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Note

Specific determination of quinidine and metabolites in biological fluids by reversed-phase high-performance liquid chromatography

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As in the case of other drugs with a narrow therapeutic index, the determination of quinidine in biological fluids is justified by the good correlation between the zero, therapeutic or toxic effects of this antiarrhythmic drug and the blood levels. That a specific assay is needed has been shown elsewhere by the presence of pharmacologically active metabolites [3(*S*)-hydroxyquinidine, 2′-quinidone, quinidine N-oxide] which may be present in high concentration in the plasma of treated subjects [1—3], as also is dihydroquinidine, an impurity that occurs in the preparation of quinidine and which can amount to 20% of the administered dose [4]. In comparison to spectrofluorimetric assays that use protein precipitation—fluorescence (PPF) [5, 6] or extraction—fluorescence (EF) [7—9] techniques, chromatographic methods have the advantage of being more sensitive and specific. However, thin-layer chromatography (TLC) [10—13] and gas—liquid chromatography (GLC) [14—18] are tedious, time-consuming, and difficult. An extraction phase is necessary, and the choice of polarity of the solvent influences the co-extraction of the metabolites both qualitatively and quantitatively. Lastly, in GLC methods, derivatisation using flash-methylation is often carried out [14—16]. Recent publications

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about quinidine assays in biological fluids or in pharmaceutical formulations describe high-performance liquid chromatographid (HPLC) procedures because of the obvious advantages: rapidity, specificity and sensitivity. These methods indicate either the use of a normal-phase column with alkaline extraction, UV [19—22] or fluorimetric [23] detection, or the use of a reversed-phase column with UV [24—28] or fluorimetric [29—35] detection. In the latter, injection may be carried out either with plasma direct [29], or with a supernatant obtained after protein precipitation and centrifugation [25, 33, 34], or with an aliquot of residue reconstituted in the mobile phase after alkaline extraction with an appropriate solvent and evaporation to dryness [24, 26—28, 30—32, 35].

The HPLC method described here uses direct protein precipitation of samples with acetonitrile, a C_{18} reversed-phase column and fluorimetric detection. The choice of mobile phase allows internal standardization with quinine, whose fluorescence characteristics are similar to those of quinidine and dihydroquinidine. Excellent separation of these three drugs and of polar quinidine metabolites using a rapid and easy technique with sensitive detection represents the advantage of this method.

MATERIALS AND METHODS

Reagents and standards

All solvents used are analytical ragent grade (E. Merck, Darmstadt, G.F.R.): acetonitrile for spectroscopy, methanol, acetic acid 100%. Phosphate buffer (0.05 *M*, pH 7.4) is made up in distilled water. Pure standards of quinidine, 3(*S*)-hydroxyquinidine, dihydroquinidine (as bases) are obtained from Nativelle (Paris, France), and quinine was in sulfate form ($Qn_2 \cdot H_2SO_4 \cdot 2H_2O$).

Standard solutions

Stock solutions of quinidine, 3(*S*)-hydroxyquinidine and dihydroxyquinidine were prepared in methanol at concentrations of 1 g/l (stable for 2 months at 4°C) and 100 mg/l (stable for 15 days at 4°C). Working solutions, at concentrations between 0 and 5 mg/l, were prepared by dilution of the stock solution in a mixture (1:1, v/v) of acetonitrile and aqueous phosphate buffer (0.05 *M*, pH 7.4). Stock solution of quinine in methanol at a concentration of 1 g/l (as base) is prepared with quinine sulfate and is used to prepare a standard solution of quinine at 2.5 mg/l in acetonitrile.

Chromatographic conditions

The technique is carried out on a Chromatem apparatus, equipped with an Altex 210 pump, a Rheodyne 7010 injector with a 20-μl loop, a Waters C_{18} μBondapak column 30 cm × 3.9 mm (particle size 10 μm), a Schoeffel FS 970 spectrofluorimeter (λ_{ex} = 340 nm, λ_{em} = cut-off filter of 418 nm), set at 0.5 μA full-scale sensitivity and a time-constant of 6 sec. The mobile phase was a degassed mixture of acetonitrile—acetic acid—water (10:4:86) with a flow-rate of 2.5 ml/min.

Procedure

To precipitate proteins, 100 μl of plasma are added to 100 μl of a solution of quinine (2.5 mg/l) in acetonitrile. After closing with Parafilm, the mixture is gently mixed on a Vortex for about 1 min, and centrifuged at 1200 *g* for 10 min; 10 μl of the resultant clear supernatant are injected directly on the column.

RESULTS AND DISCUSSION

Using the conditions described, the chromatographic resolution of the different compounds is achieved in 10 min, with the following retention times (Fig. 1a): 3(*S*)-hydroxyquinidine t_R = 2.5 min, quinidine t_R = 6.2 min, quinine t_R = 7.7 min, dihydroquinidine t_R = 8.8 min. On typical chromatograms obtained from plasma of quinidine-treated subjects (Fig. 1b), a first peak (t_R = 1.8 min) is found which corresponds to one (or several) unidentified metabolite(s). A second peak (t_R = 4.8 min) coming just before the quinidine could, according to Reece and Peikert [33] be tentatively assigned to the recently identified N-oxide [3] whose complete structure has not yet been established. In the chromatographic conditions described, we cannot detect 2'-quinidinone because of its weak fluorescence and its low plasma concentrations [30].

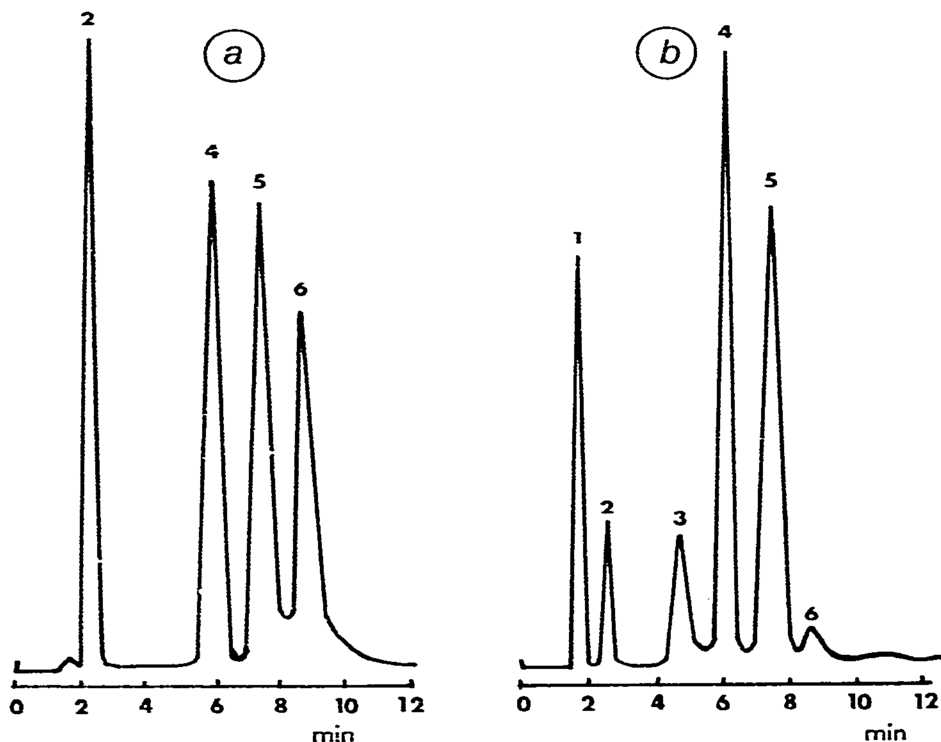

Fig. 1. Chromatograms of (a) loaded plasma at a concentration of 2 mg/l in 2, 4 and 6, and (b) plasma from a treated patient receiving 4 × 165 mg of quinidine base every 24 h in arabogalactane sulfate form (Longacor). 1 and 3 = unidentified metabolites, 2 = 3(*S*)-hydroquinidine (0.47 mg/l), 4 = quinidine (2.58 mg/l), 5 = quinine (2.5 mg/l), 6 = dihydroquinidine.

However, if a specific extraction [36] of plasma or urine is used, this metabolite can be detected (t_R = 16.2 min).

Standard curves are based on loaded plasma samples treated with acetonitrile including the quinine internal standard, chosen for its structural relationship and its identical fluorescence with quinidine, and for its suitable retention time. As internal standard, other tested compounds [cinchonine, cinchonidine, 3(*R*)-hydroxyquinidine, dihydroquinidinone] show unsatisfactory resolution or their fluorescence is too weak.

Standard curves for quinidine, dihydroquinidine and 3(*S*)-hydroxyquinidine were linear for concentrations up to 5 mg/l, verified by measuring the peak height ratio. The calibration graphs can be expressed by the equations $y = 9.172x - 0.111$, $y = 7.054x - 0.214$, and $y = 17.391x + 0.122$, respectively. Correlation coefficients were typically 0.996, 0.997 and 0.995, respectively. Recovery is 100 ± 3%, when the concentrations of plasma loaded with different compounds are compared with standard solutions in a mixture (1:1) of acetonitrile and aqueous 0.05 *M* phosphate buffer. This shows that there is no loss by adsorption on the precipitate. The sensitivity for each component was 50 ng/ml.

Intra-assay reproducibility for the assay of quinine, quinidine and metabolites was determined by assaying six replicate plasma samples containing added amounts of drug and metabolites at concentrations ranging from 0.5 to 5 mg/l. For each concentration the within-run precision was determined with a coefficient of variation of less than 3%. The day-to-day precision was determined on six consecutive days using frozen samples of plasma at levels ranging from 0.5 to 5 mg/l (Table I).

TABLE I

VARIATION IN REPLICATE STANDARDS FROM SPIKED PLASMA

	Within-run (n = 6)				Day-to-day (n = 6)			
	Concentration (mg/l)				Concentration (mg/l)			
	0.5	1	2.5	5	0.5	1	2.5	5
Quinine S.D.			0.15				0.30	
C.V. (%)			1.9				1.9	
Quinidine S.D.	0.06	0.09	0.22	0.32	0.15	0.31	1.55	2.43
C.V. (%)	1.2	1	1.8	1.3	3.3	3.5	6.7	5.3
Dihydroquinidine S.D.	0.07	0.09	0.09	0.2	0.13	0.23	0.89	1.9
C.V. (%)	2	1.3	1.1	1.2	3.9	3.4	5.1	5.4
3(S)-Hydroxyquinidine S.D.	0.14	0.12	0.12		0.36	0.7	2.3	
C.V. (%)	1.5	0.6	0.5		4.1	4	5.2	

The excitation wavelength (340 nm) was chosen to avoid possible interference at 240 nm by other endogenous or exogenous plasma compounds, and to obtain in a greater fluorescence intensity that at 280 nm. With a 418-nm cut-off filter, the four compounds [3(*S*)-hydroxyquinidine, quinidine, quinine, dihydroquinidine] have, at the same concentration, equivalent fluorescence [30, 33], and injection of drug-free plasma shows only a small front peak.

In comparison with other reversed-phase techniques, we use direct protein precipitation of samples; thus risk of clogging the column with plasma injection [29] or long and non-quantitative extraction steps are avoided [24, 26–28,

30—32, 35]. As deproteinizing agent, acetonitrile, a component of the mobile phase, is suitable [33], but when cinchonine or cinchonidine have bad fluorescence characteristics and high impurity concentrations [30, 33], quinine seems to be the most convenient internal standard. Its good resolution with quinidine and dihydroquinidine is possible in a simple isocratic system on a non-thermostated C_{18} column, when others methods show interference between these drugs [24, 25, 31, 33], have only external standardization [25, 29, 32], or use dihydroquinidine, a common impurity of quinidine [34], as internal standard.

Recently, enzyme immunoassay (EIA) has been compared with fluorescence spectroscopy [37] and HPLC [38, 39], and if a good correlation exists between EIA and EF, higher quinidine concentrations are obtained with EIA compared with HPLC, which is explained by cross-reactions of antibody with quinidine metabolites or dihydroquinidine.

The use of a specific method for quinidine assay is necessary for pharmacokinetic studies [40, 41] as well as for drug monitoring. In a steady-state situation blood concentrations of metabolites may be high and differences between non-specific (PPF, EF) and specific methods (HPLC, TLC, GLC) may be as high as 157% [42] because of co-extraction of metabolites (of the order of 60% for 3(*S*)-hydroxyquinidine, and 10% for quinidine N-oxide [22, 30].

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