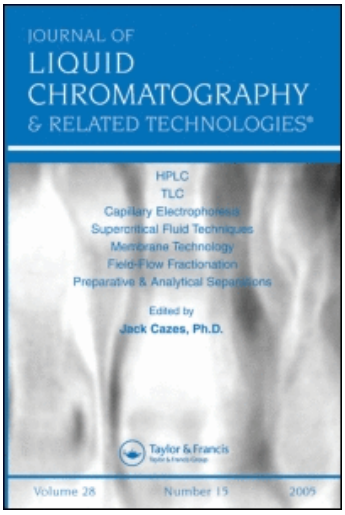

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SPECIFIC DETERMINATION OF HYDROQUINIDINE AND ITS MAJOR METABOLITES IN BIOLOGICAL FLUIDS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A specific assay have been developed for the determination of dihydroquinidine (DHQ) and its major metabolites : 11-hydroxydihydroquinidine (11-OH DHQ), 3 (S) 3-hydroxydihydroquinidine (3-OH DHQ), dihydroquinidine 1-N-oxide (DHQ N-oxide) in human urine and plasma. The assay uses a reverse phase high performance liquid chromatographic system with fluorescence detection ; the mobile phase consists of acetonitrile-acetic acid-water (7:4:89). After adding internal standard , biological samples (0.5ml) are extracted at alkaline pH by methylene chloride-isopropanol (8:2). The organic extract is dehydrated and evaporated, the residue dissolved in 150 µl mobile phase and an aliquot injected onto the column. Lower limits of sensitivity range from

1 ng of 11-OH DHQ to 15 ng of DHQ. The standard curves are linear and results are reproducible over the concentration ranges : DHQ 0.1 to 3 µg/ml, 11-OH DHQ 2.5 to 250 ng/ml, 3-OH DHQ 10 to 1000 ng/ml and DHQ N-oxide 5 to 500 ng/ml. The assays have been tested successfully in pharmacokinetic and metabolic studies of dihydroquinidine.

INTRODUCTION

Dihydroquinidine (DHQ) is an antiarrhythmic drug used in European countries as a chlorhydrate salt in a conventional tablet or a sustained release capsule (1). Commercially available quinidine preparations contain also DHQ as an impurity, Pharmacopoeias (USP or Ph. EUR.) include a limit for content of DHQ of 15 to 20% in quinidine salts.

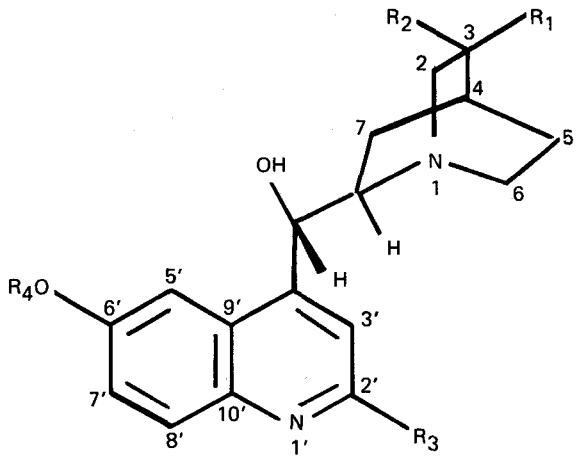
No studies have been published about DHQ metabolism in animal or in man. Measurement of DHQ alone in plasma was reported in pharmacokinetic studies of DHQ (2, 3, 4) or in determination of quinidine metabolites (5, 6, 7, 8).

In this report, a method for the separation of the main DHQ metabolites, synthesized in our laboratory and observed in treated patients plasma and urinary samples (9, 10) (figure 1), is presented, which allows plasma and urinary determinations.

MATERIALS AND METHODS

Reagents and chemicals :

Ammonium carbonate, sodium acetate, sulfuric acid, chlorhydric acid (R.P. Normapur), acetic acid (HPLC GRADE) were obtained from PROLABO. Sodium hydroxide, anhydrous sodium sulfate, isopropanol (Pro analysis grade), acetonitrile (HPLC grade) were obtained from MERCK. Methanol and methylene chloride (HPLC grade) were obtained from CARLO ERBA. Water was MilliQ (Millipore Waters).



R1	R2	R3	R4	Substances
H	CH2-CH3	H	CH3	Dihydroquinidine (DHQ)
CH2-CH2OH	H	H	CH3	11-hydroxy DHQ (M1)
OH	CH2-CH3	H	CH3	(3S)3-hydroxy DHQ (M2)
H	CH2-CH3	H	CH3	DHQ 1-N-oxide (M3)
H	CH2-CH3	H	H	O-desmethyl DHQ (M4)
H	CH2-CH3	OH ↕ C=O	CH3	2'-dihydroquinidinone (M5)
CHOH-CH3	H	H	CH3	(3S)10-hydroxy DHQ

FIGURE 1 : Structure of metabolites

Dihydroquinidine hydrochloride (DHQ) was supplied by Dr. LARCHEVEQUE, HOUDE ISH Paris. We synthesized DHQ metabolites: 11-hydroxy dihydroquinidine (11-OH DHQ or M1), (3S) 3-hydroxy dihydroquinidine ((3S) 3-OH DHQ or M2), dihydroquinidine N-oxide-1 (DHQ N-Ox or M3), O-desmethyl dihydroquinidine (M4), 2'-dihydroquinidinone (M5), (3S) 10-hydroxy dihydroquinidine (10-OH DHQ or M8) (figure 1) (9).

Internal standards were quinine sulfate (Prolabo) or cupreine (O-desmethylquinine) synthesized in our laboratory.

Drug and metabolites solutions :

A stock solution of DHQ or each metabolite was prepared at 100 µg/ml in methanol-water (20-80 v/v) and stored at +4°C. Appropriate working dilutions of DHQ and metabolites in blank plasma or urine were stored at -20°C until further dilutions for calibration.

Internal standard solutions (IS) :

A stock solution of quinine sulfate or cupreine was prepared at 100 µg/ml sulfuric acid 0.1N and stored at +4°C. A working dilution of 10 or 25 µg/ml water were prepared from the stock solutions.

Mobile phase :

The mobile phase consisted in acetonitrile-acetic acid-water (7:4:89 v/v). The solvent system was degassed by filtration through 0.45 micron Durapore filter (Millipore).

Quantitation :

Standard curves were constructed for plasma and urine at several concentrations function of dosing. For plasma determinations, after a single administration of a sustained release formulation of DHQ (Serecor°, 300 mg DHQ hydrochloride), six

concentrations were prepared ranging from 0.1 to 1 µg/ml for DHQ, from 2.5 to 50 ng/ml for M1, from 10 to 200 ng/ml for M2 and from 50 to 500 ng/ml for M3.

For drug monitoring in patients on Serecor° therapy, the standard curve concentrations ranged from 0.5 to 3 µg/ml for DHQ, from 25 to 250 ng/ml for M1, from 100 to 1000 ng/ml for M2 and from 50 to 500 ng/ml for M3.

In urine, standard curves ranged from 1.25 to 20 µg/ml for DHQ, 0.05 to 0.4 µg/ml for M1, 0.25 to 2.0 µg/ml for M2, 0.25 to 4 µg/ml for M3 and M4, and 5 to 30 µg/ml for M5.

The peak heights were measured and the ratios (drug/internal standard and metabolite/internal standard) were calculated and were plotted against concentrations in micrograms per milliliter of plasma or urine.

Recovery :

Samples (plasma or urine) containing 2.0 µg drug or metabolite/ml were carried through the analysis, after evaporation the residue was dissolved in 150 µl of an IS dilution in mobile phase and the peak heights of drug or metabolite and IS were measured. An equivalent amount of drug or metabolite was added to 150 µl of the same IS dilution in mobile phase, the peak heights of drug or metabolite and IS were measured for comparison with the extracted samples to estimate percent recovery.

Quality control samples (QC) :

Plasma and urine were spiked with known concentrations of drug or metabolite solutions prepared in methanol-water. The samples were stored at -20°C and used to determine within-run and day-to-day precision. After the samples were brought to room temperature, the samples were carried through the plasma or urine assays. The amount of drug or metabolite in the QC

samples was calculated by comparison to a standard curve prepared daily.

Instrumental conditions :

The HPLC system consisted of a Beckmann pump equipped with a Wisp autoinjector (Waters) and a stainless steel column (μ Bondapack C18, 10um, 30 cm x 4.7 mm I.D.) and a guard column (4 cm x 0.2 mm I.D.) filled with the same phase.

A Schoeffel fluorescence detector model SF 970 or a Schoeffel spectrophotometer model SF 773 was used to detect the compounds and the response was recorded on an Icap 10 integrator (Delsi Instrument).

The effluent was monitored at excitation wavelength 250 nm with a 418 nm emission cut-off filter or at 250 nm with a sensitivity of 0.002 A.U.F.S.. All separations were made at room temperature (25°C) with a flow rate of 2 ml/min.

Procedure :

Plasma assay: 0.1 ml of internal standard solution (cupreine 25 μg/ml or quinine 10 μg/ml) were added to 0.5 ml of plasma in a glass stoppered tube. Samples were alcalinized with 0.5 ml of sodium hydroxide 0.1 N, vortexed for 10sec and 10 ml of methylene chloride - isopropanol (8:2 v/v) were added. The mixture was shaken for 20 min on an alternative agitator, then centrifuged at 2,500 g for 10 min. The aqueous layer was discarded and the organic layer was dried with anhydrous sodium sulfate, centrifuged at 2,500 g for 5 min . The organic layer was transferred into 5 ml glass tube , and evaporated to dryness at 70°C under a gentle stream of nitrogen. The residue was dissolved in 150 μl of mobile phase, vortexed for 30 sec and 50 μl of the solution was injected into the column.

Urine assay: Procedure was the same as described for plasma samples but samples were alcalinized with 1 ml of ammonium carbonate saturated solution and internal standard solution was at 100 µg/ml of cupreine or quinine.

RESULTS

Figure 2 shows chromatograms of main metabolites and DHQ supplemented plasma using quinine as internal standard and two typical patient's plasmas using either quinine or cupreine as internal standard. The elution sequence (and retention time, in seconds) was : 11-hydroxy DHQ (224), (3S)3-hydroxy DHQ (270), IS (cupreine) (380), DHQ N-oxide (664), IS (quinine) (805) and DHQ (970). (3S) 10-hydroxyDHQ is a minor metabolite.

The standard curves for DHQ and metabolites are represented in table 1. The standard curves show little day-to-day variability in slopes and intercepts as well as good linearity ($r \geq 0.998$) over the concentration range studied (table 1).

Estimates of precision by within-run and day-to-day reproducibility are shown in tables 2 and 3. The limit of detection for each compound in treated sample was 15 ng for DHQ, 1 ng for 11-OH DHQ, 5 ng for (3S) 3-OH DHQ and DHQ N-oxide.

The recovery of drugs from biological samples was higher than 80% for the main metabolites (table 4).

DISCUSSION

This method is accurate and sensitive. The assay permits estimation of DHQ and all major metabolites after a single oral dose of a sustained-release formulation (300mg DHQ) ; Figure 3 illustrates the plasma concentrations profile of DHQ and its metabolites in one patient.

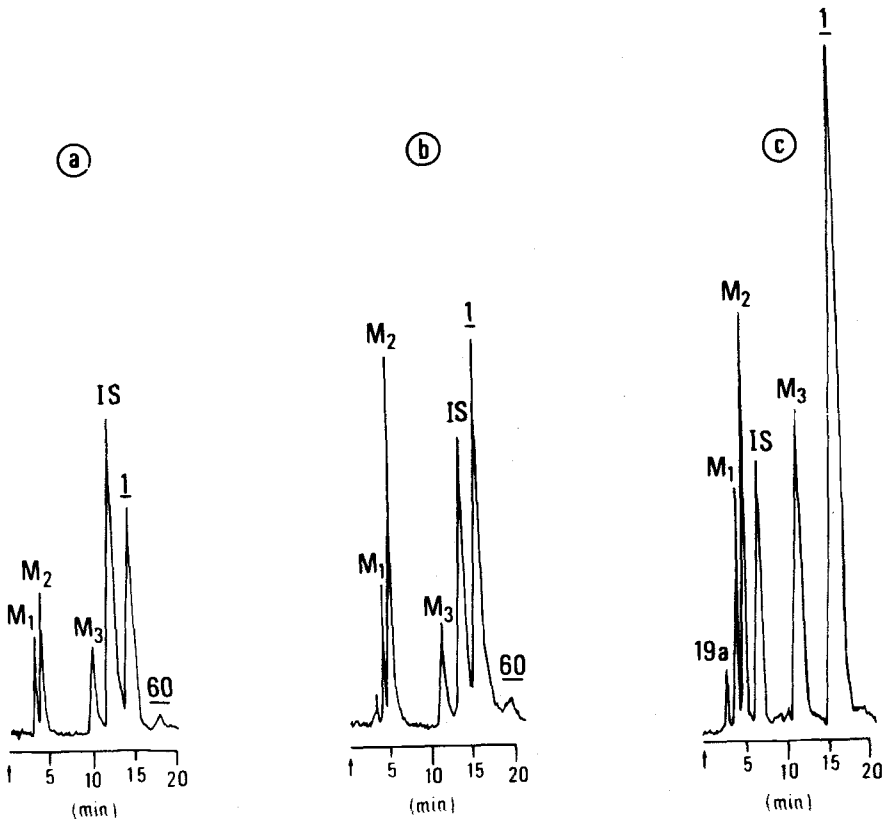


FIGURE 2 : Chromatograms of :
(a) : supplemented plasma with 11-hydroxy DHQ (M1), (3S)3-hydroxy DHQ (M_2), DHQ N-oxide (M_3), DHQ (1);
(b) and (c) : patient's plasmas ;
internal standard (IS) is quinine (a) and (b) or cupreine (c).
fluorescence detection : 250 nm, cut-off 418 nm.
60 is dihydroquinine (impurity of quinine) and 19a is (3S)10-hydroxyDHQ (M_8).

TABLE 1 : Values of the Slopes and the Intercepts of Peak Height Ratio (y) versus Concentrations (x) for Dihydroquinidine and its Metabolites in Plama (y = ax + i)

Low levels :

Substances	Slope (a)	Intercept (i)	r	n
DHQ	2.0668	0.0040	0.999	20
M_1	0.032	0.0039	0.999	11
M_2	0.0114	0.0092	0.999	11
M_3	0.0123	0.0084	0.998	11

High levels :

Substances	Slope (a)	Intercept (i)	r	n
DHQ	0.936	0.014	0.999	15
M_1	3.52×10^{-3}	0.62×10^{-3}	0.999	15
M_2	1.25×10^{-3}	6.07×10^{-3}	0.999	15
M_3	1.53×10^{-3}	0.49×10^{-3}	0.999	15

n = number of determinations

TABLE 2 : Within-run Precision of Measurements of Dihydroquinidine and its Metabolites in Plasma or Urine.

Substances	Spiked	Measured		Precision	n
		Mean	sd	CV (%)	
DHQ ($\mu g.ml^{-1}$)	0.25	0.27	0.003	1.4	10
	0.5	0.53	0.014	2.6	10
	0.70	0.75	0.005	0.7	10
	1	1.06	0.012	1.1	10
	2	1.99	0.04	2.1	10
M_1 ($ng.ml^{-1}$)	7.5	7.2	0.4	5.1	10
	30	30.9	1.2	3.7	10
	37.5	38.3	1.3	3.5	10
	100	97.9	2.8	2.8	10
M_2 ($ng.ml^{-1}$)	30	29.3	1.2	4.0	10
	120	122.0	2.9	2.4	10
	150	155.3	7.6	4.9	10
	400	388.4	13.8	3.5	10
M_3 ($ng.ml^{-1}$)	15	13.2	0.5	3.9	10
	60	58.8	1.0	1.8	10
	75	77.8	4.4	5.7	10
	200	205.5	5.4	2.6	10

TABLE 3 : Day-to-day Precision of Measurements of Dihydroquinidine and its Metabolites in Plasma or Urine.

Substances	Spiked	Measured		Precision	n
		Mean	sd	CV (%)	
DHQ ($\mu g.ml^{-1}$)	0.05	0.07	0.008	10.9	20
	0.25	0.30	0.009	3.0	20
	0.70	0.79	0.022	2.7	20
	2.5	2.55	0.075	2.9	16
M_1 ($ng.ml^{-1}$)	7.5	7.95	0.39	4.9	11
	30	32.7	0.93	2.9	11
	37.5	37.7	2.50	6.6	15
	100	97.5	4.97	5.1	15
M_2 ($ng.ml^{-1}$)	30	31	1.64	5.3	11
	120	128.8	3.8	2.9	11
	150	150.3	10.2	6.6	15
	400	397.0	11.58	2.9	15
M_3 ($ng.ml^{-1}$)	15	14.6	1.84	12.6	11
	60	63.6	3.19	5.0	11
	75	73.4	3.74	5.1	16
	200	194.0	9.89	5.1	15

Alcalinization of plasma samples by sodium hydroxide gave a good linearity and reproducibility, but in urine sample an unreproducibility in high levels of O-desmethyl DHQ was observed. Addition of a saturated ammonium carbonate solution to urine sample gave an excellent recovery of all metabolites.

TABLE 4 : Recovery (%) of Drugs Extraction in Plasma and Urine.

Substances	Plasma (%)	Urines (%)
Dihydroquinidine	95	95
Quinidine	90	88
Quinine	90	90
11-Hydroxy DHQ (M_1)	85	80
(3S)3-Hydroxy DHQ (M_2)	80	80
DHQ 1-N-oxide (M_3)	95	80
O-desméthyl DHQ (M_4)	95	95
2'-DHQone (M_5)	98	97

We investigated a number of columns to find the one that best separates DHQ and all metabolites. An "end-capping" treated stationnary phase (such as μBondapack C18) was necessary to obtain a good separation and to permit good performance during a six-mounth period.

Mobile phase was a ternary system (acetonitrile-acetic acid-water) as used by several authors for quinidine and metabolites separation (7, 11, 12). Acetic acid is necessary for quinine and quinidine separation.

Influence of acetonitrile concentrations in 4% acetic acid solution was studied on the elution of polar metabolites ;

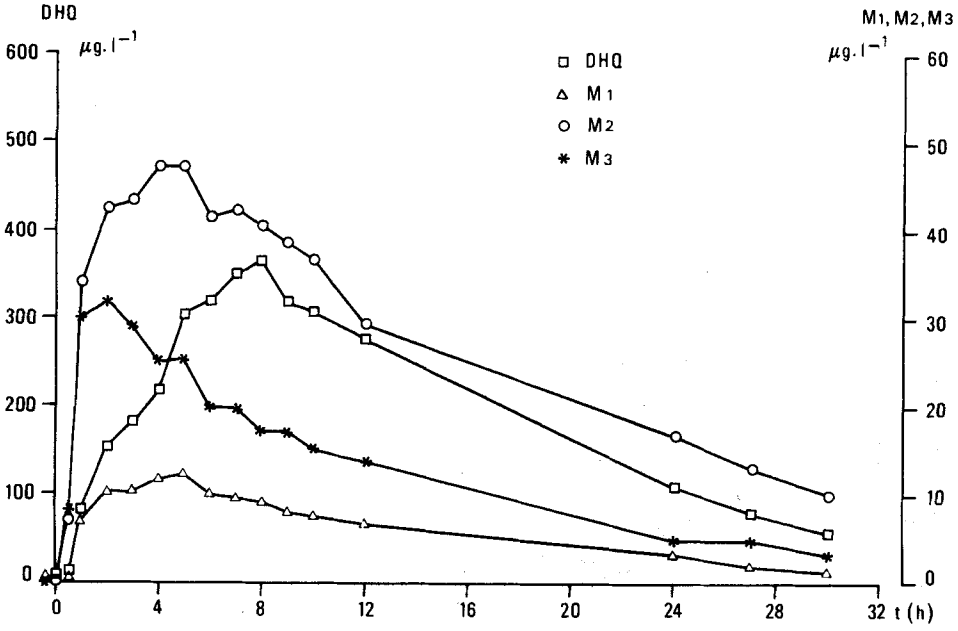


FIGURE 3 : Plasma concentrations profile of DHQ and its metabolites in one patient, after administration of a single dose of a sustained release formulation (300 mg DHQ-hydrochloride).

results are shown in figure 4 and 5, these figures shown all the metabolites observed after oral administration of DHQ (13). With fluorometer detector it is possible to measure on elution mobile phase with 7% of acetonitrile because interference peaks from biological material with short retention times were not detected ; but with a spectrophotometer it is necessary to use a 4% acetonitrile phase.

All of DHQ metabolites are highly fluorescent but two urine metabolites (O-desmethyl DHQ and 2' dihydroquinidinone) have very low fluorescence sensitivity, their detection being better with an ultraviolet detector at 250 nm.

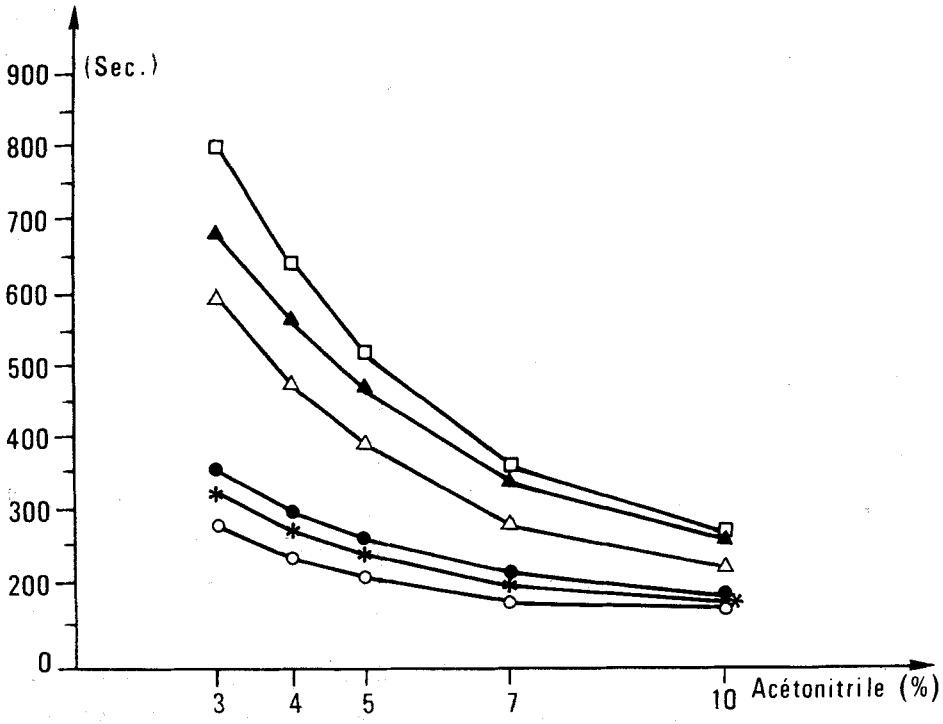


FIGURE 4 : Influence of acetonitrile concentrations on the separation of DHQ potential metabolites hydroxylated on the side chain: 10,11-dihydroxyquinidine (○ and ✳), (3S)10-hydroxy DHQ (●), 11-hydroxy-DHQ (△), (3S)3-hydroxy DHQ (▲), (3R)10-hydroxy-DHQ (□).

The assay was performed with a fluorometer (deuterium lamp), permitting excitation at 245 nm and a cutoff-filter at 418 nm, but when a fluorometer equipped with a glass xenon lamp was used, excitation was set at 350 nm and monochromator emission at 440 nm was used ; in these conditions, cupreine internal standard and O-desmethyl DHQ were undetectable.

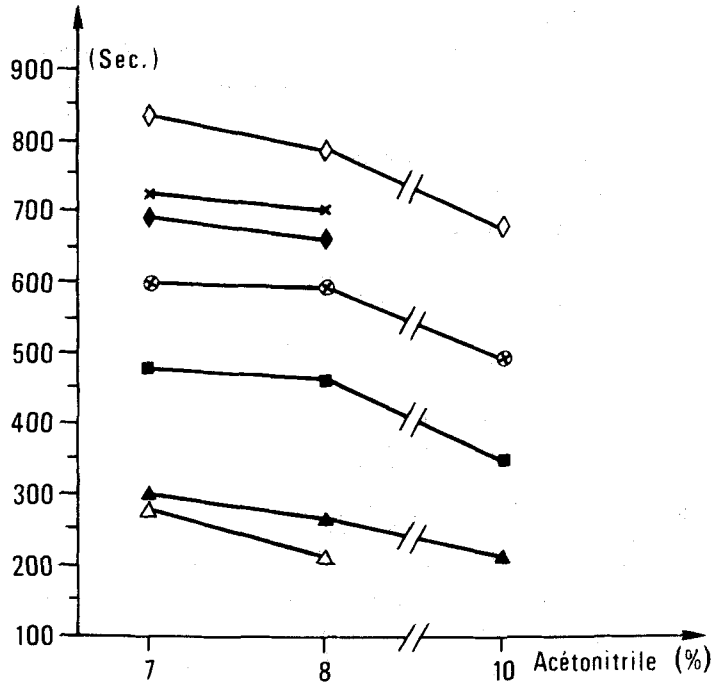


FIGURE 5 : Influence of acetonitrile concentrations on the separation of DHQ potential metabolites and quinine : 11-hydroxy DHQ (Δ), (3S)3-hydroxy DHQ (▲), O-desmethyl DHQ (■), DHQ 1-N-oxide (⊗), 9-dihydroquinidinone (♦), dihydroquinidine (◊), quinine (×).

The specificity was studied with several drugs used in cardiac treatments. No interference was observed between DHQ or its main metabolites and propranolol, acebutolol and diacetolol, atenolol, metoprolol, pindolol, oxprenolol, disopyramide and its N-dealkylmetabolite, procainamide and N-acetylprocainamide, amiodarone, prazosine, mexiletine, amiloride, furosemide, salicylic acid.

Under conditions described above, quinidine showns the same retention time as DHQ N-oxide, but in therapeutic drug monitoring it is not a real problem because these two antiarrythmics are never prescribed simultaneously. If DHQ can be an impurity of quinidine, its amounts are too low for giving a detectable concentration of DHQ N-oxide, this metabolite only exhibits 10% of DHQ plasma amounts (13).

This method is specific, sensitive, accurate and permitted the determination of pharmacokinetic studies.

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