We report a thin-layer-chromatographic micro-analysis for quinidine in serum, with detection by fluorescence densitometry. Quinidine is extracted from 20 μL of serum at pH 13 into 3 mL of hexane/aceton solution (80/20 by vol) containing N-(1-naphthyl)ethylenediamine as internal standard. The extract is concentrated and applied to silica-gel-imregnated plates for conventional thin-layer chromatography. Quinidine is identified from its Rf value and quantified from the peak-height ratio between quinidine and the internal standard, relative to that of simultaneously extracted serum standards. The proposed assay is sensitive (to 0.2 mg/L), specific for unmetabolized quinidine, precise (between-run coefficients of variation <6%), and readily adaptable to large-scale "batch" analysis. Peak-height ratio is linearly related to concentration to at least 20 mg/L. Quinidine concentrations in the serum of patients, as measured by the proposed method (x) and by a traditional double-extraction spectrofluorometric assay (y), were related as follows: y = 0.994x + 0.276 (r = 0.989, n = 20).

Additional Keyphrases: fluorometry · drug assay · N-(1-naphthyl)ethylenediamine as internal standard

Quinidine has been widely used since 1918 as an effective antiarrhythmic agent, and therapeutic monitoring of the drug has been of established value for more than 20 years. It has been measured by spectrofluorometry (1-4), thin-layer chromatography (5-8), gas-liquid chromatography (9), liquid chromatography (10-13), and nonisotopic immunoassay (14, 15).

Some fluorometric methods (3, 4) require at least 0.5 mL of sample and thus preclude micro-analysis. Others (1, 2) suffer from interference by inactive metabolites of quinidine, which are particularly prominent in patients with congestive heart failure and (or) poor renal function (16). Even the popular double-extraction fluorimetric analysis of Cramer and Isaksson (3) reportedly gives results approximately 20% greater than do some liquid-chromatographic methods (14). Gas-liquid and liquid chromatographic methods (9-13), although very specific, require laborious extractions, large sample volumes, or both. Furthermore, these chromatographic techniques are inherently slow because only one sample can be measured at a time. Homogeneous immunoassays are quickly and easily performed, but the reagents are generally expensive and the assays usually yield results about 25% greater than chromatographic methods because of the cross-reactivity of the antibody with dihydroquinidine, (3s)-3-hydroxyquinidine, and quinidine-N-oxide (14, 15). Thin-layer chromatography is both specific and sensitive for measurement of unmetabolized quinidine, and it has the additional feature of permitting large-scale "batch" analysis. However, previous thin-layer chromatographic methods (5-8) require large sample volumes, lack an internal standard to control for losses, lack validation on serum from patients, and (or) require laborious elution of the drug from the plate before measurement.

We describe here a quantitative, high-throughput, thin-layer-chromatographic micro-analysis for unmetabolized quinidine that overcomes these difficulties. We compare this method with the double-extraction fluorescence assay for use with serum from patients receiving quinidine.

Materials and Methods

Apparatus

We used commercially available thin-layer chromatography plates, 10 cm × 20 cm, coated with 0.25 mm of Silica Gel 60 F254 (EM Reagents, E. Merck, Darmstadt, F.R.G.). Before use the plates were heat-activated (80°C) and were cut to 10 cm × 10 cm. After sample application, the plates were developed in glass battery jars, then scanned with a densitometer ("Auto Scanner Flur-Vis"; Helena Laboratories, Beaumont, TX 77704) in the fluorescence mode (366-nm excitation) with slit size 0.2 mm × 5 mm, scan speed 0.13 cm/s, and chart speed 1.25 cm/s. The "auto-gain" mode was used to set at full scale the most intensely fluorescent spot in each chromatogram.

Reagents and Standards

All reagents were AR grade.

Sodium hydroxide, 0.45 mol/L, in water. Store in a polyethylene bottle at room temperature.

Hexane/aceton, 80/20 by vol.

Chromatography solvent. Mix 170 mL of ethyl acetate, 20 mL of methanol, and 10 mL of ammonium hydroxide. Allow to equilibrate in the chromatography tank for at least 30 min before use. Prepare freshly each day.

Stock standards in methanol. Store all standards at -20°C.

N-(1-naphthyl)ethylenediamine dihydrochloride (NED), the internal standard, 120 mg/L. Dissolve 2.4 mg of NED (Matheson Coleman & Bell, Cincinnati, OH 45212) in 20 mL of methanol. To facilitate solution, mix with 10 μL of 0.45 mol/L sodium hydroxide. Store in an amber-colored glass tube. Prepare freshly each month.

Quinidine, 500 mg/L. Dissolve 12 mg of quinidine sulfate dihydrate (equivalent to 10 mg of quinidine base) in 20 mL of methanol. Store in an amber-colored glass tube. Prepare freshly each week.

Dilute internal standard, 0.6 mg/L. Dissolve 0.5 mL (0.06 mg) of the NED stock standard in each 100 mL of hexane/acetone solution used for extraction. This provides 0.09 mg of NED per milliliter of serum extracted. Store in an amber-colored glass bottle at room temperature. Prepare freshly each week.

Working standards in serum. Dilute the quinidine stock standard appropriately with drug-free serum to prepare 0-10 mg/L solutions. Analyze at least two concentrations (5 and 10 mg/L) with each run. Prepare freshly each day.
Serum control, 5 mg/L. Dissolve 0.5 mL (0.25 mg) of separately prepared quinidine stock standard in drug-free serum and dilute to 50 mL with drug-free serum. Store in small aliquots at -20 °C, protected from light. Prepare freshly each day, and check-cross with the previously prepared serum control. Alternatively, a suitable commercial lyophilized control may be used, such as Quinidine Serum Toxicology Control (UTAK Laboratories, Saugus, CA 91350).

Procedure

Pipet 0.5 mL of 0.45 mol/L sodium hydroxide into 15-mL conical glass tubes equipped with Teflon-lined screw caps. Add 20 µL of serum (standards, control, and each unknown) to the sodium hydroxide and mix. Pipet 3 mL of the hexane/acetone mixture, containing the internal standard, into each tube and vortex-mix for 45 s. Centrifuge (3000 × g) for 3 min and transfer the organic extract (upper phase) to a 5-mL conical glass tube. Evaporate the extract in a water bath at 50–60 °C under a gentle current of air. Carefully dissolve the residue in 50 µL of methanol, and apply the entire volume to a thin-layer chromatography plate. Extracted standards and controls should be included on each plate used. Develop the plate until the solvent rises to within 1 cm of the top of the plate (about 10 min). Dry the plate under a hood at room temperature with a gentle current of air until the odor of ammonium hydroxide is no longer detected.

If the plate cannot be scanned immediately, protect it from light by covering it with aluminum foil. Scan the plate with the fluorescence densitometer. Identify quinidine and NED from their RF values (0.58 and 0.46, respectively), using the standards for comparison. For each sample calculate the peak-height ratio (quinidine:NED). Plot the ratio for each extracted standard against its concentration. Quantify quinidine in the unknowns and control by using their peak-height ratios and the standard plot.

Results

A standard curve was prepared by supplementing drug-free serum with quinidine. A plot of peak-height ratio (x) vs concentration (y) was linear over the range of eight concentrations studied (0–20 mg/L); y = 8.036x – 0.500 (r = 0.996). As little as 0.2 mg of quinidine per liter could be measured in serum. The uncorrected analytical recovery over this concentration range was 60–65%, determined by comparing peak heights of extracted serum standards with those of nonextracted methanolic standards. We corrected for this incomplete recovery by using simultaneously extracted serum standards in each analysis.

Serum from 10 drug-free volunteers, taken through the proposed method, showed no interfering peaks in the chromatograms at the RF of either quinidine or NED. The within-run precision was estimated by analysis of drug-free serum supplemented with quinidine to concentrations of 2.5 and 5.0 mg/L, respectively. The coefficients of variation within-run were 8.6% (2.5 mg/L, n = 12) and 8.5% (5.0 mg/L, n = 12). Between-run precision was estimated by analysis of these sera on each of six days. The between-run coefficients of variation were 5.5% (2.5 mg/L, n = 10) and 3.4% (5.0 mg/L, n = 10). Quinidine concentrations in serum declined after six days of storage (-20 °C) in the dark.

The developed thin-layer-chromatographic spots were stable for at least 4 h when the plates were stored in the dark. However, when the plates were exposed to light, the peak-height ratios began to decline within 1 h.

We evaluated 33 bases for potential interference in the proposed assay: N-acetylprocainamide, amitriptyline, caffeine, chlordiazepoxide, chloroquine, chlorpromazine, chlorpromazine sulfoxide, desipramine, diazepam, dibucaine, digoxin, flurazepam, furosemide, haloperidol, imipramine, lidocaine, metaprotrophenol, morphine, nicotine, nifedipine, nortriptiline, oxazepam, perphenazine, primaquine, procaïnamide, prochlorperazine, promazine, propranolol, terbutaline, tetracycline, thioridazine, triamterene, and warfarin. These compounds were initially screened for interference by submitting their methanolic solutions to thin-layer chromatography with fluorescence densitometry. Those compounds exhibiting fluorescence and RF values in the range 0.15–0.65 were re-studied by adding them to drug-free serum to give a concentration of 10 mg/L. When we analyzed these supplemented sera by the proposed procedure, only chlordiazepoxide, chlorpromazine sulfoxide, and oxazepam showed any interference at this concentration (equivalent to 0.3 mg of quinidine per liter).

Quinine is an optical isomer of quinidine, so it will interfere in both the proposed analysis and in spectrofluorometric procedures. However, as an antimalarial agent, it has been largely replaced by other, more efficacious medications. It is present in the beverage tonic ("quinine") water in concentrations ranging from 29 to 80 mg/L (17), but concentrations of quinine in serum 45 min after ingestion of 240 mL of tonic water are <0.1 mg/L (17), well below the limit of sensitivity of the proposed assay. Therefore this is a negligible source of interference.

Twenty sera from 12 different patients were analyzed on the same days by both the proposed method (x) and a modification of the widely used double-extraction method (y) of Cramer and Isaksson (3) (Table 1). The latter method involves extraction of quinidine from 0.5 mL of serum at alkaline pH into toluene followed by back-extraction into dilute sulfuric acid and spectrofluorometry. The "least-squares" regression equation was: y = 0.994x + 0.276 (r = 0.989). In addition to quinidine, these patients were receiv-

<table>
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<th>Table 1. Quinidine Concentrations in Serum of Patients</th>
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<td><strong>Quinidine concn, mg/L</strong></td>
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Key: 1, fluorocortisone; 2, terbutaline, 3, haloperidol; 4, sulfamethoxazole; 5, trimethoprim; 6, heparin; 7, digoxin; 8, furosemide; 9, warfarin; 10, verapamil; 11, acetaminophen; 12, nitroglycerin; 13, codeine; 14, theophylline; 15, metaprotrophenol; 16, atenolol; 17, methadone; 18, morphine; 19, diazepam; 20, diphenhydramine; 21, albuterol; 22, nifedipine; 23, trimetazidine; 24, acetazolamide; 25, metizozone; 26, diltiazem; 27, phenytoin; 28, flurazepam; 29, chlorpromazine; 30, metoprolol; 31, hydralazine; 32, levothyroixin; 33, cloni- dine.

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ing a total of 33 other drugs (Table 1), none of which (or their metabolites) interfered.

Discussion

In the proposed assay a micro-volume (20 μL) of sample is used. The assay is sufficiently sensitive to detect therapeutic and sub-therapeutic concentrations of quinidine, and will permit large-scale “batch” analysis of samples. Unlike its counterpart, “high-performance” thin-layer chromatography (HPTLC), conventional thin-layer chromatography as used here requires relatively inexpensive reagents, and the necessary supplies and equipment are routinely available in most clinical laboratories.

Although chlordiazepoxide, chlorpromazine sulfoxide, and oxazepam at concentrations of 10 mg/L yielded apparent “quinidine” concentrations of 0.3 mg/L, these compounds would not usually be found at 10 mg/L in serum after therapeutic doses (18, 19). Furthermore, a quinidine concentration of 0.3 mg/L is sufficiently below the reported (16) reference range (2.3–5.0 mg/L) as to be clinically insignificant in most cases.

Although it is not structurally similar to quinidine, we chose to use NED as the internal standard because it has extraction characteristics similar to those of quinidine, has an $R_F$ value (0.46) near that (0.58) of quinidine, and exhibits intense fluorescence. Furthermore, it is not a drug. Our finding that quinidine in serum deteriorates after six days of storage (−20 °C) in the dark is in contrast to a previously published report indicating stability in serum for three weeks (20).

Comparison of results obtained with the proposed method and with the double-extraction fluorescence method showed the latter to yield concentrations that averaged 12% higher (Table 1). This difference cannot be explained by the reported simultaneous measurement of dihydroquinidine with the double-extraction method (4) because the present chromatographic method did not reveal the presence of dihydroquinidine (at $R_F$ 0.52) in any of our patients except for a trace of the compound in patient no. 9 (Table 1), who had the highest quinidine concentration in the series. The lack of measurable dihydroquinidine is consistent with the observation of Powers and Sadee (11), who found that the compound, present as a pharmacologically active contaminant in most commercial quinidine preparations, accounts for only a negligible (<10%) proportion of the total drug in serum. In addition, we noted no other fluorescent spots in the chromatograms of hexane/acetone extracts of sera from our patients.

Our experience with the proposed thin-layer chromatographic micro-analysis of quinidine leads us to recommend it for routine use in the clinical laboratory, particularly when many samples must be analyzed simultaneously.

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References