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Short Communication

Simultaneous assay of propranolol, diltiazem and metabolites of diltiazem in human plasma by liquid chromatography

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Keywords: *Diltiazem; diltiazem-metabolites; propranolol; HPLC-assay; plasma concentrations; pharmacokinetic interaction.*

Introduction

Diltiazem, a calcium-channel blocking agent, has been shown to be a potent coronary vasodilator, and is used in the treatment of various forms of angina [1]. Calcium-channel blockers are sometimes administered in combination with β-adrenergic blocking agents. However, this combination may result in a variety of side effects [2]. The nature of these side effects is such that it is difficult to determine which drug is causing the adverse reaction. Although an additive pharmacologic action of the drugs has been proposed, it is also possible that an important pharmacokinetic interaction is responsible for the development of these effects [3, 4]. Diltiazem and propranolol are both known to change liver blood flow. Both drugs are metabolized by the liver with a high extraction ratio [3, 6]. Propranolol reduces whereas diltiazem increases hepatic blood flow [5, 6]. Therefore, alterations in hepatic blood flow could lead to significant changes in the pharmacokinetics of both drugs when administered concurrently.

To determine pharmacokinetic interactions between propranolol and diltiazem, a selective and sensitive method is required which allows the simultaneous quantitation of diltiazem, diltiazem-metabolites and propranolol in human plasma. Deacetyldiltiazem shows a pharmacological activity of about 50% that of the parent drug [7]. The chemical structure of the parent drug and its major metabolites are shown in Fig. 1. Several methods have been described for monitoring diltiazem and its metabolites [8, 9] as well as propranolol [10, 11] in human plasma, but a method to analyse diltiazem and diltiazem-metabolites in the presence of propranolol has not been published.

Experimental

Materials

All solvents and reagents were of analytical grade except acetonitrile and methanol which were HPLC grade. The following chemicals were used: acetonitrile, methanol, *n*-butanol, hexane, boric acid, phosphoric acid 85% (g/g), ammonium chloride, triethylamine and tetrabutylammonium hydroxide (Fisher Scientific Co., Fairlawn, NJ), diltiazem, deacetyldiltiazem and *N*-demethyldiltiazem (Marion Laboratories, Kansas City, MO), 1-octane sulphonic acid sodium salt (Eastman Kodak Company, Rochester, NY), (±)-propranolol HCl and imipramine HCl (Sigma, St. Louis, MO).

Instrumentation

The following instruments were used for the HPLC assay: a high-pressure, constant volume

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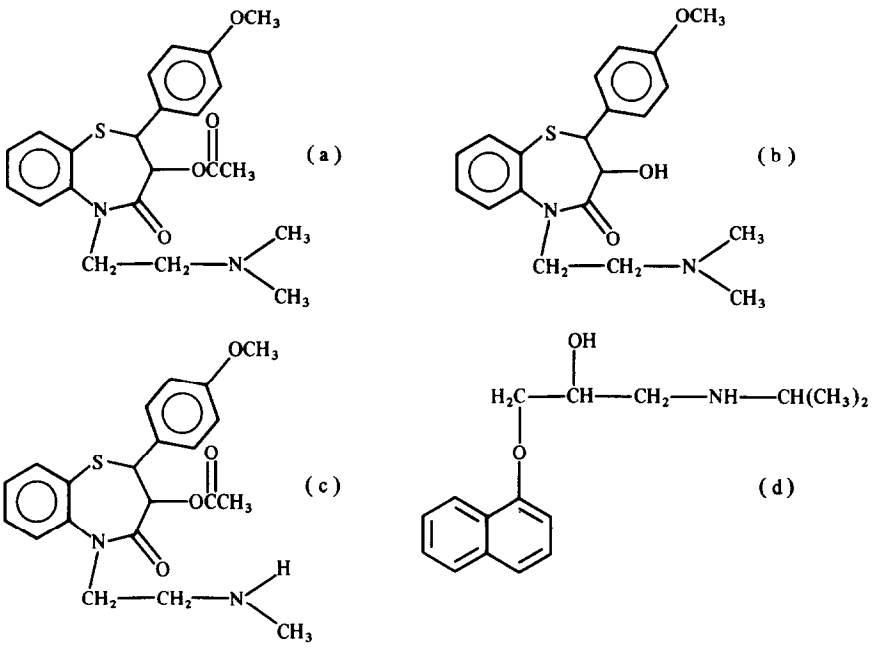

Figure 1
Chemical structures of diltiazem (a), its major metabolites: deacetyldiltiazem (b) and *N*-demethyldiltiazem (c) and propranolol (d).

pump (Constametric II, LDC/Milton Roy, Riviera Beach, FL), a variable wavelength UV detector (SpectroMonitor D, LDC/Milton Roy), an integrator (model 3390 A, Hewlett-Packard, Avondale, PA), an autosampler (Perkin Elmer ISS-100, Perkin Elmer Corp., Norwalk, CT), a Nucleosil C_{18} column (5 μm; 150 × 4.6 mm) (Chromtec, Lake Worth, FL), a pre-column (Du Pont 5 cm × 4.5 mm i.d. packed with Zorbax® ODS Chromatography Packing, E.I. Du Pont De Nemours, Wilmington, DE). A linear, mechanical shaker (Eberbach, Ann Arbor, MI) and a laboratory centrifuge (Dynac II, Clay Adams) were used for the extraction.

Chromatographic conditions

Each sample was analysed by liquid chromatography using a Nucleosil C_{18} column and UV-detection at 238 nm and at 295 nm. The mobile phase consisted of acetonitrile–methanol–ammonium chloride (0.04 M)–triethylamine (24:40:36:0.08, v/v/v/v). After the four mobile-phase components were mixed, phosphoric acid (85%, g/g) was used to adjust the pH* to 6.9. The flow rate was 1.0 ml min^{-1} and imipramine used as internal standard.

Sample preparation

Plasma, 1 ml, was alkalinized with 0.6 ml borate buffer (0.1 M, pH 8.9) and 0.5 g sodium chloride, 50 μl imipramine HCl (10 μg ml^{-1}) and 6 ml hexane–*n*-butanol (96:4, v/v) were then added. The tubes were shaken for 10 min and then centrifuged for 10 min at 1500*g*. The organic phase was removed and back-extracted with 180 μl sulphuric acid (5 mM). After shaking (10 min) and centrifugation (10 min, 1500*g*) 20 μl of the acidic aqueous phase were analysed by LC.

Assay validation

Calibration curves were derived from pooled human plasma by adding appropriate amounts of a mixture of diltiazem, deacetyldiltiazem, *N*-demethyldiltiazem and propranolol in acetonitrile–water (33:67, v/v) and 50 μl of internal standard solution (10 μg ml^{-1}). Calibration curves were obtained by plotting peak-height ratios of drug to the internal standard versus concentration of the drug. (The use of area measurements gave equivalent results.) The concentration ranges of the calibration curves were 5–200 ng ml^{-1} for diltiazem, 5–100 ng ml^{-1} for deacetyldiltiazem, 10–200 ng ml^{-1} for *N*-demethyldiltiazem and 5–100 ng ml^{-1} for propranolol. The intra- and interday variability (as the RSD values) were evaluated by repetitive analysis of spiked human plasma samples at different drug concentrations. For

the interday variability these spiked plasma samples were analysed every other day over two weeks.

Application

One healthy male subject received 60 mg diltiazem (Cardizem®) and 40 mg propranolol (Inderal®) four times a day for three days. On the fourth day only one dose of the drug combination was administered and blood samples were obtained 0, 0.5, 1, 2, 3, 4, 6, 8, 12 and 24 h after dosing. The blood was centrifuged and the plasma was frozen at −20°C until assayed.

Results and Discussion

Extraction

Diltiazem and its metabolites (pK_a 7.7) as well as propranolol (pK_a 9.4) are lipophilic weak bases, which could be extracted simultaneously from alkalinized plasma (pH 8.9 borate buffer). With hexane–*n*-butanol (96:4, v/v) as the extraction solvent in high extraction efficiency was achieved, but an additional clean-up step was necessary because of background interferences observed during chromatographic analysis. Dilute sulphuric acid (5 mM) was used to back-extract propranolol, diltiazem and diltiazem metabolites from the organic solvent phase. Addition of sodium chloride to the plasma increased the extraction efficiency to 90% for *N*-demethyldiltiazem and 95% for deacetyldiltiazem, diltiazem, propranolol and imipramine. Diltiazem and its metabolites are not stable below a pH of 2 or above a pH of 9 [8], and pH values outside this range should be avoided during the extraction process.

LC assay development

After the back-extraction, two 20 µl injections of the samples were analysed by LC. During the first run the eluent was monitored at a wavelength of 238 nm, where all four compounds and the internal standard could be detected (Fig. 2). In plasma samples from patients receiving diltiazem alone, an unidentified diltiazem metabolite was observed, which had the same retention time as propranolol. It was not possible to separate this peak chromatographically from propranolol. The unknown metabolite had no UV absorbance at 295 nm (Fig. 3), therefore, quantitation of propranolol was accomplished at 295 nm (Fig. 4). This

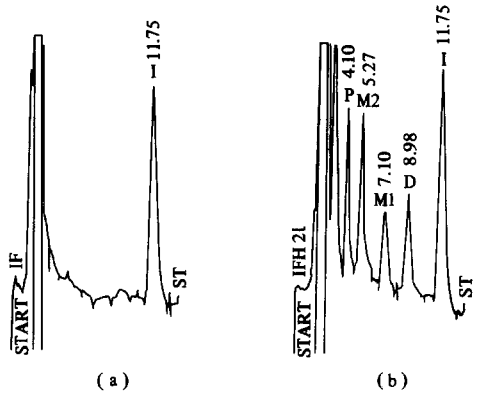

Figure 2
Chromatograms after extraction of (a) blank plasma and (b) spiked plasma (P = propranolol, M1 = deacetyldiltiazem, M2 = *N*-demethyldiltiazem, D = diltiazem, I = internal standard).

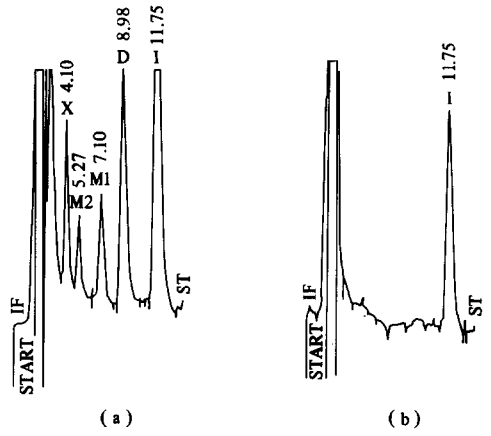

Figure 3
Chromatograms after extraction of plasma from a patient, who received only diltiazem at detection wavelength (a) 238 nm and (b) 295 nm (X = unidentified metabolite, M1 = deacetyldiltiazem, M2 = *N*-demethyldiltiazem, D = diltiazem, I = internal standard).

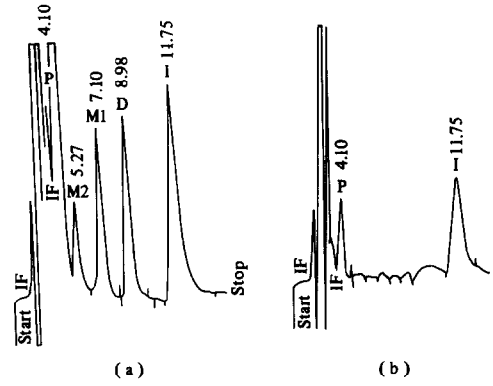

Figure 4
Chromatograms after extraction of plasma from a patient, who received diltiazem and propranolol at detection wavelength (a) 238 nm and (b) 295 nm (P = propranolol, M1 = deacetyldiltiazem, M2 = *N*-demethyldiltiazem, D = diltiazem, I = internal standard).

Table 1
Intraday variability

Actual concentration (ng ml^{-1})	Assayed concentration (mean ± SD) (ng ml^{-1})	*n*	RSD (%)
Diltiazem			
5	4.9 ± 0.44	5	8.9
10	10.1 ± 0.80	5	8.0
20	20.0 ± 0.83	5	4.2
40	39.2 ± 2.64	5	6.6
100	101.2 ± 2.48	5	2.5
200	199.8 ± 10.34	5	5.2
Deacetyldiltiazem			
10	10.1 ± 0.26	5	2.6
20	20.2 ± 1.39	5	6.9
50	49.7 ± 2.16	5	4.3
100	100.1 ± 4.37	5	4.4
N-Demethyldiltiazem			
10	10.1 ± 0.89	5	8.8
20	20.0 ± 1.21	5	6.1
40	40.9 ± 2.28	5	5.6
100	98.5 ± 4.51	5	4.6
200	200.5 ± 10.29	5	5.1
Propranolol			
5	5.2 ± 0.43	5	8.3
10	10.0 ± 0.61	5	6.1
20	20.4 ± 1.79	5	8.8
50	49.4 ± 3.40	5	6.9
100	100.2 ± 5.67	5	5.7

Table 2
Interday variability

Actual concentration (ng ml^{-1})	Assayed concentration (mean ± SD) (ng ml^{-1})	*n*	RSD (%)
Diltiazem			
5	4.9 ± 0.52	7	10.4
10	9.7 ± 0.97	7	9.7
50	51.1 ± 2.75	7	5.5
100	100.1 ± 1.92	7	1.9
200	200.1 ± 0.93	7	0.5
Deacetyldiltiazem			
5	5.4 ± 0.78	7	15.6
25	25.2 ± 1.07	7	4.2
50	50.2 ± 1.19	7	2.4
100	100.0 ± 1.10	7	1.1
N-Demethyldiltiazem			
10	10.1 ± 1.38	7	13.8
50	48.1 ± 2.80	7	5.8
100	99.9 ± 3.10	7	3.1
200	198.8 ± 4.9	7	2.5
200			
Propranolol			
10	10.5 ± 0.49	7	4.9
50	48.0 ± 2.90	7	6.0
150	145.3 ± 4.80	7	3.2
200	202.5 ± 4.50	7	2.3

required the assay of a second sample or the use of a dual-wavelength detector.

Assay validation

The intra- and interday variability were determined at the plasma concentration expected after oral administration four times a day of 60 mg diltiazem and 40 mg propranolol. Results obtained from intraday assays are shown in Table 1. The method gave an average RSD of 5.9% for diltiazem, 4.6% for deacetyldiltiazem, 6.0% for *N*-demethyldiltiazem and 7.2% for propranolol. Table 2 demonstrates the interday variabilities, which were, as expected, slightly higher. The limits of detection were 1 ng ml^{-1} for propranolol, deacetyldiltiazem, diltiazem and 5 ng ml^{-1} for *N*-demethyldiltiazem (RSD ≥30%). Calibration curves were linear over the concentration range analysed for all compounds with correlation coefficients ≥0.994.

Application

The method was applied to the determination of the plasma concentrations in a healthy subject, who received 60 mg diltiazem and 40 mg propranolol four times a day for three days to achieve steady state. On the fourth day only one dose was given and the plasma concentrations were determined. Plasma profiles for the four analytes of interest are presented in Fig. 5 which demonstrates the applicability of the assay.

The decrease in plasma concentrations of diltiazem and its metabolites during the first hour was attributed to the continued elimination of the previous dose and the delayed absorption of the new dose. The plasma concentrations of the diltiazem-metabolites were lower than the plasma concentration of the parent drug. Earlier studies have shown both higher [12] and lower [8] plasma concentrations for the diltiazem-metabolites, compared with diltiazem. The elimination half-life in this subject was 1.5 h for propranolol and 4.3 h for diltiazem.

The method presented is being used in this laboratory to investigate the pharmacokinetic interactions between diltiazem and propranolol in more detail and the results of these studies will be presented elsewhere.

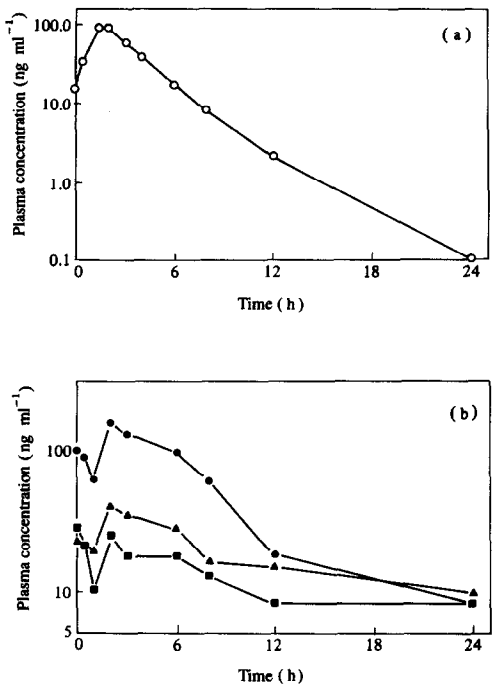

Figure 5
Plasma concentration–time curve of (a) propranolol (b) diltiazem and diltiazem-metabolites in a healthy subject after oral administration of 40 mg propranolol and 60 mg diltiazem at steady state: (○) propranolol; (●) diltiazem; (▲) deacetyldiltiazem; (■) *N*-demethyldiltiazem.

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