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High-performance liquid chromatographic method for the quantitation of quinidine and selected quinidine metabolites

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ABSTRACT

A specific and sensitive assay for the separation and quantitation of quinidine, 3-hydroxyquinidine, quinidine-N-oxide, O-desmethylquinidine and dihydroquinidine is presented. The assay is shown to be sensitive to concentrations of 0.1 μg/ml for all the above compounds when using a serum sample of 0.1 ml. The standard curve demonstrates linearity at concentrations from 0.1 to 5 μg/ml. The extraction procedure consists of adjusting the serum to an alkaline pH and extracting once with a mixture of methanol–dichloromethane (15:85). The organic extract is dried and the residue is solubilized in mobile phase. The chromatographic conditions are an isocratic delivery of the mobile phase 0.01 *M* K_2HPO_4–acetonitrile (96:4) through a C_{18} column at ambient temperature. Detection of the compounds of interest is by ultraviolet absorption at a wavelength of 210 nm. For each compound the inter-assay variation is less than 10% and the intra-assay variation is less than 15%. No interfering compounds were detected when a commercially prepared serum spiked with 28 commonly used therapeutic compounds was assayed by this method. The analytical method presented here for the isolation and quantitation of quinidine, several active metabolites, and dihydroquinidine has adequate sensitivity and specificity for monitoring the concentration of quinidine and quinidine metabolites in patient samples.

INTRODUCTION

Quinidine, a type 1-A antiarrhythmic drug derived from the bark of the chinchona tree, is the oldest antiarrhythmic drug used for ventricular and supraventricular arrhythmias. The majority of quinidine is metabolized by the liver, however, 13–27% of quinidine is excreted unchanged by the kidneys [1,2]. Many metabolites of quinidine have been identified including 3-hydroxyquinidine, 2′-oxoquinidinone, quinidine-N-oxide, quinidine 10,11-dihydrodiol and O-desmethylquinidine [3–6]. Several metabolites, including 3-hydroxyquinidine, quini-

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dine-N-oxide, and O-desmethylquinidine, have exhibited antiarrhythmic activity when studied alone or in combination with quinidine [7–10]. Drugs that effect hepatic enzymes can change the elimination half-life of quinidine [11–13]. The clearance of quinidine and quinidine metabolites correlates with creatinine clearance [14], is pH-dependent, and occurs by glomerular filtration [15]. Quinidine and metabolites are not significantly removed from the circulation by hemodialysis or peritoneal dialysis. The pharmacokinetics of quinidine and quinidine metabolites can vary greatly between individuals depending on many factors including age, disease state, and concomitant drug therapy [16–20]. These factors may result in quinidine concentrations that fall below or rise above the therapeutic range causing decreased efficacy or an increase in adverse reactions, respectively. This has resulted in the routine clinical monitoring of quinidine concentrations in order to maximize therapeutic response while minimizing toxic effects [15,21].

In clinical situations quinidine concentrations are often determined by enzyme multiplied immunoassay technique (EMIT/ACA) [22] fluorescence polarization immunoassay (FPIA/TDx) [23], or enzyme immunoassay (EIA) [24]. Currently high-performance liquid chromatography (HPLC) is the only assay method that can separate and quantitate the metabolites of quinidine, whereas the assays listed previously are not specific for quinidine alone, but also interact with quinidine metabolites. Studies that quantitated quinidine and quinidine metabolites by HPLC and compared concentrations to those determined by other assay methods found that methods similar to commonly used clinical assays overestimate the levels of quinidine [22,24–26].

Many HPLC assays for quinidine and some quinidine metabolites have been previously published. When analyzing a large number of samples the length of time necessary for the completion of the chromatographic run may be a limiting factor. Several of the previously published HPLC assays that quantitate quinidine and quinidine metabolites take longer than 15 min to complete [27–29]. Other assays utilize fluorescence detection [26,30], which lack sensitivity for the O-desmethylquinidine and 2′-oxoquinidinone metabolites. One assay methodology [31] requires a gradient pump and another assay [32] has not been shown to be effective in discriminating quinidine from its metabolites nor has it been used to quantitate quinidine in human serum.

It would be desirable to be able to quantitate quinidine and its metabolites in order to better meet the specific requirements of the patient. This paper presents an HPLC assay for quinidine , dihydroquinidine, the major contaminant of quinidine, and three active metabolites of quinidine. This method avoids several of the problems associated with other HPLC assays, can quantitate low concentrations, has a short chromatographic run time, and uses very little organic solvent in the mobile phase.

EXPERIMENTAL

Chemicals

Quinidine free base and quinine free base were purchased from Sigma (St. Louis, MO, USA). Dihydroquinidine hydrochloride and nonylamine were purchased from Aldrich (Milwaukee, WI, USA). 3-Hydroxyquinidine was donated by A. H. Robbins, Research Laboratories (Richmond, VA, USA). O-Desmethylquinidine was generously supplied by AFC Chemie (Maarssen, Netherlands). Quinidine-N-oxide was provided by Laboratoire Nativelle (Paris, France). All organic solvents were HPLC grade including acetonitrile, dichloromethane, and methanol (Baker, Phillipsburg, NJ, USA). Water used in this assay was glass-distilled, treated by ion exchange, charcoal-filtered, and subsequently filtered through a 0.45-μm pore Nylon filter.

Potassium phosphate (EM Scientific, Gibbstown, NJ, USA) and nonylamine (Aldrich) used to make the mobile phase were reagent-grade chemicals.

Instruments and chromatographic conditions

The liquid chromatographic system consisted of a Rheodyne Model 7010 (Rheodyne, Reno, NV. USA) manual injection valve and a Hewlett Packard Model 1090 liquid chromatograph (Hewlett Packard, Oxnard, CA, USA) with a diode-array detector. A Hewlett Packard Model 85B personal computer controlled the chromatograph and detector, in addition to recording and analyzing spectrographic data on quinine, quinidine and metabolites of quinidine. Printed chromatograms and integration of peak height or area were obtained from a Hewlett Packard 3392A recording integrator. An Altex (Beckman, San Ramon, CA, USA) 25 cm × 4 mm I.D. reversed-phase column packed with 5 μm octadecylsilane (ODS–C_{18}) stationary phase was used for the chromatographic separation. The mobile phase consisted of 0.01 *M* dibasic potassium phosphate K_2HPO_4) adjusted to pH 2.4 (using phosphoric acid), with 0.375 ml of nonylamine added per liter, and acetonitrile (96:4) delivered at a constant flow rate of 2 ml/min. The column and mobile phase were maintained at ambient temperature. Prior to analysis of samples or standards, a conditioning injection of 5 μg each of quinidine, quinine, and the quinidine metabolites was made to assure complete elution of the compounds of interest. The diode-array detector was set to detect absorbance at 235 nm with a 4-nm window.

Standards

Quinidine, 3-hydroxyquinidine, O-desmethylquinidine, quinidine-N-oxide, dihydroquinidine, and quinine powders were individually weighed and solubilized in water to yield a concentration of 100 μg/ml. Structures of the compounds are shown in Fig. 1. Quinine, the internal standard, was diluted in water to a final concentration of 5 μg/ml. Aliquots of each solution, except for quinine, were combined and diluted with pooled human serum to produce final concentrations

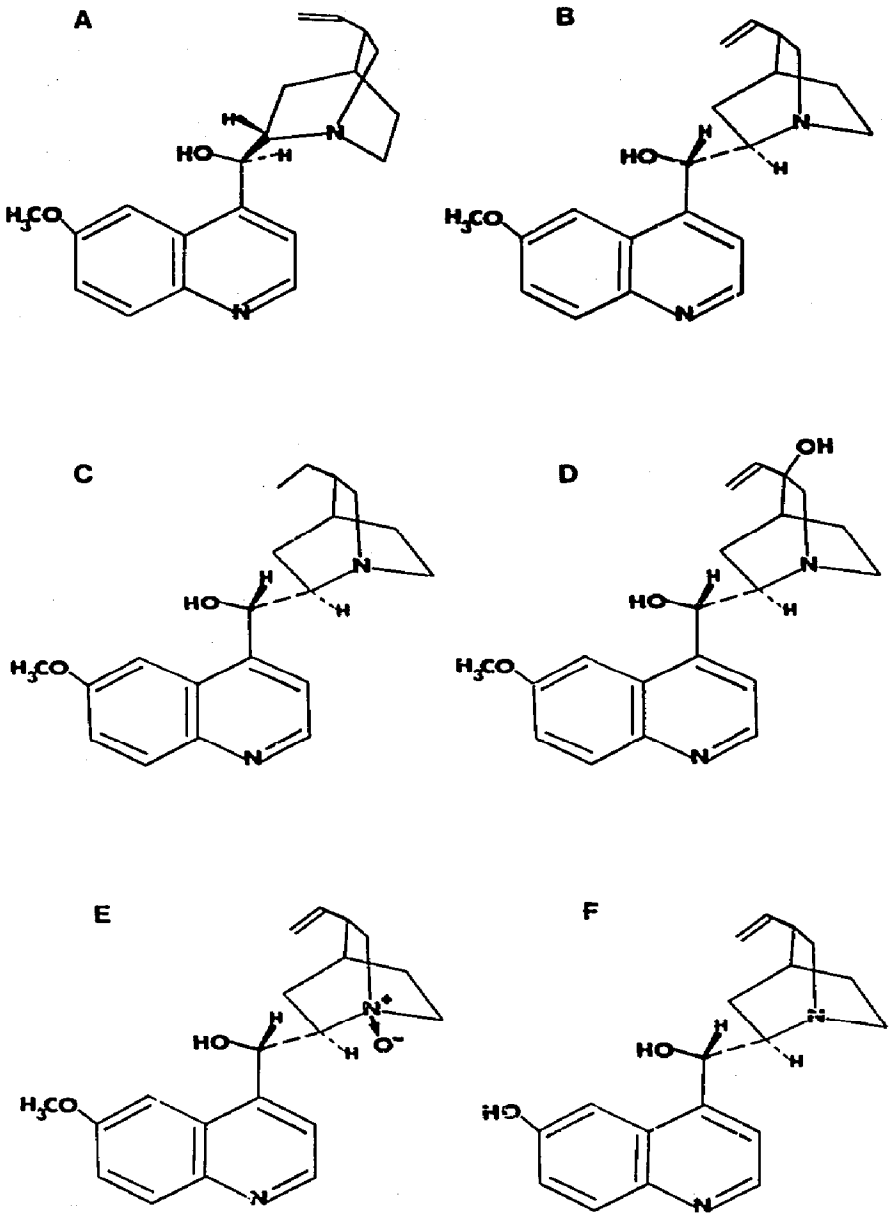

Fig. 1. Structures of compounds used in this assay: (A) quinine; (B) quinidine; (C) dihydroquinidine; (D) 3-hydroxyquinidine; (E) quinidine-N-oxide; (F) O-desmethylquinidine.

of 5.0, 3.0, 1.0, 0.5, and 0.1 μg/ml of each compound. The various standard concentrations were divided into 1.25-ml portions and frozen for future use in the assay. Spiked serum controls, pooled human serum with a known amount of quinidine, dihydroquinidine, and quinidine metabolites (0.7 and 3.0 μg/ml), were used as a control to determine the inter- and intra-assay variation of the method. For each-day analyses one portion of each standard concentration and spiked

serum control was thawed and duplicate 100-μl aliquots were pipetted into labeled screw-cap tubes. In addition, duplicate 100-μl aliquots of pooled human serum, with no quinidine, dihydroquinidine, or quinidine metabolites added, were pipetted into labeled test tubes to serve as blank (zero) controls in order to determine if there were any inferences from normal serum components.

Serum extraction

Duplicate samples of spiked serum, blank pooled serum, standards, and patient serum were extracted by liquid–liquid extraction. To each tube, and 100-μl aliquot of serum, were added 100 μl of 0.5 *M* NaOH to adjust the pH of the serum and 100 μl of quinine solution (internal standard). If a smaller amount of serum is used, the amount of 0.5 *M* NaOH utilized should be adjusted to equal the amount of serum. The tubes were briefly vortex-mixed and 5 ml of the extraction mixture (dichloromethane–methanol, 85:15) were added to each tube; they were capped and shaken for 10 min. The tubes were centrifuged at 1000 *g* for 10 min to separate the organic (lower) phase from the aqueous (upper) phase. The aqueous phase was removed and discarded. A 4-ml volume of the organic phase was transferred to a conical centrifuge tube and dried under a nitrogen gas stream in a warm water-bath (40°C). The dried extract was reconstituted in 200 μl of mobile phase, and 100 μl were injected onto the column.

Assay validation

Several controls were used to validate the reproducibility of the assay. Intra-assay variation was determined by analyzing seven aliquots of spiked serum, containing either 0.75 or 2.5 μg/ml of each compound on one day and determining the coefficient of variation. Inter-assay variation was determined by comparing the results of the analysis of aliquots of spiked serum containing either 0.7 or 3.0 μg/ml on seven different days and computing the coefficient of variation. The efficiency of the extraction method was determined by comparing the peak height of a known concentration of each compound, injected directly onto the column, with the peak height of an extract of an identical concentration of the same compound in serum. After corrections for volume differences the percentage of each compound extracted (recovered) was determined.

In order to establish the accuracy and precision of the proposed assay we assayed pooled serum spiked with predetermined concentrations of quinidine, dihydroquinidine, and quinidine metabolites. These samples were extracted, analyzed, and the concentrations where established by determining the assayed values from the standard curve for the respective day of analysis. Potential interference from several antiarrhythmics and other commonly prescribed drugs was tested by extracting and analyzing therapeutic drug monitoring (TDC) serum (Fischer Scientific, Pittsburgh, PA, USA), a commercially prepared spiked serum.

RESULTS

Fig. 2 presents a comparison of chromatograms produced during the development of this assay. The peaks identified as 3-hydroxyquinidine, O-desmethylquinidine, quinidine-N-oxide, quinidine, quinine, and dihydroquinidine elute at 1.8, 2.0, 2.8, 3.1, 3.8, and 4.4 min, respectively. Although no standard for dihydroquinine was utilized the peak at approximately 6.0 min may represent this compound due to its association with quinine. The figure shows the lack of interfering substances in normal pooled human serum (A), the separation of 1 μg/ml each of quinidine, dihydroquinidine, 3-hydroxyquinidine, quinidine-N-oxide, O-desmethylquinidine, and quinine (B), and a chromatogram of an extract of patient serum (C). During the analysis of 100 patient samples no apparent interferences from other drugs or natural constituents of serum have been observed. The chromatograms show adequate separation between 3-hydroxyquinidine and O-desmethylquinidine. The least separation is between quinidine-N-oxide and quinidine; however, there is sufficient separation to detect and quantitate quinidine-N-oxide at concentrations to 0.1 μg/ml. The intra-assay and inter-assay mean values, standard deviations, and coefficients of variation (C.V.) at concentrations of 0.75 and 2.5 μg/ml are shown in Table I and Table II, respectively. Intra-assay coefficients of variation ranged from 3.4 to 9.8%. Inter-assay coefficients of variations range from 2.1 to 10.4%.

This assay has been repeatedly shown to be linear for all the compounds concerned. A standard curve of duplicate concentrations in the range 0.1–5.0 μg/ml was used during each assay session. Standard curves were obtained for each compound by plotting the compound/internal standard peak-height ratio *versus* the concentration and were linear throughout the range studied. The assay was able to detect all compounds down to a level of at least 0.1 μg/ml.This

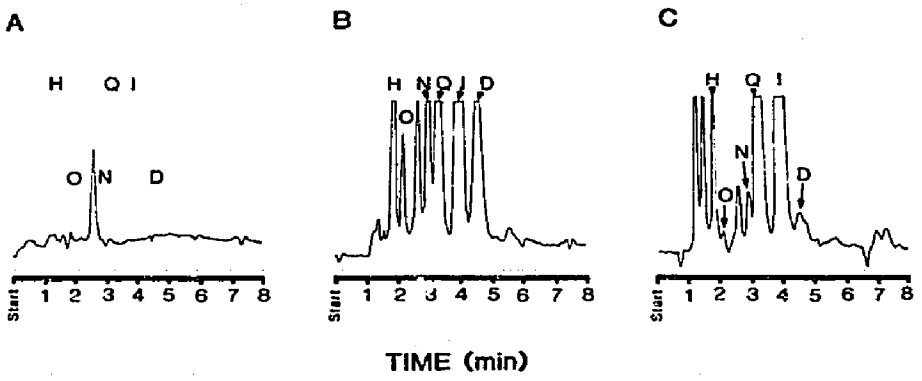

Fig. 2. Chromatograms of (A) blank pooled serum, (B) blank pooled serum spiked with 1 μg/ml each of 3-hydroxyquinidine (H), O-desmethylquinidine (O), quinidine-N-oxide (N), quinidine (Q), dihydroquinidine (D), and 200 ng internal standard (I), and (C) patient serum spiked with 200 ng internal standard (I) with peaks identified as 3-hydroxyquinidine (H), O-desmethylquinidine (O), quinidine-N-oxide (N), quinidine (Q), and dihydroquinidine (D).

TABLE I

INTRA-ASSAY ACCURACY AND COEFFICIENTS OF VARIATION (n = 7)

Compound	2.5 μg/ml		0.75 μg/ml	
	$\bar{x}^a$ ± S.D. (μg/ml)	C.V. (%)	$\bar{x}^a$ ± S.D. (μg/ml)	C.V. (%)
Quinidine	2.74 ± 0.140	5.1	0.80 ± 0.0272	3.4
3-Hydroxyquinidine	2.66 ± 0.223	8.4	0.75 ± 0.0645	8.6
O-Desmethylquinidine	2.70 ± 0.265	9.8	0.76 ± 0.0616	8.1
Quinidine-N-oxide	2.55 ± 0.194	7.6	0.75 ± 0.0623	8.3
Dihydroquinidine	2.72 ± 0.095	3.5	0.78 ± 0.0335	4.3

[a] $\bar{x}$ = the average calculated concentration.

quantitation limit, determined by the range of the standard curve, was accomplished utilizing 100 μl of serum as the sample aliquot and without having the detector attenuation adjusted to a high sensitivity. Direct injection of each compound at the most sensitive attenuation has shown that the limit of detection, as determined as a peak height three times the baseline noise, was: quinidine, 1 ng; O-desmethylquinidine, 5 ng; quinidine-N-oxide, 1 ng; 3-hydroxyquinidine, 2 ng; and dihydroquinidine, 5 ng. The range of the standard curve, 0.1–5.0 μg/ml, was chosen to be adequate for the quantitation of most patient samples and low enough to quantitate the quinidine metabolites in pateints receiving a normal regimen of quinidine. During the analysis of 150 patient samples none had quinidine levels greater than 5 μg/ml.

The extraction efficiency was 71.6% for 3-hydroxyquinidine, 24.8% for O-

TABLE II

INTER-ASSAY ACCURACY AND COEFFICIENTS OF VARIATION (n = 7)

Compound	2.5 μg/ml		0.75 μg/ml	
	$\bar{x}^a$ ± S.D. (μg/ml)	C.V. (%)	$\bar{x}^a$ ± S.D. (μg/ml)	C.V. (%)
Quinidine	2.64 ± 0.055	2.1	0.78 ± 0.057	7.5
3-Hydroxyquinidine	2.68 ± 0.165	6.2	0.76 ± 0.073	9.6
O-Desmethylquinidine	2.45 ± 0.214	8.7	0.74 ± 0.077	10.4
Quinidine-N-oxide	2.56 ± 0.187	7.3	0.76 ± 0.058	7.6
Dihydroquinidine	2.65 ± 0.081	3.1	0.75 ± 0.053	7.1

[a] $\bar{x}$ = the average calculated concentration.

TABLE III

DRUGS TESTED FOR INTERFERENCE WITH QUINIDINE, 3-HYDROXYQUINIDINE, O-DESMETHYLQUINIDINE, QUINIDINE-N-OXIDE, DIHYDROQUINIDINE, AND QUININE

Antiarrhythmics	Other drugs	
Amiodarone	Acetaminophen	Methotrexate
Desethylamiodarone	Amikacin	Netilmicin
Digoxin	Amitriptyline	Nortriptyline
5-Hydroxypropafenone	Carbamazepine	Phenobarbital
Lidocaine	Chloramphenicol	Primidone
Mexiletine	Desipramine	Salicylate
N-Acetylprocainamide	Ethosuximide	Theophyline
Phenytoin	Gentamicin	Tobramycin
Pirmenol	Lithium	Vancomycin
Procainamide		
Propafenone		
Sotalol		

desmethylquinidine, 84.8% for quinidine-N-oxide, 55.2% for quinidine, and 59.2% for dihydroquinidine. The addition of 15% methanol to the the extraction liquid was necessary to decrease extraction of interfering compounds found in pooled human serum. The methanol resulted in lower extraction efficiencies for most of the compounds of interest, however, the sensitivity displayed in this assay was sufficient to compensate for the decreased extraction efficiency. A scanning fluorescence detector was used in order to determine if fluorescence could be used for the determination of quinidine and quinidine metabolite concentrations. It was found that O-desmethylquinidine fluoresced poorly. Therefore, detection using UV absorbance was more effective even though this resulted in overall decreased sensitivity. The extraction and injection of the FDC serum demonstrates that the drugs contained in the mixture do not interfere with the detection or quantitation of quinidine, dihydroquinidine, or quinidine metabolites (Table III). Furthermore, after the analysis of 150 patient samples no direct interferences from other drugs were detected.

DISCUSSION

This paper describes a method for the isolation, detection, and quantitation of quinidine, dihydroquinidine, 3-hydroquinidine, O-desmethylquinidine, and quinidine-N-oxide. This method has demonstrated good sensitivity, selectivity, and linearity over the range of the assay. The assay time is relatively short, less than 7 min if dihydroquinidine is included and less than 6 min if dihydroquinidine is not quantitated. A fluorescence detector is not required because detection of quinidine, quinidine metabolites, and dihydroquinidine at levels found in patients is

adequate by UV absorbance. The separation of quinidine and its metabolites is based on isocratic delivery of mobile phase and therefore requires only one pump for solvent delivery. The normal sample size is 100 μl of serum; however, detection of lower levels of quinidine metabolites may be enhanced by increasing the amount of serum used. The lower limit of detection, as determined on our instrumentation and our column, was found to range from 1 ng for quinidine and quinidine-N-oxide to 5 ng for dihydroquinidine and O-desmethylquinidine when injected directly on column. There are several other metabolites of quinidine, including quinidine 10,11-dihydrodiol and 2-oxoquinidinone, that were not included in this assay due to the lack of availability of standards.

The extraction efficiency was quite variable for the various compounds of interest, ranging from 25% for O-desmethylquinidine to 85% for quinidine-N-oxide. The variability of the extraction efficiency and volume losses results in discrepancies between the level of a compound that can be detected upon direct injection *versus* the level than can be detected upon extraction from serum. Although the recovery of any one compound was not maximized for this assay, the sensitivity allows the quantitation of all compounds to 0.1 μg/ml. No interference from the drugs listed in Table II were noted, in addition, no interfering compounds were noted in the patient samples analyzed. Occasionally a trailing peak would cause a rise in the baseline of subsequent samples. These trailing peaks were not necessarily associated with a particular patient or batch of pooled human serum. It is likely that the trailing peaks may be be attributed to the transference of a small amount of aqueous phase with the organic phase after the extraction. Another possible source of these "ghost" peaks could be the column itself. For this assay we used a column that was also used for six other assays and potentially some of the rising baselines noted during the quinidine assay may be due to contaminants that were left on the column from previous assays. Normally after each day's analysis the column is subjected to a wash using a gradient of water–acetonitrile (0 to 96% acetonitrile in 35 min, then 96 to 50% in 10 min) and stored in water–acetonitrile (1:1). Due to the extreme use of the column (> 700 h) a conditioning injection containing 5 μg/ml of each compound was made prior to an analytical run in order to ensure that any active sites on the column that may bind with quinidine or quinidine metabolites would be occupied prior to the start of analysis. Because of the other demands on the column used in this assay, no determination of the effect of this assay on the performance or column life has been made.

Several metabolites of quinidine have been identified [3–6], including 3-hydroxyquinidine, O-desmethylquinidine, and quinidine-N-oxide. These three metabolites have been shown to be active in various preparations [7–10]. The metabolism of quinidine, and therefore the formation of metabolites, is extremely variable in patients and healthy individuals [15,21]. Several studies have investigated quinidine metabolites and their relative abundance. The 3-hydroxyquinidine metabolite of quinidine has been determined to be the major metabolite in

most individuals [3,8]. The quinidine 10,11-dihydrodiol metabolite has also been listed as a major metabolite [3], however, other researchers have found that quinidine-N-oxide and 2-oxoquinidione comprise the other major quinidine metabolites of quinidine [8]. During the analysis of 150 patient samples we have encountered individuals in which the dominant metabolite was the 3-hydroxyquinidine, quinidine-N-oxide, or O-desmethylquinidine. Disease states, concomitant drug therapy, and other factors may effect quinidine metabolism, protein binding, and elimination [16–20]. These factors may have relevance in the efficacy or toxicity of quinidine in certain individuals. The commonly utilized antibody-based assay methods suffer from interferences from quinidine metabolites that result in an overestimation of quinidine levels without yielding information on the quinidine metabolites themselves. The assay discussed in this paper allows the separation and quantitation of quinidine and quinidine metabolites. In certain cases it may be of interest to profile a patients metabolite production for which this HPLC assay may be of use.

We have shown that the assay discussed in this manuscript is linear over the range 0.1–5.0 μg/ml for all the compounds discussed when determined from a serum sample of 0.1 ml. The assay has an inter-assay coefficient of variation of less than 11% and intra-assay coefficient of variation of less than 10% for quinidine and the quinidine metabolites. No interfering compounds were noted during the analysis of 100 patient samples or during the analysis of TDC serum. The method presented in this manuscript requires a single extraction step, has an isocratic solvent delivery, utilizes a mobile phase containing very little organic solvent, and utilizes UV absorption to detect the compounds. These aspects of this assay make it suitable for most laboratories that have HPLC capability. This assay should provide more accurate information for patient care on quinidine and quinidine metabolite concentrations than the assays currently used clinically that do not differentiate quinidine from its metabolites.

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