CHROMBIO. 449

SIMPLE AND SELECTIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR ESTIMATING PLASMA QUINIDINE LEVELS

PHILLIP A. REECE and MARGOT PEIKERT

Department of Clinical Pharmacology, The Queen Elizabeth Hospital, Woodville, S.A. 5011 (Australia)

SUMMARY

A reversed-phase, high-performance liquid chromatographic method employing fluorescence detection is described for the rapid quantification of plasma levels of quinidine, dihydroquinidine and 3-hydroxyquinidine. It involves protein precipitation with acetonitrile followed by direct injection of the supernatant into the chromatograph. For the preparation of plasma standards, pure 3-hydroxyquinidine was isolated from human urine by a simplified thin-layer chromatographic procedure. The mobile phase for the chromatography was a mixture of 1.5 mM aqueous phosphoric acid and acetonitrile (90:10) at a flow-rate of 2 ml/min. The intra-assay coefficient of variation for the assay of quinidine and 3-hydroxyquinidine over the concentration range 2.5—20 μmole/l was < 1% for both. Interassay coefficients of variation for quinidine (10 μmole/l) and 3-hydroxyquinidine (5 μmole/l) were 3.5% and 4.0% with detection limits of 50 and 25 μmole/l respectively. The method correlated well (r^2 = 0.96) with an independently developed gas—liquid chromatographic—nitrogen detection assay for quinidine which also possessed a high degree of precision. (Intra-assay coefficient of variation 3.6% at 20 μmole/l). As expected, comparison of the high-performance liquid chromatographic assay with a published protein precipitation—fluorescence assay showed poor correlation (r^2 = 0.78).

INTRODUCTION

Quinidine remains an important agent for the treatment of arrhythmias. A relationship between quinidine plasma levels and efficacy has been demonstrated [1] and hence the monitoring of plasma levels has become an important adjunct in the management of patients administered the drug. There are a number of published methods available for measuring quinidine levels with varying degrees of specificity. The original protein precipitation—fluorescence method [2] is still widely used but both that method and extraction—fluorescence

methods have been demonstrated to measure a number of metabolites in addition to quinidine [3, 4].

Recently, a summary of the available quinidine assay methods and their limitations together with a normal-phase high-performance liquid chromatographic (HPLC) assay were reported [4]. Although the reported method possessed high selectivity it lacked the convenience required for rapid emergency assays. A published reversed-phase HPLC assay [5] involving protein precipitation, direct injection and ultraviolet detection offered the advantage of considerably shorter assay time but had limited sensitivity. A recently reported HPLC assay employing extraction and post-column acidification with fluorescence detection [6] did not use internal standardization.

In the present report, a simple reversed-phase HPLC assay for quinidine, 3-hydroxyquinidine and dihydroquinidine employing protein precipitation with direct injection and fluorescence detection is described. Comparisons with a gas chromatographic—nitrogen detection method and a published protein precipitation—fluorescence method are also described.

MATERIALS AND METHODS

Reagents

All reagents were analytical grade and aqueous solutions were prepared using glass distilled water. Specially purified acetonitrile (Unichrom, Ajax Chemicals, Sydney, Australia) was used for high-performance liquid chromatography. The chloroform was nanograde from Mallinckrodt (St. Louis, Mo., U.S.A.) and the methanol, spectrofluorescence grade (Uvasol, Merck, Darmstadt, G.F.R.). Quinidine sulphate was obtained from Burroughs Wellcome (Detroit, Mich., U.S.A.) and contained 9% dihydroquinidine. Commercially available cinchonidine from Koch-Light (Colnbrook, Great Britain) contained traces of quinidine and was recrystallized four times from methanol—water (1 : 1) before use. The cinchonidine also contained traces of dihydrocinchonidine in unknown quantity. Dihydroquinidine was obtained from ICN, Irvine, Calif., U.S.A.

Standards

Stock solutions of quinidine sulphate and 3-hydroxyquinidine were prepared in glass distilled water at concentrations of 200 and 100 μmole/l, respectively. These solutions were stable for at least 2 months at 4°. For the HPLC assay a solution of recrystallized cinchonidine (internal standard) was prepared in acetonitrile (170 μmole/l). For the gas—liquid chromatographic (GLC) assay the cinchonidine solution (68 μmole/l) was prepared in water. These solutions were also stable for at least 2 months at 4°. Plasma standards containing the required amounts of quinidine and 3-hydroxyquinidine were prepared and stored at 4° for no longer than 2 weeks. Peak area ratios of the drug and metabolites to the internal standard were determined for plasma standards and unknowns and quantification performed by reading unknown values from a plotted standard curve.

Isolation of 3-hydroxyquinidine from urine

Urine (20 ml) was collected from a patient taking oral quinidine at steady-

state. It was basified (pH 14) with 1 N aqueous sodium hydroxide and extracted twice with chloroform (20 ml). The phases were separated by centrifugation and the aqueous layer discarded. The chloroform extracts were pooled and evaporated under nitrogen (45°) and the residue reconstituted in methanol (1 ml). A 250-μl amount of the methanol solution was chromatographed on each of 4 preparative thin-layer plates (silica gel 60 (20 × 20 cm), Merck) using methanol as the solvent. The fluorescent bands were identified under ultraviolet light and those for quinidine and dihydroquinidine selected by comparison of the R_F values with those of authentic samples of the compounds. Two other bands with larger R_F values (0.8, 0.65) were scraped from each of the plates, separately pooled and extracted with methanol (three times). The extracts were evaporated and the residue treated with diethyl ether (0.5 ml twice) and separated by centrifugation. The diethyl ether was evaporated and a small portion of each residue reconstituted in mobile phase and injected into the high-performance liquid chromatograph. In this way each band was assigned to a peak in the HPLC chromatogram.

The major metabolite isolated (210 μg) (R_F = 0.65) was recrystallized once from methanol—diethyl ether by adding diethyl ether dropwise to seed crystallization. It had a melting point (210—211°) and mass spectrum (m/e 340, 267, 189, 173, 152; determined at 20 eV, probe temperature 170° using a Model AEI MS-30 mass spectrometer) identical with that published for 3-hydroxyquinidine [7] and different from that of the recently identified N-oxide metabolite of quinidine [8] which in contrast showed the characteristic fragmentation of an N-oxide. There was insufficient material to obtain a meaningful proton magnetic resonance spectrum. The isolated 3-hydroxyquinidine was used to prepare plasma standards for the estimation of plasma levels in patient samples. Insufficient quantities of the second metabolite were obtained to allow satisfactory recrystallization.

High-performance liquid chromatographic assay

An aliquot of the internal standard solution (cinchonidine in acetonitrile, 170 μmole/l) was added to an equal volume of patient plasma or plasma standard (20—200 μl) and vortexed at high speed for 30 sec. After standing at room temperature for 15 min the mixture was centrifuged and 10 μl of the supernatant injected into the high-performance liquid chromatograph using a 10-μl injector loop.

The chromatograph used was a Spectra-Physics Model SP8000 equipped with a ternary solvent system, helium degassing and automatic data reduction facilities. A 10-μm alkyl phenyl reversed-phase column (μBondapak/Phenyl from Waters Assoc., Milford, Mass., U.S.A.) was used at a column temperature of 50°. The mobile phase was a mixture of 1.5 mM aqueous phosphoric acid and acetonitrile (90 : 10) at a flow-rate of 2 ml/min and all solvent lines from the column to the detector were carefully thermally insulated. The effluent was monitored using a fluorescence detector (Schoeffel Model 970) at an excitation wavelength of 320 nm with an emission cut-off filter allowing 90% transmission at 418 nm. The fluorimeter sensitivity setting was 3.5, range 0.4 μA full-scale and time constant 5.0 sec. On completion of an assay run, the column was automatically washed with water for 30 min at a flow-rate of 2 ml/min, fol-

lowed by a 5-h acetonitrile wash at a flow-rate of 0.2 ml/min.

The levels of quinidine and 3-hydroxyquinidine in unkowns were determined from plotted standard curves. Dihydroquinidine had identical fluorescence to quinidine and was determined using the quinidine standard curve. For comparison with the published precipitation fluorescence method [2], other fluorescent quinidine metabolites eluted were assumed to have fluorescence equivalent to quinidine and quantitated as such. This provided a quinidine plus metabolites level which included 3-hydroxyquinidine and dihydroquinidine.

Gas—liquid chromatographic assay

500 μl of the internal standard solution (cinchonidine 68 μmole/l) was added to 1 ml of patient plasma and plasma standards; 2 ml of 0.1 N aqueous sodium hydroxide were added followed by 5 ml of chloroform and the mixture was shaken for 10 min at 100 r.p.m. After separation of the phases by centrifugation the aqueous layer was removed by vacuum aspiration and the organic layer transferred to a conical centrifuge tube. The solvent was evaporated under a flow of nitrogen at 45° and the residue reconstituted in 100 μl of methanol. A 2-μl aliquot of the methanol solution was injected into the gas chromatograph (Packard Model 419) equipped with a 1 m × 2 mm I.D. column containing 3% OV-17 on Gas-chrom Q (Supelco, Bellefonte, Pa., U.S.A.). Injector, column and detector temperatures were 280°, 270° and 300°, respectively. An alkali-flame detector (Packard Model 713) was used with the following flow-rates: nitrogen, 30 ml/min; hydrogen, 40 ml/min; air, 260 ml/min. Peak area ratios of quinidine to the internal standard were determined using an integrator (Spectra-Physics, Autolab System IVB).

Spectrofluorometric assay

The method of Brodie and Udenfriend was used [2] with 0.1 ml of plasma. Plasma proteins were precipitated with a solution of metaphosphoric acid and the fluorescence of the supernatant determined using an Aminco-Bowman Ratio Photometer Spectrofluorimeter. The excitation wavelength used was 320 nm and emission monitored at 418 nm. Quantification was performed by comparison of the relative fluorescence with that of plasma standards containing added amounts of quinidine.

Reproducibility and recovery

The intra-assay reproducibility for the HPLC method was determined by assaying five plasma samples containing added amounts of quinidine and 3-hydroxyquinidine (2.5, 5.0, 10 and 20 μmole/l) at each concentration. The inter-assay reproducibility was determined by assaying a previously prepared plasma sample containing quinidine (10 μmole/l) and 3-hydroxyquinidine (5 μmole/l) in each daily run (20 determinations). The intra-assay reproducibility of the GLC method was determined by assaying five plasma samples containing added quinidine (20 μmole/l). The recovery of the two methods was determined by injecting known amounts of quinidine and 3-hydroxyquinidine into the chromatograph and comparing the peak areas with those obtained for an extracted plasma standard.

Comparison of HPLC and GLC assays

Eighteen plasma samples taken from patients on oral quinidine were assayed for quinidine by both the HPLC and GLC assay methods.

Comparison of HPLC and precipitation—fluorescence assays

Twenty patient plasma samples were assayed by HPLC and by the precipitation method. Two scattergrams were plotted: (1) the HPLC quinidine level against the quinidine level obtained by the precipitation method; (2) quinidine plus metabolite levels determined by assuming that unknown metabolite peaks had fluorescence equivalent to quinidine, that is, quinidine + 3-hydroxyquinidine + dihydroquinidine + unknown metabolites plotted against the quinidine level obtained by the precipitation method.

RESULTS AND DISCCUSION

The HPLC assay for quinidine, 3-hydroxyquinidine and dihydroquinidine was sensitive and selective and sufficiently rapid for use in emergency situations. Preparation time for standards and patient samples was approximately 20—30 min and the chromatographic run time 18 min for each sample. If required, as little as 20 μl of plasma could be used for the assay and by injecting 10 μl of the resultant supernatant levels of 50 and 25 nmole/l (16 and 8.5 ng/ml) of quinidine and 3-hydroxyquinidine were detectable at peak height twice noise. The sensitivity could be enhanced by injecting a larger volume onto the column. Improved peak resolution and reproducibility of retention times was obtained using a column oven temperature of 50° rather than ambient temperature. By washing the column carefully with water and acetonitrile after each assay run several hundred injections have been made without any noticeable loss in performance.

Dihydroquinidine had fluorescence equivalent to quinidine and the ratio of the fluorescence intensity of 3-hydroxyquinidine to quinidine was 1.1. The internal standard, cinchonidine, had considerably less intense fluorescence at the excitation wavelength used for the assay (320 nm), hence a relatively high concentration was required to obtain a peak of suitable area. Under these conditions the traces of quinidine in the commercial preparation became an interference and the material required recrystallization four times before use.

Attempts to obtain samples of the metabolites from all sources was unsuccessful. However, a simplified thin-layer chromatographic procedure for the isolation of 3-hydroxyquinidine from human urine in sufficient quantities for the preparation of plasma standards was developed. The isolated material had a melting point and mass spectrum identical with those published for 3-hydroxyquinidine. Since there were no extraction losses in the HPLC assay method reported (linear recovery of 100% over the concentration 2.5—20 μmole/l), it is valid to use the fluorescence ratio of 1.1 (3-hydroxyquinidine:quinidine) to estimate plasma levels of this metabolite.

The chromatograms obtained for the assay of blank plasma, a plasma standard containing 10 μmole/l (3.2 μg/ml) of quinidine and 5 μmole/l (1.7 μg/ml) of 3-hydroxyquinidine and a patient sample are shown in Fig. 1. Three important metabolite peaks were found in all patient samples, that is, peak 1
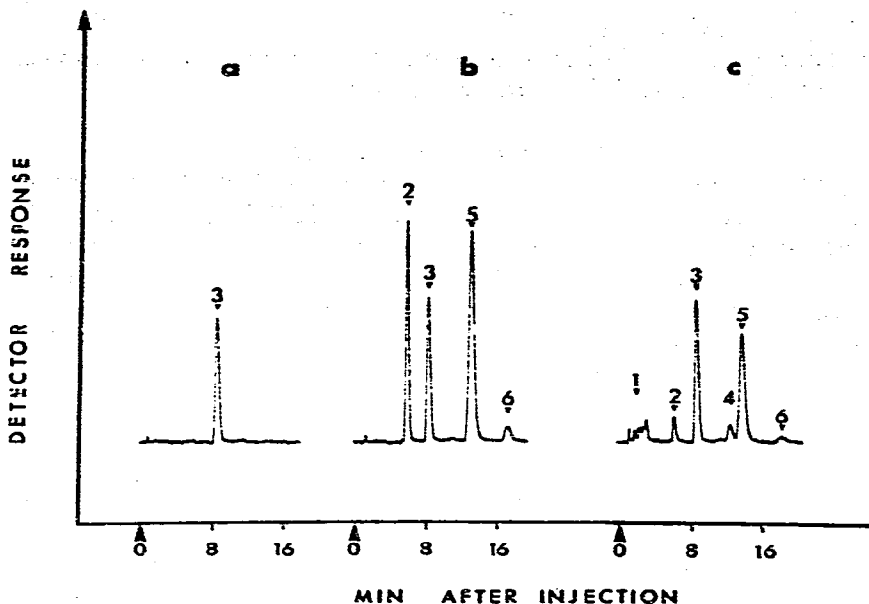
Fig. 1. Chromatograms obtained for the HPLC quinidine assay of (a) blank plasma, (b) plasma standard containing 10 μmole/l quinidine and 5.0 μmole/l 3-hydroxyquinidine and (c) plasma from a patient on chronic oral quinidine therapy containing 0.6 μmole/l 3-hydroxyquinidine, 5.2 μmole/l quinidine and 0.3 μmole/l dihydroquinidine. Deproteinated plasma (10 μl) was injected onto column. Peaks: 1 = polar metabolites; 2 = 3-hydroxyquinidine; 3 = internal standard (cinchonidine); 4 = unidentified metabolite; 5 = quinidine; 6 = dihydroquinidine.

(polar metabolites), peak 2 which was assigned to 3-hydroxyquinidine on the basis of the mass spectrum and melting point of the isolated metabolite and peak 4 which was tentatively assigned to the recently identified N-oxide metabolite [4]. Plasma levels of O-desmethylquinidine and 2'-oxoquinidine have been reported to be very low [9] and their relative fluorescence intensity under the conditions used presently has been reported to be only 0 and 15% of that quinidine [10]. Hence these metabolites will not be detected in the present method and both the major metabolites have been separated from quinidine allowing selective quantification of quinidine plasma levels.

The intra-assay coefficient of variation for the HPLC assay of quinidine and 3-hydroxyquinidine over the concentration range 2.5–20 μmole/l was < 1% in each case. Inter-assay coefficients of variation for quinidine (10 μmole/l) and 3-hydroxyquinidine (5 μmole/l) were 3.5% and 4.5%, respectively. No interference was observed from hydralazine and its metabolites, and propranolol, 4-hydroxypropranolol, imipramine, desipramine, procainamide, N-acetylprocainamide, disopyramide and tocainide in the assays for quinidine, dihydroquinidine and 3-hydroxyquinidine. However, N-desisopropylpropranolol and quinine had retention times identical to that of quinidine.

Chromatograms obtained for the GLC assay of quinidine are shown in Fig. 2. There was a greater detector response to dihydroquinidine than quinidine in this assay. Excess internal standard was used to improve the precision of quan-
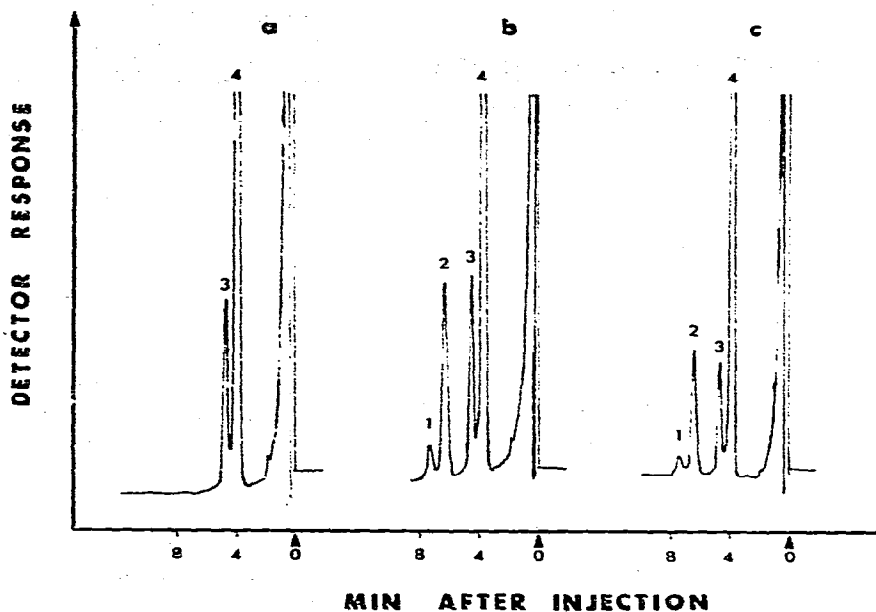
Fig. 2. Chromatograms obtained for the GLC quinidine assay of (a) blank plasma, (b) plasma standard containing 20 µmole/l quinidine and (c) plasma from a patient on chronic oral quinidine therapy. Peaks: 1 = dihydroquinidine; 2 = quinidine; 3 = dihydrocinchonidine; peak 4 = internal standard (cinchonidine). Peak areas were determined by integration.

titation. The GLC assay possessed a high degree of precision with an intra-assay coefficient of variation of 3.6% at a quinidine plasma concentration of 20 µmole/l. Recovery was 92% over the concentration range 5—20 µmole/l. Excellent correlation ($r^2 = 0.96$) was found between the HPLC and GLC assay (Fig. 3). The retention times of other drugs injected into the chromatograph are indicated in Table I. Since the HPLC and GLC methods rely on inde-
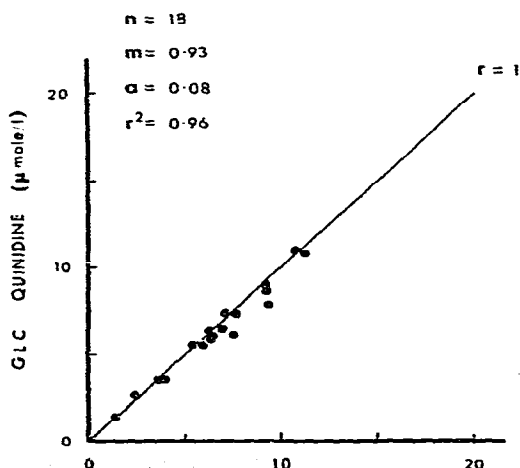

Fig. 3. Comparison of HPLC and GLC assays for plasma quinidine (m = slope, a = intercept). Samples were taken from patients on chronic oral quinidine therapy.

TABLE I

RETENTION TIMES FOR GLC ASSAY OF QUINIDINE

Compound	Retention time (sec)
Quinidine	368
Dihydroquinidine	444
Cinchonidine	222
Disopyramide	274
Procainamide	79
N-Acetylprocainamide	227

pendent means of extraction, chromatography and detection, this correlation is supportive evidence of their specificity for quinidine. The disadvantages of the GLC assay were that it did not provide an estimate of the 3-hydroxyquinidine level and required a considerably longer sample preparation time.

As expected, there was good correlation ($r^2 = 0.98$) between the quinidine plus metabolites level determined by the HPLC method and quinidine plus metabolites level determined by the precipitation method (Fig. 4). However, the correlations between the quinidine level measured by HPLC and quinidine plus metabolites level (HPLC) ($r^2 = 0.81$) (Fig. 5) and between quinidine (HPLC) and quinidine plus metabolites measured by the precipitation method ($r^2 = 0.78$) (Fig. 6) were relatively poor. This is consistent with published work [11, 12] and supports the observation that quinidine levels measured by the precipitation method contain a variable and significant contribution from polar metabolites which may be pharmacologically inactive.
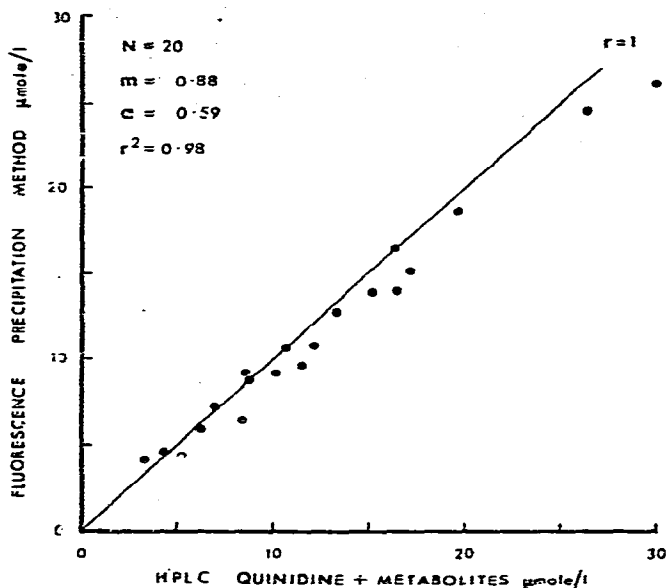

Fig. 4. Comparison of quinidine plus metabolites level determined by HPLC and levels determined by precipitation—fluorescence assay for the same plasma samples (m = slope, a = intercept).
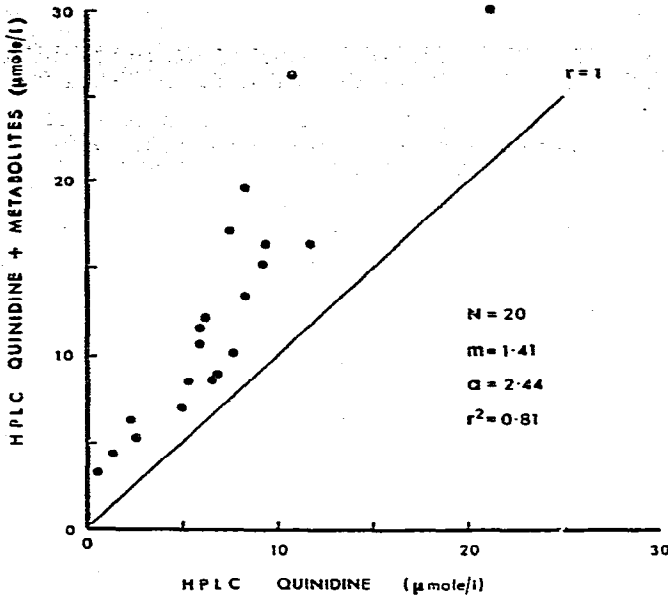
Fig. 5. Comparison of quinidine and quinidine plus metabolites plasma levels determined by HPLC (m = slope, a = intercept). Samples were taken from patients on chronic oral quinidine therapy.
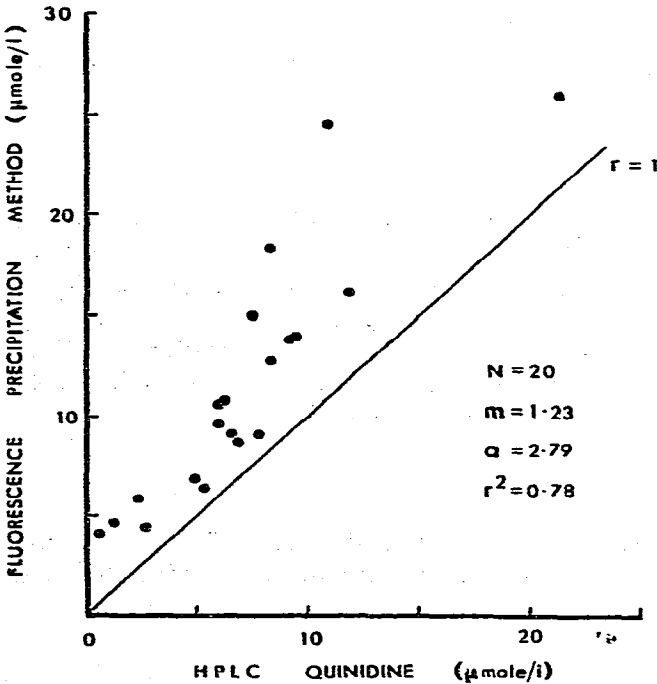

Fig. 6. Comparison of HPLC and precipitation—fluorescence assay methods for plasma quinidine (m = slope, a = intercept). Samples were taken from patients on chronic oral quinidine therapy.

TABLE II

QUINIDINE, 3-HYDROXYQUINIDINE, DIHYDROQUINIDINE AND QUINIDINE PLUS FLUORESCENT METABOLITE LEVELS (μmole/l) IN PLASMA SAMPLES FROM PATIENTS ON ORAL THERAPY

Quinidine	3-Hydroxyquinidine	Dihydroquinidine	Quinidine + metabolites[*]
7.4	1.5	0.3	10.8
11.4	2.4	0.4	16.1
22.2	3.3	1.0	29.4
30.0	5.2	1.2	42.0
5.8	1.4	0.4	10.7
15.2	2.5	0.7	21.5
2.2	0.5	0.1	3.3
9.7	0.6	0.2	11.9
10.0	1.7	0.2	13.3
10.2	1.7	0.3	13.6
12.6	1.9	0.5	19.2
3.3	0.6	0.1	4.6
6.8	0.5	0.2	8.3
16.6	1.9	0.6	22.6
8.0	0.3	0.3	9.4
4.6	1.5	0.2	9.9
18.0	2.6	0.7	24.3
6.0	2.7	0.3	14.8
0.6	0.5	< 0.1	3.4
6.6	0.9	0.2	8.6
Mean:			
10.4	1.71	0.40	14.9
(3.35 μg/ml)	(0.58 μg/ml)	(0.13 μg/ml)	

[*]Quinidine + 3-hydroxyquinidine + dihydroquinidine + other metabolites assumed to have identical fluorescence to quinidine.

The levels of quinidine, 3-hydroxyquinidine, dihydroquinidine and quinidine plus metabolites obtained for the HPLC assay of plasma samples drawn from different patients on varying oral quinidine therapy and not necessarily at steady-state are shown in Table II. The levels of 3-hydroxyquinidine and dihydroquinidine found in this mixed patient population showed similar variations to those published [3, 4, 9]. Peak 1 accounted for the majority of the additional metabolites indicated in the last column of Table II.

REFERENCES

1 I.R. Edwards, B.W. Hancock and R. Saynor, Brit. J. Clin. Pharmacol., 1 (1974) 455.
2 B.B. Brodie and S. Udenfriend, J. Pharmacol. Exp. Ther., 78 (1943) 154.
3 T.W. Guentert and S. Riegelman, Clin. Chem., 24 (1978) 2065.
4 T.W. Guentert, P.E. Coates, R.A. Upton, D.L. Combs and S. Riegelman, J. Chromatogr., 162 (1979) 59.
5 J.L. Powers and W. Sadee, Clin. Chem., 24 (1978) 1007.
6 B.J. Kline, V.A. Turner and W.H. Barr, Anal. Chem., 51 (1979) 449.
7 K.H. Palmer, B. Martin, B. Baggett and M.E. Wall, Biochem. Pharmacol., 18 (1969) 1845.
8 T.W. Guentert, Dept. of Pharmacy, University of California Medical Centre, San Francisco, personal communication.
9 D.E. Drayer, D.T. Lowenthal, K.M. Restivo, A. Schwartz, C.E. Cook and M.M. Reidenberg, Clin. Pharmacol. Ther., 24 (1978) 31.
10 D.E. Drayer, K. Restivo and M.M. Reidenberg, J. Lab. Clin. Med., 90 (1977) 816.
11 G. Cramer and B. Isaksson, Scand. J. Clin. Lab. Invest., 15 (1963) 553.
12 K.M. Kessler, D.J. Lowenthal, H. Warner, T.Gibson, W. Briggs and M. Reidenberg, New Engl. J. Med., 290 (1974) 706.