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Simultaneous determination of diltiazem and quinidine in human plasma by liquid chromatography

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Abstract

A novel method for simultaneous determination of diltiazem and quinidine in human plasma is described. Plasma is alkalinized and extracted with methyl *tert.*-butyl ether. The ether phase is separated and evaporated. The residue is reconstituted in 0.2 ml of mobile phase containing 56 m*M* octanesulfonic acid then washed twice with *n*-hexane. Aliquots are chromatographed on a silanol-deactivated reversed-phase column using a mobile phase containing aqueous H_2SO_4 (0.01 *M*, pH 2)–methanol–acetonitrile (45:45:10) and 10 m*M* octanesulfonic acid. Peaks are monitored with a UV detector set at 237 nm and a fluorescence detector using an excitation set at 247 nm and a 270 nm UV cut-off filter at the emission. Calibration and standard curves were linear from 1 to 130 ng on-column for diltiazem and from 2 to 600 ng on-column for quinidine. Limits of quantitation were 2 and 4 ng/ml for diltiazem and quinidine, respectively. Recoveries from spiked plasma were 94.0 to 102.5% (R.S.D. 6.0–11.4%) for diltiazem and 98.5% to 104.1 (R.S.D. 7.7–8.7%) for quinidine over the ranges studied. In vitro stability was studied in spiked plasma samples stored at −80°C for sixteen months. Both diltiazem and quinidine remained within 10% from nominal values. For ex vivo stability at −80°C, a plasma sample obtained from a volunteer 2 h after oral administration of diltiazem (60 mg) was analysed for two days after sampling and eighteen months later. The mean deviation from initial measured was 4.7%.

1. Introduction

The combination of a calcium channel blocker and an antiarrhythmic agent is widely used in the management of ischemic heart disease and arrhythmia. Quinidine is currently the most prescribed antiarrhythmic agent for the control of arrhythmias [1]. Diltiazem is frequently used in coronary patients for angina and hypertension [2]. Both are extensively metabolized through oxidation and demethylation (Figs. 1 and 2). A recent study has suggested that quinidine impairs the clearance of drugs by inhibiting their metabolism [3]. Similar drug–drug interactions have also been reported for diltiazem [4]. Because both drugs are metabolized through oxidative P450 3A4 drug-metabolizing enzymes, it is possible that a drug–drug interaction may occur. The inhibition of metabolism of a drug results in an increased level of the parent compound which could accumulate and cause toxicity. Because the therapeutic indices of cardiac drugs are low, such interactions are of great concern to physicians. This drug combination is common in patients with multiple cardiovascular diseases.

Quinidine has been quantified in plasma by

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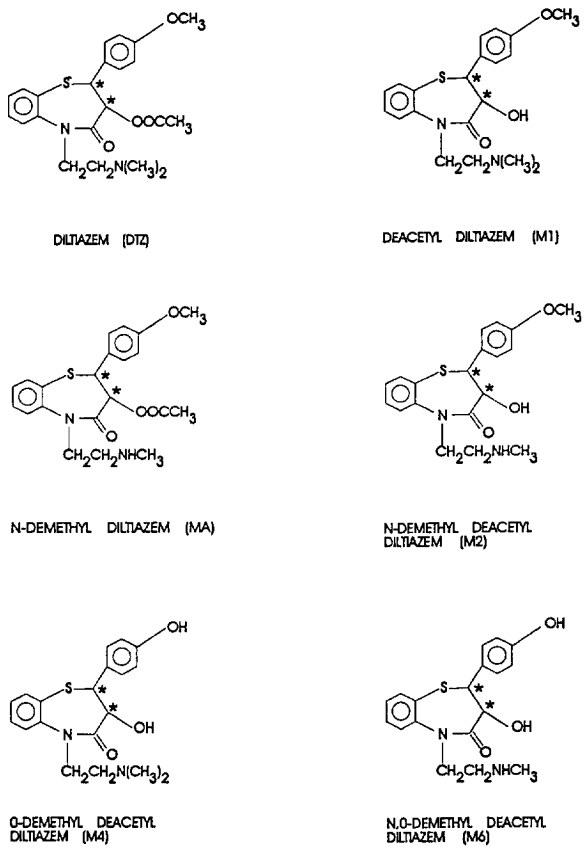

Fig. 1. Structures of diltiazem and metabolites. Chiral carbons are identified by an asterisk.

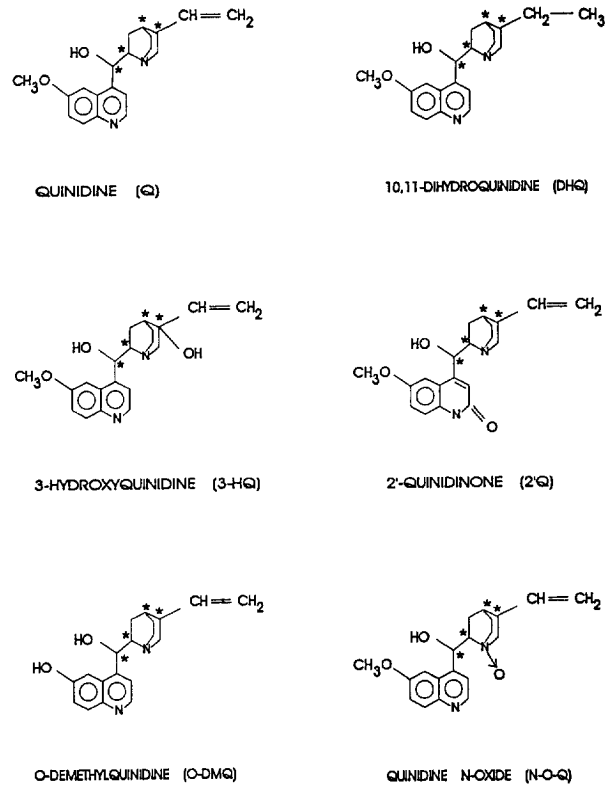

Fig. 2. Structures of quinidine and related compounds. Chiral carbons are identified by an asterisk.

fluorescence [5], enzyme immunoassay [6] and more recently HPLC coupled with UV or fluorescence detection [7–14]. Diltiazem has also been assayed using HPLC with UV detection [15–19]. The determination of both drugs in presence of each other is difficult as their respective polarity is very different and also because both drugs are highly metabolized (Figs. 1 and 2). Although both compounds exhibit asymmetric carbons, the chirality aspect was not considered in the development of this analytical method, as they are marketed as pure enantiomer. We decided to combine the specificity of the fluorescence detector for the detection of quinidine-related compounds and the addition of an ion-pairing agent to the mobile phase for the separation, to develop a quantitative analytical method for the determination of both drugs simultaneously.

2. Experimental

2.1. Reagents and chemicals

Diltiazem hydrochloride and its metabolites deacetyldiltiazem (M1), N-demethyldiltiazem (MA), N-demethyldeacetyldiltiazem (M2), O-demethyldeacetyldiltiazem (M4), N,O-demethyldeacetyldiltiazem (M6) were kindly supplied by Nordic Labs. (Laval, Canada). Quinidine and 10,11-dihydroquinidine (QHQ) were purchased from Aldrich (Milwaukee, WI, USA), whereas 3*S*-hydroxyquinidine (3-HQ) and 2′-quinidinone (2′-Q) were kindly provided by Berlex Labs. (Cedar Knolls, NJ, USA). The internal standard (I.S.) (4-methylpropranolol hydrochloride) was generously given by Wyeth-Ayerst Research (Rouses Point, NY, USA). Diltiazem, quinidine and 4-methylpropranolol were characterised in our laboratory using spec-

trometric (MS) and spectroscopic techniques (NMR and IR).

Acetonitrile, methanol, methyl *tert.*-butyl ether (Baxter, Muskegon, MI, USA) and hexane (J.T. Baker, Phillipsburg, NJ, USA) were HPLC grade. Sulfuric acid (BDH, Toronto, Canada), dipotassium hydrogenphosphate (Fisher Scientific, Fair Lawn, NJ, USA) and octanesulfonic acid sodium salt (OSA, Sigma, St. Louis, MO, USA) were reagent grade. Deionized water was purified by a Milli Q-4 system (Millipore, Bedford, MA, USA).

Methyl *tert.*-butyl ether was stored over activated charcoal and filtered immediately before use (Whatman No. 2v). Hexane was freshly purified by continuous stirring overnight four volumes with one volume of concentrated sulfuric acid, then washing with one volume of water twice. The purification of these organic solvents was necessary to improve sensitivity and reduce interference.

The reconstituting solvent consisted of a mixture of aqueous H_2SO_4 solution (0.01 *M*, pH 2)–methanol–acetonitrile (45:45:10) containing 56 m*M* octanesulfonic acid sodium salt.

Outdated frozen human plasma in a bag of 250 ml was obtained from the Canadian Red Cross (Ottawa, Canada).

2.2. Apparatus and chromatographic conditions

The liquid chromatograph consisted of a Spectroflow 400 pump (ABI Analytical, Ramsey, NJ, USA), a SP8780XR autosampler (Spectra-Physics, San Jose, CA, USA) equipped with a 200-μl loop, a Suplex pKb-100 HPLC analytical column, 150 × 4.6 mmI.D., 5 μm particle size, protected by a Suplex pkb-100 guard column, 20 × 4.6 mm I.D., 5 μm particle size (Supelco, Bellafonte, PA, USA), a Spectroflow 783 UV–Vis variable-wavelength detector (ABI Analytical) set at 237 nm, linked to an HPLC-Plus FL-750 fluorometer (McPherson, Acton, MA, USA) using an excitation wavelength of 247 nm produced by a xenon arc lamp and a 270-nm UV cut-off filter at the emission. Axxiom Chromatography Model 727 software (Calabasas, CA, USA) was used for data acquisition and integration.

The mobile phase consisted of a mixture of aqueous H_2SO_4 solution (0.01 *M*, pH 2)–methanol–acetonitrile (45:45:10) containing 10 m*M* octanesulfonic acid sodium salt and was filtered through a 0.2-μm Ultipor N_{66} membrane (Pall Trinity Micro, Cortland, NY, USA). The flow-rate was set at 1.0 ml/min.

2.3. Preparation of standards

Diltiazem and quinidine standard stock solutions were prepared separately at a concentration of 1 mg/ml base equivalent and the I.S. stock solution at a concentration of 20 μg/ml base equivalent. All methanolic stock solutions were stored at −30°C for a maximum of two months. A series of working standard solutions containing diltiazem, quinidine and I.S. (Table 1) were prepared weekly in reconstituting solvent. The solutions were used for the preparation of standard curve samples.

2.4. Quality control standard

Stock quality control samples were prepared by spiking human plasma with diltiazem and quinidine and subdividing into 1-ml aliquots. Quality control samples were analysed blindly on each analytical day in duplicate at three con-

Table 1
Standard solutions containing diltiazem and quinidine used to prepare standard curve samples

Standard solution	Diltiazem (ng/ml)	Quinidine (ng/ml)	I.S. (ng/ml)
1	–	–	500
2	10	6000	500
3	25	3000	500
4	50	1500	500
5	100	750	500
6	250	300	500
7	500	100	500
8	750	50	500
9	1250	20	500

Diluted in reconstituting solvent.

centrations. These concentrations, unknown to the analyst prior to assay, were about 2.5, 10, 55 and 5, 120, 880 ng/ml for diltiazem and quinidine, respectively.

2.5. Biological samples

Outdated frozen plasma was used for method validation, standard curve samples and quality control samples. The plasma was thawed at room temperature, transferred (1.0 ml) into 15-ml PTFE-lined screw-cap glass disposable culture tubes and refrozen at −80°C. Volunteer plasma samples obtained at the Ottawa Heart Institute (Ottawa Civic Hospital, Ottawa, Canada) were stored at −80°C until analysed.

2.6. Sample preparation

To a 1.0-ml aliquot of plasma were added 200 μl of the working standard containing only the I.S. (1, Table 1) and 1.0 ml of aqueous K_2HPO_4 (0.2 *M*, adjusted to pH 10 with 5 *M* KOH) solution and briefly mixed. Methyl *tert.*-butyl ether (5 ml) was added. The tube was capped and vigorously shaken for 10 min, then centrifuged for 10 min at 2500 *g*. A 4-ml aliquot of the upper organic phase was transferred to another culture tube and evaporated to dryness using a vortex evaporator at 30°C. Freshly purified hexane (2 ml) and 200 μl of reconstituting solvent were added, and the tube was vortex-mixed for 2 min. Most of the upper hexane layer was discarded. The aqueous phase was washed a second time with 2 ml of purified hexane. The aqueous phase was transferred into an autosampler vial, and a 100-μl aliquot was chromatographed.

2.7. Standard curve preparation

Standard curve samples were prepared by spiking 1 ml of blank plasma with 200 μl of working standard solutions containing diltiazem, quinidine and I.S. Samples were extracted as described above.

The standard curve was analyzed using the linear least-squares regression equation derived from the ratios of peak height of diltiazem or quinidine to the I.S. against concentrations using a weighting correction factor of 1/concentration^2. Concentrations of diltiazem and quinidine in samples were calculated from this regression analysis.

3. Results and discussion

A literature survey indicated that quinidine is eluted much faster than diltiazem when submitted to reversed-phase HPLC owing to more polar functional groups such as hydroxyl and aromatic nitrogen (Figs. 1 and 2). The addition of an ion-pairing agent such as octanesulfonic acid to the mobile phase complexes with ionic compounds and affects their elution on reversed-phase chromatography. The extent of this effect depends on the number, the steric hindrance and the polarity of functional groups present on the molecule. Fig. 3 shows the effect of addition of OSA on the relative retention of diltiazem to quinidine. As a result of this addition, quinidine and diltiazem exhibit similar chromatographic behaviour, with retention times being sufficiently close to allow their simultaneous HPLC determination. Conditions described in this method permit the elution of diltiazem well resolved

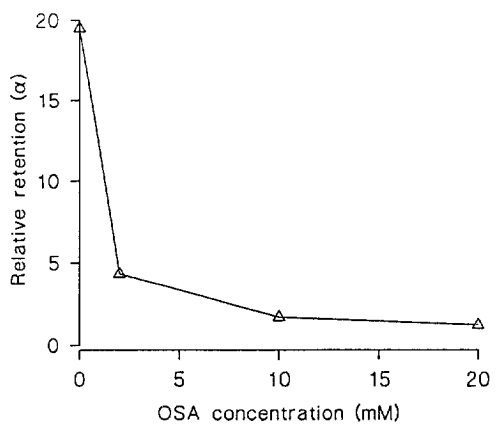

Fig. 3. Relative retention of diltiazem to quinidine ($\alpha = k'_{DTZ}/k'_{Q}$) versus octanesulfonic acid concentration added to the mobile phase, aqueous 0.01 *M* H_2SO_4 (pH 2.0)–methanol–acetonitrile (45:45:10).

from any metabolites and plasma endogenous interferences. The metabolites M1 and M2 were not completely resolved from quinidine and DHQ. However, the selectivity of fluorescence allowed the detection of all quinidine-related compounds without interference from either plasma constituents or diltiazem metabolites (Fig. 5). Figs. 4 and 5 show chromatograms from standard solution, pre-dosed and 3-h plasma samples. The retention time of 2'-Q, M4, M6, 3-HQ, quinidine, M1, DHQ, M2, diltiazem, MA and I.S. are 4.1, 5.2, 5.8, 6.3, 7.5, 7.9, 8.4, 9.1, 10.3, 11.6 and 17.1 min, respectively. An unknown peak at 13.1 min was also present in all UV-detected chromatograms.

Diltiazem was best detected using UV set at its maximum UV absorption at 237 nm. The natural fluorescence of quinidine in acidic aqueous solution was best detected using settings at 247 nm excitation wavelength and using a UV cut-off filter (CF-270) at the emission.

The 4-methyl derivative of propranolol was chosen as I.S. because its retention time was relatively close to that of diltiazem within a window free of interferences, and it showed both good UV absorption and fluorescence with the above detector settings. It was also quantitatively recovered using the above extraction procedure.

The extraction of diltiazem and quinidine was achieved by shaking buffered (K_2HPO_4, adjusted to pH 10 with 5 *M* KOH) plasma with methyl *tert.*-butyl ether. The ether phase was separated and evaporated to dryness. The residue was partitioned between *n*-hexane and reconstituting solvent. A second *n*-hexane wash was necessary to remove all endogenous interferences. Cleaner chromatograms from plasma were obtained when the methyl *tert.*-butyl ether and purified *n*-hexane were stored over activated charcoal and sulfuric acid, respectively.

In order to evaluate method performance on a daily basis, the quality control samples were analyzed in duplicate with each sample run. Samples were enclosed between two standard curves before and after the sample run, while the quality control samples were randomly inserted between the samples. On some occasion a calibration curve was also run ahead of the first standard curve.

Calibration and standard curves were linear ($r > 0.999$) over ranges of 1–130 and 2–600 ng per injection for diltiazem and quinidine, respec-

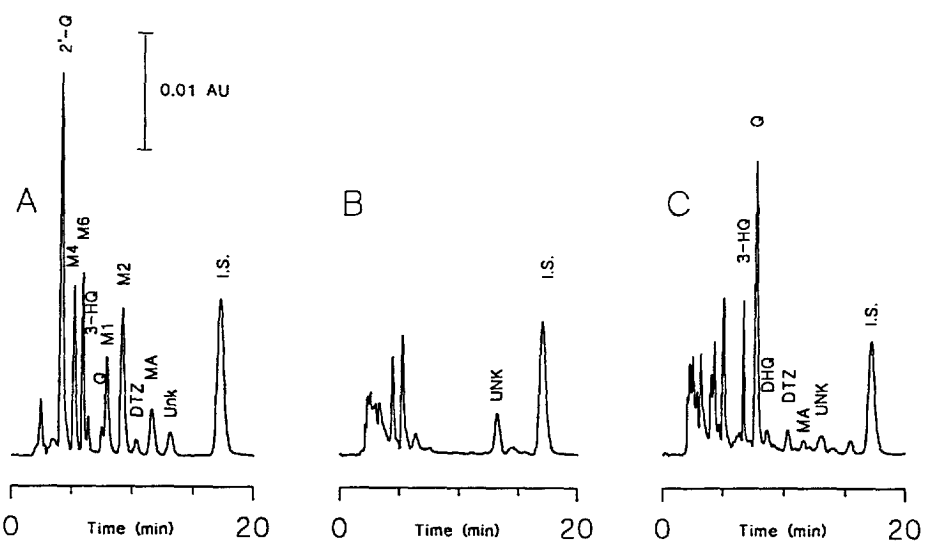

Fig. 4. Typical chromatograms using a UV detector set at 237 nm for diltiazem and quinidine determination in human plasma. (A) Mixed standard solution; (B) pre-dose human plasma; (C) 3-h sample from a volunteer given a 60-mg dose of diltiazem and a 100-mg dose of quinidine (20.6 ng/ml diltiazem, 301.0 ng/ml quinidine).

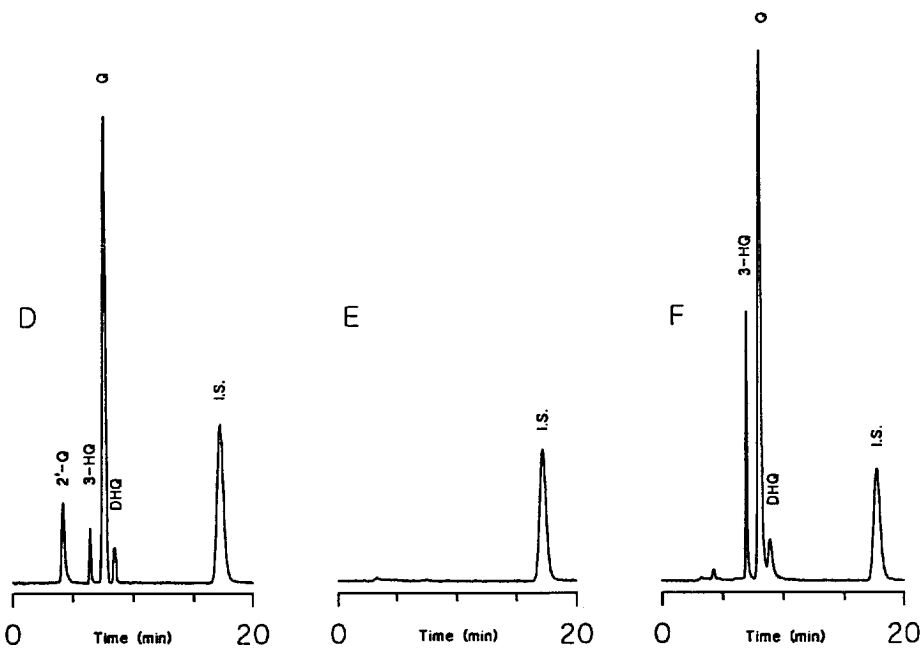

Fig. 5. Typical chromatograms using a fluorescence detector with excitation set at 247 and a 270-nm UV emission cut-off filter. (D) Mixed standard solution; (E) pre-dose human plasma; (F) 3-h sample from a volunteer given a 60-mg dose of diltiazem and a 100-mg dose of quinidine (20.6 ng/ml diltiazem, 301.0 ng/ml quinidine).

tively, corresponding to 2–260 and 4–1200 ng/ml of plasma when a 1-ml aliquot was taken. Limits of quantitation in plasma were established at 2 ng/ml for diltiazem and 4 ng/ml for quinidine. At these concentrations the R.S.D.s were less than 11.4%. A weighting factor of 1/concentration^2 gave the smallest concentration deviations from nominal over the standard curve range. All were found close to their nominal concentration with R.S.D.s less than 15%.

Absolute recoveries of diltiazem, quinidine and I.S. were assessed by direct comparison of peak height from extracted spiked plasma samples to corresponding standard solutions. Homogeneous recoveries were obtained over the range studied (2–260 and 4–1250 ng/ml for diltiazem and quinidine, respectively) (Table 2). Average recoveries were 96.9% (±6.5) for diltiazem, 96.9% (±8.6) for quinidine. Recovery of the internal standard added at a concentration of 100 ng/ml was 95.5 ± 4.7%.

Concentrations, recoveries, within-day and between-day R.S.D.s from quality control samples are summarized in Table 3. Accuracy ranged from 94 to 102.5% for diltiazem and from 98.5 to 104% for quinidine with within-day and between-day R.S.D.s better than 11.4%.

Sample extracts were stable for at least three days at room temperature. This exceeded significantly the time required for a normal analytical run (16 h). In an extension of Yeung et al. [20] reporting no significant change of diltiazem in plasma when stored at −70°C for two months, the stability of diltiazem and quinidine in frozen plasma at −80°C were assessed in spiked plasma (Table 4) and plasma from one volunteer who received a single 60-mg dose of diltiazem and from twelve volunteers who received concomitant doses of 100 mg quinidine and 60 mg diltiazem (Table 5). Clinical samples were analysed singly on each analysis day. For spiked plasma, quality control samples (diltiazem 10.5 and 57.8 ng/ml; quinidine 122.8 and 883.9 ng/ml) were assayed during the first month of preparation and sixteen months later, representing the time period from initial storage of clinical sam-

Table 2
Absolute recoveries

Diltiazem				Quinidine			
Amount added (ng)	*n*	Average recovery (%)	R.S.D. (%)	Amount added (ng)	*n*	Average recovery (%)	R.S.D. (%)
2.2	9	96.5	8.2	4.0	10	98.2	9.6
5.4	10	97.4	10.8	10.0	10	104.8	8.1
10.8	10	97.6	5.6	20.0	10	97.0	7.3
21.5	10	97.4	4.9	60.0	10	95.7	6.2
53.8	10	96.2	5.0	150.0	10	93.8	7.9
107.5	10	98.9	4.8	300.0	10	96.3	9.4
161.3	10	96.0	7.4	450.0	10	93.9	9.6
268.8	10	95.6	3.8	1000.0	10	95.6	6.9
Mean	79	97.0	6.4	Mean	80	96.9	8.6

Table 3
Precision and accuracy for the diltiazem and quinidine assay in spiked human plasma

Amount added (ng/ml)	Number of days[a]	Amount found (ng/ml)	Accuracy (%)	Within-day variation[b] (%)	Between-day variation[b] (%)
Diltiazem					
2.6	10	2.5	94.0	10.4	11.4
10.5	12	10.1	96.0	3.2	8.6
42.4	9	42.7	100.8	2.4	4.7
58.3	12	59.7	102.5	3.9	5.9
Quinidine					
5.1	11	5.3	104.1	6.4	8.5
122.8	12	121.3	100.4	3.5	7.7
883.9	12	870.6	98.5	6.3	8.8

[a] Duplicate extractions each day.
[b] Within-day (repeatability) and between-day (reproducibility) R.S.D.s were calculated using analysis of variance.

Table 4
Stability of diltiazem and quinidine in spiked plasma samples stored at −80°C

Storage (month)	*n*	Concentration (ng/ml)			
		Diltiazem		Quinidine	
		Added	Measured (mean ± S.D.)	Added	Measured (mean ± S.D.)
0	13	10.5	10.6 ± 0.9	883.9	921.3 ± 36.4
16	5	10.5	10.8 ± 0.9	883.9	847.4 ± 35.9
0	13	57.8	56.9 ± 3.1	122.8	121.8 ± 9.3
16	5	57.8	62.1 ± 4.1	122.8	122.3 ± 9.7

Table 5
Stability of diltiazem and quinidine in ex vivo plasma samples obtained from volunteers 2 and 10 h after drug administration stored at −80°C for ten and thirty-one months

Volunteer	Concentration (ng/ml)							
	Diltiazem				Quinidine			
	2 hours		10 hours		2 hours		10 hours	
	10 months	31 months	10 months	31 months	10 months	31 months	10 months	31 months
1	61.7	72.3	12.1	13.4	586.2	603.5	262.6	296.0
2	17.8	17.1	13.8	11.6	437.3	420.9	239.3	238.9
3	38.3	38.9	13.8	13.6	554.2	562.1	245.2	273.4
4	35.3	41.0	6.6	8.5	340.5	314.4	151.3	170.1
5	19.1	21.4	9.0	9.2	264.0	275.6	127.2	119.2
6	26.8	36.4	4.4	6.5	237.5	275.5	116.7	129.4
7	22.1	27.2	12.0	9.8	380.5	368.6	205.7	142.3
8	49.1	52.5	10.2	17.0	567.4	514.9	196.6	262.4
9	40.7	46.3	5.8	7.4	413.4	377.8	197.6	178.3
10	31.4	36.7	7.6	9.6	603.7	633.0	218.6	240.7
11	80.4	84.6	19.9	21.9	734.8	745.1	418.2	418.5
12	26.1	23.7	6.9	8.3	525.8	538.5	208.9	209.6
Avg	37.4	41.5[a]	10.2	11.4[b]	470.4	469.2	215.7	223.2
±S.D.	18.6	20.3	4.4	4.5	149.8	152.6	78.2	84.9

[a] $p = 0.0085$.
[b] $p = 0.046$ compared to mean values determined at ten months.

ples until processing for analysis. Diltiazem and quinidine mean recoveries remained within 10% from nominal values over the sixteen-month time period, showing no significant changes for the two spiked concentrations. For diltiazem in plasma obtained from a volunteer, a sample collected 2 h after administration of diltiazem (60 mg) was analyzed two days after sampling and eighteeen months later resulting in 27.7 ± 0.8 and 29.0 ± 3.0 ng/ml. The mean deviation from initial measured was 4.7%. Similarly, plasma samples from twelve volunteers receiving concomitant doses of 100 mg quinidine and 60 mg diltiazem were assayed ten and thirty-one months after initial blood draw. Two plasma samples were obtained 2 and 10 h after dosing from each volunteer. Paired *t*-test analysis using the SAS program showed a significant increase of the mean diltiazem concentrations in the two sets of samples. At the 2-h sampling time, the mean deviation from initial measured was 11.3 (S.D. ± 12.2, 90% confidence limit 4.5–17.2). At the 10-h sampling time, the mean deviation was 17.1 (S.D. ± 24.8, 90% confidence limit 2.8–28.0). As a possible explanation for this increase, it is worth noting that the ten-month values were obtained from twelve different runs spread over a month, while the thirty-one-month values were obtained from two runs on two consecutive days. For quinidine there was no significant change in concentration in plasma from the twelve volunteers over the same time period. Mean deviations of −0.37 (S.D. ± 7.0, 90% confidence limit −3.3 to 3.5) and 1.26 (S.D. ± 17.3, 90% confidence limit −5.7 to 11.5) were calculated from the 2-h and the 10-h time point samples, respectively. In summary, diltiazem plasma samples (spiked and ex vivo) were found to be stable for at least sixteen and eighteen months, respectively. The significant increase in diltiazem concentration resulting from extended storage at −80°C (from month 10 to month 31 after sampling) were slightly exceeding the generally accepted stability limit of ± 10% (11.0 and 11.8% for the two sets of sam-

ples). Similarly, in vitro and ex vivo quinidine samples were found to be stable for at least sixteen and thirty-one months, respectively.

This method is fast and accurate. It was used for pharmacokinetic evaluation of both drugs administered alone and in combination.

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