure and impaired renal function are treated with quinidine because of the persistence of elevated serum levels of quinidine [57] and its metabolites [2]. As concentrations of quinidine in plasma go above 2 mg/l, the QRS complex** widens progressively. This change is useful for the monitoring of patients. A 25%

*EMIT is the trade name for the enzymatic immunoassay system marketed by SYVA, Palo Alto, CA, U.S.A.

**QRS complex is the ventricular electrical activity of the heart.

increase of QRS complex is a cause for concern and with a 50% increase, the dosage should be immediately reduced. The most common adverse reactions to quinidine are nausea, vomiting and diarrhea [55,56,58]. Cinchonism, which includes tinnitus, loss of hearing, slight blurring of vision and gastrointestinal disturbances, is caused by an overdose of quinidine. If toxicity is severe enough, confusion, delirium and psychosis may occur.

Pharmacokinetic data of quinidine and its metabolites indicate that a two-compartment open model best describes the pharmacokinetics of quinidine [20,52]. The major route of metabolism appears to be in the liver since renal excretion of intact drug accounts for only 10—20% of the given dose which is 10—20 mg/kg d [14,20,21,26]. The distribution half-life is about 6—12 min and the elimination half-life is about 4—8 h [2,20,21,26,59,60]. The elimination half-life does not appear to vary in patients with congestive heart failure [2,26] or poor renal function [2,61]. The apparent volume of ditribution is about 3 l/kg and the central-compartment volume is 0.9 l/kg [14,20,26]. The total body clearance is about 4.5 ml/min/kg with great variation being observed among patients [6,20,26,50,60]. Some of the metabolites of quinidine that have been identified are 3-hydroxyquinidine [62], 2'-quinidinone [13,62,63], O-desmethylquinidine [4,13,25,62,64], and quinidine-N-oxide [6,36]. The major end-products of quinidine metabolism are 3-hydroxyquinidine and 2'-quinidinone. About 1—2% of quinidine goes to O-desmethylquinidine and is excreted in the urine [4]. Studies have revealed that some of the metabolites have anti-arrhythmic activity but definitive human data is questionable [25,27,50].

With the acceptance of newer analytical methodology for quinidine, investigators are re-evaluating the pharmacokinetic data, the interaction of quinidine and other drugs such as digoxin [65—69], digitoxin [70,71], propranolol [72], and phenytoin [73], and the effect of other drugs in the presence of quinidine [40,74].

In this study four analytical methods for quinidine are compared. Two of the methods, the fluorometric ones, are currently used in our laboratories. The HPLC method is a recently developed method by our laboratory and the fluorescence scanning—TLC method that uses a spectrodensitometer with dual-channel reflectance fluorescence capabilities was developed during this investigation.

MATERIALS AND METHODS

Apparatus

A Model J4-8963 ellipsoidal condensing system spectrofluorometer and a Model J10-280 photomultiplier microphotometer equipped with a 1P21 photomultiplier tube (all from American Instrument, Silver Spring, MD, U.S.A.) were used. A Model SD 3000 scanning spectrodensitometer equipped with a QPM 30 quartz prism monochromator, an SDA-335 reflection mode assembly and a Model SDC 300 density computer (all from Schoeffel Instrument Division, Kratos, Westwood, NJ, U.S.A.) were used for the fluorescence TLC measurements.

For high-performance liquid chromatography (HPLC) we used a Model

6000-A solvent-delivery system, Model 600 solvent programmer, Model 440 fixed-wavelength absorbance detector (254 nm), Model 450 variable-wavelength absorbance detector, Model U6K universal liquid chromatograph injector (all from Waters Assoc., Milford, MA, U.S.A.) and a Model B5217-1 dual-pen recorder (Houston Instrument Division, Bausch and Lomb, Austin, TX, U.S.A.). HPLC separations were done on a prepacked 10-μm particle size μ-Bondapak C_{18} (300 × 3.9 mm I.D.) reversed-phase column from Waters Assoc.

TLC separations were done on 20 × 20 cm, Type LK5D, precoated silica gel plates with a preadsorbent area (Whatman, Clifton, NJ, U.S.A.). Tanks were equilibrated with the solvent prior to use.

Reagents

All solutions were prepared in glass-distilled de-ionized water from analytical or spectral grade reagents and solvents (Burdick and Jackson, Muskegon, MI, U.S.A.) unless otherwise stated.

Metaphosphoric acid, 20% (w/v): dissolve 20 g of metaphosphoric acid in 80 ml of water. Sulfuric acid, 0.05 *M*: dilute 2.8 ml of concentrated sulfuric acid to 1 l with water. Sulfuric acid in methanol, 0.05 *M*: dilute 0.28 ml of concentrated sulfuric acid in 40 ml of water, then dilute to 100 ml with methanol. Sulfuric acid, 10% (v/v): mix 10 ml of concentrated sulfuric acid with 90 ml of water. Sodium hydroxide, 0.5 *M*: dissolve 20 g of sodium hydroxide pellets in 1 l of water. Isoamyl alcohol in *n*-heptane, 1.5% (v/v): dissolve 1.5 ml of isoamyl alcohol in 100 ml of *n*-heptane (HPLC grade). Other solutions were: diethyl ether (anhydrous), methanol (HPLC grade), ethyl acetate, ethanol (absolute), 1-butanol, and ammonium hydroxide (concentrated).

HPLC mobile phase: 1-octanesulfonic acid 0.005 *M* in methanol—water (60:40, v/v). Dissolve 2.16 g of 1-octanesulfonic acid sodium salt (Eastman Organic Chemicals, Eastman Kodak, Rochester, NY, U.S.A.) in 800 ml of water and then filter through a Type HA Millipore filter (Millipore, Bedford, MA, U.S.A.). Dilute the filtered solution to 2 l with methanol previously filtered through a Type LS filter, also from Millipore. Adjust the pH to 3.5 with 0.05 *M* sulfuric acid in methanol.

TLC developing solvent: ethyl acetate—absolute ethanol—1-butanol—concentrated ammonium hydroxide (56:28:4:0.5, v/v).

Standards

Quinidine sulfate (Sigma, St. Louis, MO, U.S.A.) and loxapine succinate (Lederle Laboratory Division, American Cyanamid, Pearl River, NY, U.S.A.) were used as the salts; however, all concentrations are expressed as the free base.

Quinidine stock standard, 100 mg/l: dissolve 115 mg of quinidine sulfate in 1 l of 0.05 *M* sulfuric acid. Quinidine working standards, 2, 4, and 8 mg/l: dilute 2, 4, and 8 ml of quinidine stock standard to 100 ml with water. These aqueous standards were used to reconstitute unassayed Normal Serum Control (Ortho Diagnostic Systems, Raritan, NJ, U.S.A.). Loxapine stock standard, 1 g/l: dissolve 34 mg of loxapine in 25 ml of ethanol. Loxapine (internal

standard), 15 mg/l: dilute 1.5 ml of loxapine stock solution to 100 ml with water.

Operating conditions

For spectrofluorometric analysis, the excitation and the emission wavelengths were 360 and 450 nm, respectively. The ellipsoidal condensing system slit width was 3 mm and all other slit widths were 2 mm. Sensitivity setting was between 90 and 100.

For TLC fluorescence scanning, the excitation and the emission wavelengths were 364 and 440 nm, respectively. The QPM monochromator slit was 0.5 mm. The spectrodensitometer was set in the reflectance mode. The TLC plates were scanned at 10 cm/min and the chart speed was set at 10.2 cm/min.

For HPLC analysis, the fixed and the variable wavelengths were 254 and 330 nm, respectively. The mobile phase was set at a flow-rate of 1.5 ml/min and the chart speed was set at 0.25 cm/min.

Procedures

Direct precipitation method. Metaphosphoric acid is added to diluted serum to precipitate the serum proteins and the fluorescence of the supernatant is read in a spectrofluorometer.

To a 50-ml glass-stoppered centrifuge tube were added 0.5 ml of serum, 19.5 ml of water, and 5.0 ml of 20% metaphosphoric acid. The mixture was shaken on a mechanical shaker (Eberbach Model 6000) for 15 min, then centrifuged for 30 min and the fluorescence of the supernatant was measured with a spectrofluorometer. The fluorescence of the patients' sera, the control sera, and the quinidine serum standards were corrected by the subtraction of the fluorescence of a serum blank which was taken through the procedure.

Extraction method. Quinidine is extracted from alkaline serum into an organic solvent and then back extracted into sulfuric acid. The fluorescence of the acidic extract is measured spectrofluorometrically. A micro-aliquot of the extract is submitted to TLC separation and fluorescence is measured with a TLC fluorescence scanner. A second aliquot of the acidic extract is made basic and then extracted with an organic solvent which is evaporated. The residue is redissolved in acidic methanol and analyzed by HPLC.

To a 50-mL glass-stoppered centrifuge tube were added 1.0 ml of serum, 2.0 ml of 0.5 *M* sodium hydroxide, and 15 ml of 1.5% isoamyl alcohol in *n*-heptane. The mixture was shaken on a mechanical shaker for 5 min and then centrifuged for 5 min. A 10-ml aliquot of the organic layer was transferred to a 15-ml screw-capped centrifuge tube containing 4.0 ml of 0.05 *M* sulfuric acid and the mixture was shaken on a mechanical shaker for 5 min. After 5 min of centrifugation, the organic layer was discarded and the fluorescence of the acidic aqueous phase was measured with a spectrofluorometer (single-extraction method). This acidic aqueous phase was also submitted to TLC and HPLC analyses.

A 3.0-ml aliquot of the acidic aqueous phase was transferred to 1 15-ml screw-capped centrifuge tube to which 2.0 ml of 0.5 *M* sodium hydroxide, 0.2 ml of 15 mg/l loxapine internal standard, and 10 ml of ethyl ether were added. The mixture was shaken on a mechanical shaker (low speed) for 2 min

and then centrifuged for 2 min. An 8-ml aliquot of the ether layer was transferred to a 12-ml screw-capped test tube and evaporated to dryness at 40°C. The residue was redissolved in 50 μl of 0.05 *M* sulfuric acid in methanol and a 25-μl aliquot was submitted to HPLC analysis.

A 20-μl aliquot of the acidic aqueous phase was spotted on a silica gel TLC plate and developed to 10 cm in a mixture of ethyl acetate—ethanol—1-butanol—ammonium hydroxide (56:28:4:0.5, v/v). The plate was then sprayed lightly with 10% sulfuric acid and heated to 70°C for 5 min. TLC—fluorescence scans of the plate were made with the spectrodensitometer set in the fluorescence reflectance mode.

For all three analyses, spectrofluorometry, TLC—fluorescence scanning and HPLC, standard curves were obtained by analyzing serum standards containing known amounts of quinidine and in the case of HPLC, a known amount of loxapine internal standard. For each set of patients' sera that were analyzed, a serum blank, serum controls and three quinidine serum standards (2, 4 and 8 mg/l) were analyzed. The results of the serum standards were used to draw the standard curve as a check on linearity. However, quinidine concentrations of the patients' sera were not obtained from the curve, but rather by using the nearest serum standard which was usually the 2 mg/l serum standard.

For HPLC analysis the ratios of the peak heights of quinidine to that of loxapine (the internal standard) were used to calculate quinidine concentrations. For the TLC fluorescence analysis, the peak heights for quinidine in the patients' sera were compared with those of the serum standards, and in the spectrofluorometric analysis the relative fluorescence intensities were compared after correcting for background interferences.

RESULTS

Spectrofluorometric analysis

Direct precipitation method. When the relative fluorescence intensity of quinidine serum standards, 3—6 mg/l, were plotted against concentration the resulting line had a slope of 1.33, an *y*-intercept of 0.02, a standard error of estimate (S_{yx}) of 0.008 and a correlation coefficient of 1.0. Within-run precision at the 3 mg/l concentration ($n=5$) averaged 1.6% while between-run precision averaged 5% ($n=29$).

Single-extraction method. Duplicate quinidine serum standards were determined by the single-organic extraction—fluorescence method using isoamyl alcohol in *n*-heptane. When the relative fluorescence intensity of quinidine serum standards were plotted against concentration (0.4—10 mg/l), the resulting line had a slope of 3.48, an *y*-intercept of 0.051, a standard error of estimate of 0.15, and a correlation coefficient of 0.999 ($n=15$).

The daily, within-run, and the day-to-day, between-run, variability of the single-organic extraction—fluorescence method was 1% ($n=6$) and 3.5% ($n=18$) at the 3 mg/l concentration. The absolute percent recovery was 59 ± 1% while the relative percent recovery using serum standards was 100 ± 1% ($n=8$).

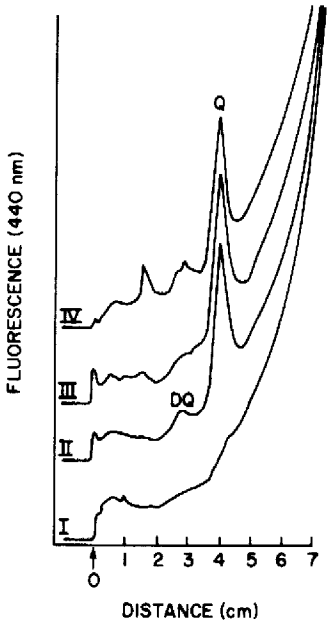

Fig. 1. TLC fluorescence scans of a serum blank (I), a serum standard with 2 mg/l of quinidine (II), a serum control with 2.1 mg/l of quinidine (III) and a patient's serum with 1.5 mg/l of quinidine (IV). Arrow marks origin of TLC plate. Q = quinidine, DQ = dihydroquinidine.

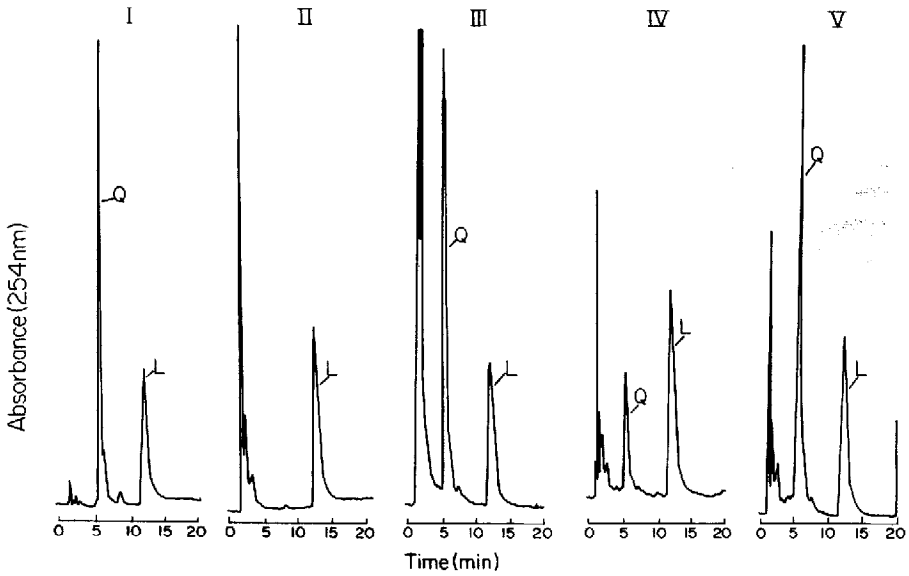

Fig. 2. Liquid chromatograms of a pure 0.1 g/l mixture of quinidine (Q) and loxapine (L) (I), a serum blank (II), a serum standard with 3 mg/l of quinidine (III), a patient's serum with 0.6 mg/l of quinidine (IV) and a patient's serum with 2.7 mg/l of quinidine (V). Q = quinidine, L = loxapine internal standard (0.8 mg/l). Conditions: C-18 reversed-phase column; mobile phase, methanol—water (60:40, v/v) with 5 mmol octanesulfonic acid; flow-rate, 1.5 ml/min; 25 μl injected.

TLC fluorescence scanning

TLC fluorescence scans of a serum blank, a quinidine serum standard, a serum control, and a patient's serum are shown in Fig. 1. R_F values are 0.46 and 0.59 for dihydroquinidine and quinidine, respectively.

When peak heights of quinidine serum standards, subjected to single organic extraction and then thin-layer chromatography followed by fluorescence reflectance scanning, were plotted against concentration (0.4—10 mg/l), the resulting line had a slope of 10.0, an y-intercept of −3.75, a standard error of estimate of 3.25, and a correlation coefficient of 0.995 (n=14).

The daily, within-run, precision of the TLC—fluorescence method (n=5) was 5.2% while the day-to-day, between-run, variability (n=7) was 9.2% at the 3 mg/l concentration. The relative percent recovery using serum standards was 95 ± 14% (n=5).

HPLC analysis

High-performance liquid chromatograms at 254 nm of a pure mixture, a serum blank, a serum standard and two patients' sera are given in Fig. 2. Similar results were observed at 330 nm. Retention times are 0.49 and 0.55 for quinidine and dihydroquinidine, relative to loxapine, 13.0 min.

Peak height ratios of quinidine to that of loxapine (the internal standard) were calculated for quinidine serum standards subjected to the double-organic extraction HPLC method using n-heptane isoamyl alcohol and then ethyl ether. When peak height ratios determined at 254 nm were plotted against the concentration of quinidine serum standards (0.8—10 mg/l), the resulting line had a slope of 0.98, an y-intercept of 0.38, a standard error of estimate of 0.27, and a correlation coefficient of 0.996 (n=6). If peak height ratios measured at 330 nm were used, then the resulting line had a slope of 0.81, an y-intercept of 0.74, a standard error of estimate of 0.38, and a correlation coefficient of 0.987 (n=5).

The daily and day-to-day precision of the double-organic extraction HPLC method as determined by using a 3 mg/l serum control was 3% (n=6), 6% (n=17) and 4% (n=5), 5% (n=15) at 254 and 330 nm, respectively. The relative percent recovery using serum standards was 100 ± 7% (n=6).

Interference studies

Blank serum samples, toxicology serum controls (Hyland Diagnostics, Deerfield, IL, U.S.A.), and eighteen serum samples from patients who were not being treated with quinidine were analyzed in the same manner as the serum standards and sera from quinidine-treated patients. The HPLC analysis of one patient's serum (quinidine untreated) gave a peak where quinidine was usually found when detected at 254 nm. The peak was equivalent to 2.3 mg/l of quinidine. However, when calculations were made from the same peak detected at 330 nm, the peak was equivalent to 0.9 mg/l of quinidine. The use of two wavelengths to check for HPLC interference is therefore very important. The following drugs interfere with quinidine by HPLC: oxazepam, methaqualone, nordiazepam and p-chlorodisopyramide. The following drugs will not interfere by HPLC, TLC spectrofluorometry, and EMIT: procainamide, salicylate, diazepam, sulfanilamide, ethchlorvynol, meprobamate, chlordiazepoxide,

TABLE I

STATISTICAL ANALYSIS OF THE COMPARISON BETWEEN QUINIDINE METHODS

PPT—Fluores = protein precipitation—fluorometric method; SE—Fluores = single-organic extraction—fluorometric method; DE—HPLC = double-organic extraction—HPLC method; SE—TLC Fluores = single-organic extraction—TLC fluorescence scanning method; EMIT = enzyme multiplied immunoassay technique.

Method (x) vs. method (y)	n	Regression equation ($y = bx+a$)	Standard error of estimate (S_{yx})	Correlation coefficient (r)
PPT—Fluores vs. SE—Fluores	92	$y = 0.58x - 0.31$	0.65	0.87
PPT—Fluores vs. DE—HPLC (254 nm)	62	$y = 0.52x - 0.40$	0.65	0.83
PPT—Fluores vs. SE—TLC Fluores	36	$y = 0.389x - 0.088$	0.595	0.74
SE—Fluores vs. DE—HPLC (254 nm)	59	$y = 0.998x - 0.175$	0.30	0.96
SE—Fluores vs. SE—TLC Fluores	36	$y = 0.887x - 0.034$	0.396	0.90
DE—HPLC vs. SE—TLC Fluores	36	$y = 0.837x + 0.152$	0.294	0.94
EMIT vs. DE—HPLC (254 nm)	17	$y = 0.90x - 0.27$	0.49	0.95
SE—Fluores vs. EMIT	17	$y = 1.15x - 0.072$	0.28	0.98

glutethimide, hydantoin, propoxyphene, amobarbital, phenobarbital, secobarbital, pentobarbital, flurazepam, amitriptyline and doxepin. The best solution to interference problems is to use another technique for analysis at the end of the sample preparation step as previously described [75].

Comparison of methods

Sera of patients receiving quinidine were analyzed by the four methods and the levels of quinidine were compared. Least-squares analysis was used to calculate the slope, y-intercept, standard error of estimate, S_{yx}, and correlation coefficient for each pair of methods. A summary of the statistical analysis is given in Table I. Included in Table I are also the results of patients' analysis using the enzymatic immunoassay technique (EMIT). Excellent agreement was observed between the fluorescence extraction method and HPLC, TLC, and EMIT (correlation coefficients, r, of 0.96, 0.90 and 0.98). Comparison of patients' sera between HPLC and TLC, and EMIT and HPLC, gave also a high correlation (r = 0.94 and 0.95).

DISCUSSION

In this study a comparison was made of four different analytical methods for the determination of quinidine in serum. The methods varied in the preparation

of the sample for analysis and in the analytical instrumentation for the detection and quantitation of quinidine. Three of the methods, HPLC, TLC and extraction fluorescence required two or more liquid—liquid extractions whereas the fourth method required the serum sample to be deproteinated with metaphosphoric acid and the resulting supernatant to be analyzed by spectrofluorometry. Traditionally, this method is called the direct fluorometric method of Brodie and Udenfriend [8], but in this report an abbreviated identification (PPT—Fluores method) is used. Quinidine levels determined by this method are known as Q_P values.

Two of the quinidine methods were identical in sample preparation, but the instrumentation which was used for quantitation was different. Quinidine was extracted from alkalinized serum with an organic solvent and then re-extracted into acid. If the acidic solution was analyzed by spectrofluorometry, then the procedure is called the single-organic extraction—fluorometric (SE—Fluores) method and the quinidine concentrations obtained are known as Q_E values. In the literature, this method is often called the double-extraction—fluorometric method of Cramer and Isaksson [9]. The original method used benzene as the extraction organic solvent. Because benzene is a substance posing a potential occupational carcinogenic risk and is currently regulated by the Occupational Safety and Health Administration (OSHA), the SE—Fluores method in this study employed isoamyl alcohol in *n*-heptane as an alternative to the carcinogen benzene. Isoamyl in *n*-heptane has not been previously used as an extraction solvent for quinidine. In order to ascertain its analytical accuracy as an extraction solvent, we used two chromatographic techniques, TLC and HPLC after the extraction procedure to measure quinidine concentrations in serum.

If the above acidic solution was subjected to thin-layer chromatography and the quinidine on the TLC plate was measured by a spectrodensitometer in the reflectance fluorescence mode, then the procedure is identified as the single-extraction—TLC fluorescence scanning (SE—TLC Fluores) method. Quinidine levels obtained by this method are referred to as Q_{TLC} values.

The fourth method in this study was an extension of the single-organic extraction method previously described. An aliquot of the resulting acidic solution from those procedures was made basic and extracted with diethyl ether. The extract was concentrated and analyzed by HPLC with ultraviolet absorbance detection. In this report, this procedure is known as the double-organic extraction—HPLC (DE—HPLC) method and its quinidine concentrations are given as Q_{HPLC} values. Lastly, we compared our method to the EMIT system since it is a commonly used technique for quinidine analysis.

Precision was estimated for three of the methods based on the statistical analysis of the concentration of quinidine in a serum control. The mean, standard deviation, and coefficient of variation (C.V.) were calculated for each of three daily (within-run) and day-to-day (between-run) determinations on five and seventeen identical serum controls, respectively. For the SE—Fluores method, the daily C.V. was in a range of 0.7—1.7% whereas the day-to-day C.V. was 2.2%. The DE—HPLC method gave a daily C.V. range of 3.0—6.0% and a day-to-day C.V. of 6.0%. For the SE—TLC Fluor method the daily C.V. was in a range of 5.2—8.9% and the day-to-day C.V. was 9.2%. These results

indicated that the SE—Fluores method gives the least variation of all the analytical methods studied.

Quinidine concentrations in the sera of patients receiving quinidine therapy were determined simultaneously by the four methods described in this report and EMIT. The results obtained were evaluated by the least-squares linear regression method (Table I). The statistical analysis included the calculation of a slope (b), an y-intercept (a), a standard error of estimate (S_{yx}) and a correlation coefficient (r). The PPT—Fluores method when compared with the three extraction methods gave correlation coefficients between 0.74 and 0.87. Good agreement, reflected by a correlation coefficient of 0.96 and a slope of 0.99, was noted between the SE—Fluores and DE—HPLC methods. A correlation coefficient of 0.94 and standard error of estimate of 0.294 were associated with the comparison between the DE—HPLC and SE—TLC Fluores methods. Consistently high correlations were observed between EMIT and our extraction procedure: 0.95 and 0.98 (Table I).

The above results confirm that our extraction fluorescence procedure with isoamyl alcohol *n*-heptane gives comparable results to HPLC, EMIT and TLC. As expected, a poorer correlation was observed between the extraction procedure and the direct precipitation method since the later measures quinidine and its metabolites. What is interesting, however, is the fact that a significant correlation of 0.87 was observed between the non-selective precipitation method and the extraction procedure, which suggests that in patients with normal renal function the concentration of quinidine metabolites present in serum are constant. The high correlation between the extraction fluorescence method and HPLC, 0.96 or TLC, 0.90, confirms that the extraction procedure extracts primarily quinidine and dihydroquinidine and none of the other metabolites. The R_F values for the isolated quinidine and dihydroquinidine by TLC fluorescence scanning were 0.59 and 0.46, respectively. The retention times for quinidine and dihydroquinidine by HPLC were 6.4 and 7.2, respectively. Using *n*-heptane and isoamyl alcohol as an extraction solvent therefore eliminates interference from metabolites and serves as a selective solvent for the analysis of quinidine in serum. The use of HPLC and TLC after the extraction procedure does not add any specificity to the procedure except for the separation of quinidine from dihydroquinidine.

Although fluorometry, TLC, and HPLC have been used to measure quinidine, this study has incorporated several improvements. The double-organic extraction methods will leave behind 3-hydroxyquinidine and other metabolites, but not dihydroquinidine. The use of isoamyl alcohol in *n*-heptane as an extracting solvent is not as biohazardous as benzene. The use of loxapine as an internal standard and 1-octanesulfonic acid in a methanol—water mixture as a mobile phase in the HPLC analysis, and the use of the TLC conditions with reflectance fluorescence scanning to quantitate quinidine have not been previously reported.

In the past, quantitative TLC analysis for quinidine has been limited because of a lack of appropriate instrumentation, the use of involved sample preparation steps, and difficulties in the standardization of the results. Some of the early problems were caused by the coating materials and the plates. Poor reproducibility occurred due to non-uniform plate thickness and the varying

consistency of the coating materials. After TLC separation, the absorbent had to be scraped from the plate, the compounds of interest had to be eluted from the absorbent, and, finally, the eluant was subjected to spectrophotometry for quantitation. This technique was time-consuming and required a large sample size. With the advent of scanning densitometers, the ultraviolet absorbance or density was measured directly from the plate without removal of the chromatographed compounds from the plate.

In this study, a TLC analysis for quinidine was formulated that surmounted these obstacles. The chromatographic aspect of the analysis was improved by the use of precoated silica gel plates with a preadsorbent area and nineteen channels premarked on the plate. Development time for 10 cm was 30 min. The preadsorbent allows the developing solvent to extract and concentrate the applied sample and thus presents the sample to the silica gel adsorbent layer as a uniform, concentrated band. Improved quantitation was possible because the scanning densitometer consists of photomultipliers in the reference channel as well as the sample channel that intercept emitted light from the surface of the TLC plate being scanned. This interception takes place at 45° to the normal angle of incidence of the exciting radiation. This type of instrument configuration, TLC reflectance spectrofluorescence scanning, will minimize any differences on the TLC plate and any variation that occurs with the lamp power supply or the characteristics of the lamp itself. The above instrumentation and TLC plates resulted in an inexpensive, sensitive, selective and precise procedure for the determination of quinidine in serum.

The choice of which method to use for the determination of quinidine in serum should be determined on the basis of the particular analytical need; sample size, turn around time, equipment required, costs, personnel requirements, and likely interferents. For example, the spectrofluorometric precipitation method is simple, fast and does not require sophisticated instrumentation, but is non-specific. Even with a single or double organic extraction, quinidine and dihydroquinidine are not separated. On the other hand, the HPLC analysis which is more selective, is time-consuming, expensive, requires sophisticated instrumentation, but does separate quinidine from dihydroquinidine and other interferents. The double extraction affords a cleaner sample to analyze than the protein precipitation method. The TLC method has advantages over both previously described methods. Quantitative TLC is not unreasonably long or involved; the spectrodensitometer is moderately priced and simple to operate. Quinidine is separated from the dihydroquinidine and other interferents. The selection of both excitation and emission wavelengths gives improved selectivity whereas fluorescence gives an increased sensitivity. In TLC each sample is separated on new absorbent, but in HPLC the column is used many times with the expectation that all of the excess endogenous materials can be removed. The ability to remove the developing solvent before scanning the TLC plate eliminates the incompatibility that may be found between the mobile phase and the ultraviolet detector of an HPLC system. TLC analysis time can be reduced by scanning only the area of interest on the TLC plate. TLC scanning is not without some disadvantages such as the small range of linear correlation between sample concentration and detection signal. Also, 10 cm may not be enough distance to separate interfering substances from the compound of interest.

Interference studies showed that various basic drugs will not interfere with the present procedures (TLC, EMIT and spectrofluorometry), except for oxazepam, methaqualone, nordiazepam and *p*-chlorodisopyramide which will interfere with quinidine by HPLC. A scheme to the analysis of quinidine in serum from patients on quinidine therapy may initially include the determination of the drug by a protein precipitation fluorometric method. If the result is above the therapeutic range of 4—8 mg/l, then one may wish to consider an alternative method such as the single-organic extraction method followed by spectrofluorometry, although the TLC procedure is warranted. If the new result is still high, then the double organic extraction and HPLC analysis may be in order before making a decision to reduce the therapeutic dose in a patient. In our experience the direct precipitation method and the organic extraction procedure are simple to perform and give more useful information on parent drug and metabolite concentration than either procedure by itself. One would rarely have to go beyond these two procedures for monitoring of quinidine therapy.

ACKNOWLEDGEMENT

We wish to thank Clara Bonaquest for typing the manuscript.

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