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Short Communication

Liquid chromatographic determination of celiprolol, diltiazem, desmethyldiltiazem and deacetyldiltiazem in plasma using a short alkyl chain silanol deactivated column

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Introduction

There has been an increased interest in using calcium channel antagonists (e.g. diltiazem) together with beta adrenergic blocking agents (e.g. celiprolol) for management of hypertension and/or angina (Fig. 1). Their mechanisms of action are different, so theoretically the combination should improve overall cardiovascular pharmacotherapy for these diseases while decreasing the potential for adverse events [1–8]. In fact, physicians frequently prescribe these agents together.

In the liver, diltiazem is metabolized to pharmacologically active compounds, N-desmethyldiltiazem and O-deacetyldiltiazem (Fig.

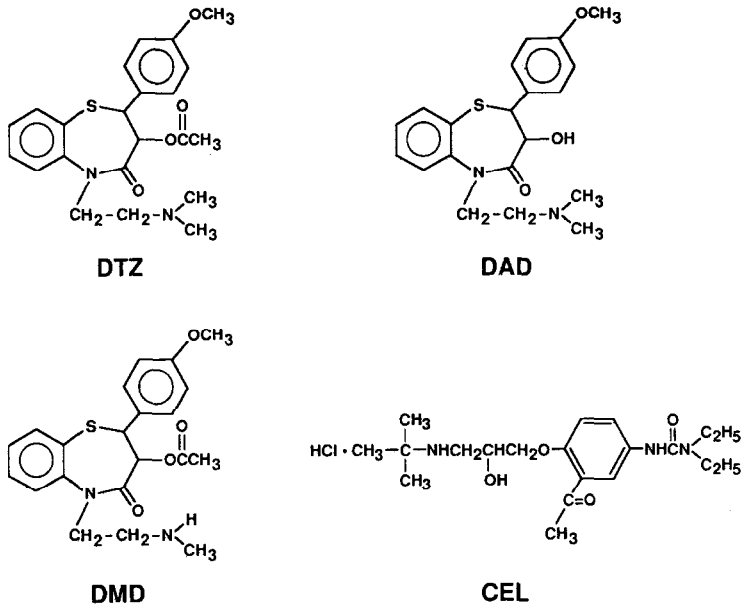

Figure 1
Chemical structures for diltiazem (DTZ), deacetyldiltiazem (DAD), desmethyldiltiazem (DMD) and celiprolol (CEL).

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1). These metabolites undergo further biotransformation to yield relatively inactive metabolites, O-demethyldesacetyldiltiazem, N-demethyldesacetyldiltiazem and N,O-didemethyldesacetyldiltiazem [9, 10]. Celiprolol is a third generation beta adrenergic receptor blocker with beta-1 antagonist and beta-2 agonist properties [7, 8]. There are properties of celiprolol that many clinicians believe make it an attractive beta adrenergic receptor blocker to be used in combination with calcium channel blockers. For example, it is not extensively metabolized and consequently, there are no clinically relevant metabolites [11]. Its beta-2 adrenergic receptor agonist properties make it relatively more acceptable in patients with varying degrees of pulmonary function. Celiprolol is also expected to have fewer adverse effects on peripheral blood flow, insulin secretion, lipids, and exercise [7, 8].

Several liquid chromatography (LC) methods have been published for either diltiazem [12–24] or celiprolol [25–28]. None have been reported for the simultaneous determination of these agents which could then be useful in therapeutic monitoring or compliance testing.

Experimental

Reagents and chemicals

Diltiazem, deacetyldiltiazem and desmethyldiltiazem were supplied by Marion Merrell Dow (Kansas City, MO, USA). Celiprolol hydrochloride and verapamil hydrochloride were supplied by Rhone-Poulenc Rorer (Collegeville, PA, USA) and Knoll Pharmaceutical (Whippany, NJ, USA), respectively. The following chemicals were purchased from Fisher Scientific (Fairlawn, NJ, USA) and were of analytical grade: boric acid, potassium phosphate, phosphoric acid (85% v/v), sodium chloride, sulphuric acid (5 mM). HPLC grade solvents were used either for extraction (e.g. hexane, isopropanol) or in the mobile phase (e.g. methanol) and were purchased from Sigma (St Louis, MO, USA). Milli-Q-filtered water was used for all experiments.

Instrumentation

The HPLC system consisted of a Spectra Physics autosampler model S8875 (San Jose, CA, USA), a Waters constant flow reciprocating pump model 510 solvent delivery system (Milford, MA, USA), and a Spectromonitor 3100 variable wavelength UV detector (Milton Roy, Riveria Beach, FL, USA). The eluate was monitored continuously for absorbance at 237 nm and the detector output was recorded on a Waters Data Module Integrator model 710 (Milford, MA, USA). The separation system was a new 25 cm × 4.6 mm i.d. stainless-steel reversed-phase, short alkyl chain, silanol deactivated column (SCD 100) obtained from SynChrom (Lafayette, IN, USA) and used at ambient room temperature.

Chromatographic conditions

Each sample was analysed by liquid chromatography using the short alkyl chain, silanol deactivated support with UV detection at 237 nm. Mobile phase consisted of methanol–buffer (50:50, v/v). The buffer was dibasic potassium phosphate (0.04 M). The pH of the aqueous solution was adjusted to 5.5 with phosphoric acid 85%. The solvent flow rate was 1 ml min^{-1} which produced a column pressure of 1500 psi. Stock solutions of celiprolol, diltiazem, and diltiazem metabolites were made in Milli-Q-filtered water at a concentration of 200.0 μg ml^{-1}. Dilutions of these were utilized to prepare plasma standards needed to construct calibration curves. Verapamil hydrochloride (5 μg ml^{-1}) was used as the internal standard. Retention times were determined by injecting an aliquot of the aqueous standard solution into the HPLC system. Chromatography from this system is illustrated in Fig. 2.

Extraction procedure

Celiprolol, diltiazem and its two major metabolites were extracted from plasma using a simple, two-step extraction procedure. An aliquot (50 μl) of the internal standard solution (5 μg ml^{-1}) was added to 1.0 ml of plasma. One millilitre of a borate buffer (pH 8.9) and 1.0 g of NaCl were then added and the mixture was vortexed for 30 s. Six millilitres of a hexane–isopropanol (95:5, v/v) mixture was then added and the tubes vortexed for 30 s and shaken for 10 min. Each sample was centrifuged at 700 ***g*** for 10 min and the organic layer separated and back extracted with 200 μl sulphuric acid (5 mM). These tubes were then vortexed for 30 s, shaken for 10 min and centrifuged for an additional 5 min at 700 ***g***. For LC analysis, a portion (50 μl) of the acidic, aqueous solution was injected.

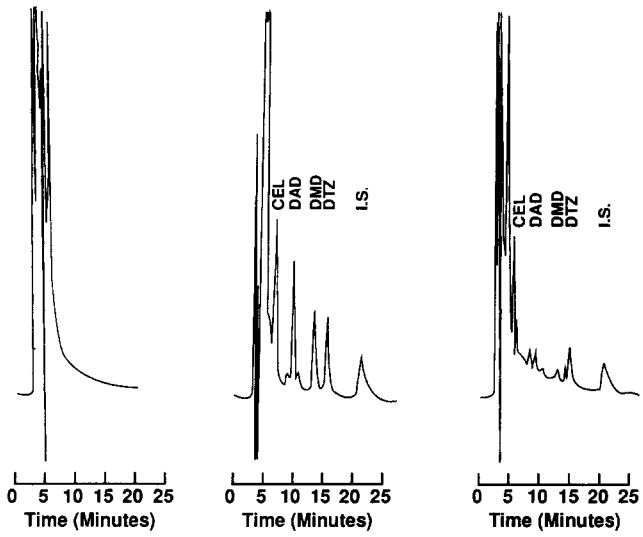

Figure 2
Chromatograms obtained on analysis of a drug-free sample (left), a 300 ng ml^{-1} extracted plasma standard (middle), and a 4-h sample from a research subject (right) [40.9 ng ml^{-1} {CEL}, 32.0 ng ml^{-1} {DAD}, 74.0 ng ml^{-1} {DMD}, 307.0 ng ml^{-1} {DTZ}]. For conditions see Table 1 footnote. Peaks: CEL = celiprolol, DAD = deacetyldiltiazem, DMD = desmethyldiltiazem, DTZ = diltiazem, I.S. = internal standard, verapamil.

Validation study

Plasma standards were prepared by adding aliquots of the respective stock solutions of celiprolol, diltiazem, deacetyldiltiazem and desmethyldiltiazem to culture tubes in amounts to give the six appropriate final concentrations in drug-free human plasma. Plasma samples for celiprolol were assessed at 50.0, 100.0, 500.0, 1000.0, 2000.0 and 3000.0 ng ml^{-1} [25–28]. Diltiazem, deacetyldiltiazem and desmethyldiltiazem were analysed at concentrations of 20.0, 40.0, 100.0, 200.0, 400.0 and 500.0 ng ml^{-1}. Peak-height ratios were then plotted as a function of the concentrations of the analyte, e.g. celiprolol/internal standard, diltiazem/internal standard.

To test accuracy and precision of this assay, plasma samples were prepared. Celiprolol concentrations were 200 and 1500 ng ml^{-1} and the diltiazem and its metabolite concentrations were 75.0 and 300.0 ng ml^{-1}. Within-day (n = 10) and between-day (n = 10) variations were evaluated by repetitive analysis of these spiked plasma samples. Concentrations of drug and metabolite in these samples, as well as the unknowns, were obtained through linear regression analyses of the six plasma standards. All samples were measured and analysed using peak-height ratios.

Lower limits of sensitivity were determined using aqueous stock solutions, with the limit of sensitivity defined as a signal-to-noise ratio of 4:1. Percentage extraction recovery (n = 3) was determined by comparing peak-height ratios of chromatograms obtained from extracted plasma samples to those of the standard stock aqueous solutions at celiprolol concentration of 200.0 ng ml^{-1} and at diltiazem and its metabolite concentration of 200.0 ng ml^{-1}. Plasma samples were prepared fresh and comparisons of the peak-height ratios for celi-

Table 1
Capacity ratios (k') for compounds commonly administered

Compound	Plasma conc.	k'*
Atenolol	50 ng ml^{-1}	0.0
Aspirin	100 μg ml^{-1}	0.0
Caffeine	10 μg ml^{-1}	0.0
Ibuprofen	20 μg ml^{-1}	0.0
Lidocaine	5.0 μg ml^{-1}	0.0
Metoprolol	50 ng ml^{-1}	0.0
Nifedipine	25 ng ml^{-1}	0.0
Celiprolol	200 ng ml^{-1}	2.2
Propranolol	50 ng ml^{-1}	2.3
Deacetyldiltiazem	200 ng ml^{-1}	3.6
Desipramine	250 ng ml^{-1}	5.0
Desmethyldiltiazem	200 ng ml^{-1}	5.1
Diltiazem	200 ng ml^{-1}	6.1
Imipramine	250 ng ml^{-1}	6.4
Verapamil	500 ng ml^{-1}	8.2

*The column was a 25 cm × 4.6 mm i.d. stainless steel reversed-phase, short chain silanol deactivated support (SCD-100). The mobile phase consisted of methanol–buffer (50:50, v/v). The buffer was 0.04 M basic potassium phosphate and the pH was adjusted to 5.5 with phosphoric acid (85%). The solvent flow rate was 1 ml min^{-1} which produced a column pressure of 1500 psi.

prolol, diltiazem and its metabolites were made to the ratio from samples frozen at −20°C for a period of 30 days. Potential interfering substances were added to drug-free plasma and extracted.

Interfering substances

To assess the potential for chromatographic interference from agents that are commonly coadministered with diltiazem in the clinical setting, we added and extracted from plasma a number of such representative drugs (Table 1). Concentrations were chosen to represent those commonly encountered in clinical practice.

Stability

Stability was assessed by comparing peak height ratios of each compound from freshly prepared plasma samples to those obtained from plasma frozen at −20°C for a period of 30 days.

Application of method

A white male volunteer (37 years) was given a once-a-day formulation of diltiazem (240 mg) and a cardioselective beta adrenergic receptor blocker, celiprolol (200 mg). A baseline blood sample was obtained, followed by the oral administration of diltiazem. Thirty minutes later, celiprolol was taken. Blood samples were obtained during normal working hours (8 h).

Results

Figure 2 shows typical chromatograms of an extracted drug-free blank plasma sample, a 300 ng ml^{-1} extracted plasma sample, and the 4-h sample from the volunteer. The elution order is celiprolol, deacetyldiltiazem, desmethyldiltiazem, diltiazem, followed by the internal standard, verapamil. There were no interfering peaks detected in the pooled blank plasma sample. In addition, the peaks looked symmetrical, and well resolved from each other.

Analysis of 10 standard curves revealed that the curves for celiprolol and diltiazem or its two major metabolites were linear over the concentration ranges, 50.0–3000.0 ng ml^{-1} and 20.0–400 ng ml^{-1}, respectively (Fig. 3). All correlation coefficients were >0.998. Slope values were calculated to be 0.69, 1.0, 0.46 and 0.5 for celiprolol, deacetyldiltiazem, desmethyldiltiazem and diltiazem, respectively. The *y*-intercept values for were found to be

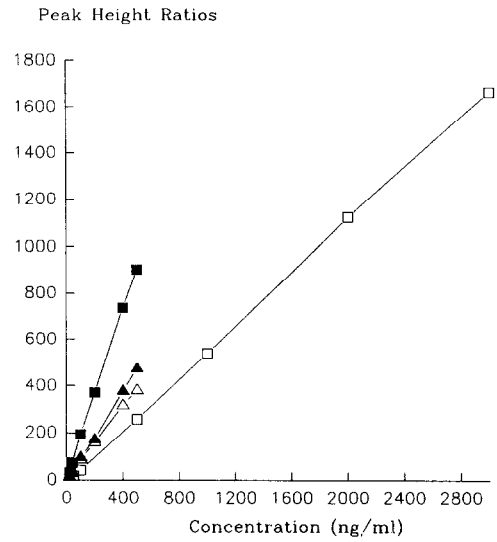

Figure 3
Standard curves for celiprolol (□), diltiazem (▲), desmethyldiltiazem (△) and deacetyldiltiazem (■). For conditions see Table 1 footnote.

−5.1, −8.4, −7.8 and −10.7 for celiprolol, deacetyldiltiazem, desmethyldiltiazem, and diltiazem, respectively. From these equations, concentrations of analytes were determined.

Accuracy and precision

Reproducibility of the procedure was evaluated by analysing 10 replicate samples containing celiprolol (200.0 and 1500 ng ml^{-1}), diltiazem and its two metabolites (75.0 and 300.0 ng ml^{-1}) on the same day (n = 10) and over 10 consecutive days (Table 2). Relative standard deviations were less than 10% for both high and low values.

Mean extraction efficiencies at 200.0 ng ml^{-1} for celiprolol, diltiazem, deacetyldiltiazem, desmethyldiltiazem and verapamil were 17.5% ± 2.0, 64.4% ± 5.0, 62.5% ± 4.1, 62.5% ± 5.6 and 83.9% ± 5.0, respectively. The relatively low extraction efficiency for celiprolol is partially explained by the use of borate buffer at a pH of 8.9 in the extraction procedure. This pH value was chosen because it does not cause hydrolysis of diltiazem, but it is lower than the pK_a of celiprolol which is 9.7. Increasing the extraction buffer to more alkaline pH values increases the extraction efficiencies for celiprolol at the expense of diltiazem stability. A potential limitation from low extraction efficiencies is there may be variability between samples taken from large, diverse populations.

Table 2
Assay accuracy, repeatability and reproducibility*

Compound	Conc. added (ng ml^{-1})	Within-day Conc. found (ng ml^{-1})	Within-day RSD† (%)	Between-day Conc. found (ng ml^{-1})	Between-day RSD† (%)
Celiprolol	200	202.5 ± 16.6	8.2	205.8 ± 19.6	9.6
	1500	1451.5 ± 61.7	4.4	1524.0 ± 100.3	6.6
Diltiazem	75	72.0 ± 4.3	6.0	78.9 ± 4.8	6.1
	300	294.3 ± 10.0	3.4	297.1 ± 12.2	4.1
Deacetyldiltiazem	75	75.9 ± 4.6	6.0	76.9 ± 4.9	6.4
	300	307.5 ± 15.6	5.1	302.1 ± 16.1	5.3
Desmethyldiltiazem	75	75.0 ± 4.5	6.1	77.7 ± 5.0	6.5
	300	299.8 ± 12.9	4.3	293.0 ± 16.1	5.5

* For conditions see footnote for Table 1.
† $n = 10$.

Limits of detection from aqueous stock solutions for celiprolol, deacetyldiltiazem, desmethyldiltiazem and diltiazem were calculated to be 4.5, 1.5, 3.0 and 3.0 ng ml^{-1}, respectively. Incorporating the extraction efficiencies, lower limits of detection from plasma samples were estimated to be 25.7, 2.3, 4.8 and 4.8 ng ml^{-1} for celiprolol, deacetyldiltiazem, desmethyldiltiazem and diltiazem, respectively.

Comparison of peak-height ratios for celiprolol, diltiazem and its metabolites from freshly prepared plasma samples (200 ng ml^{-1}) to the ratio from samples frozen at −20°C for a period of 30 days showed no differences, consistent with previous reports. Diltiazem and celiprolol changed by less than 2%. This was accompanied by an increase in deacetyldiltiazem and desmethyldiltiazem, 7 and 2.2%, respectively. Samples drawn for monitoring purposes can therefore be analysed within 30 days.

No endogenous sources of interference were observed (Fig. 2). To assess potential for chromatographic interference from drugs that are commonly coadministered with either celiprolol or diltiazem, we evaluated a number of representative compounds (Table 1). Propranolol, desipramine and imipramine were detected if present at therapeutic concentrations and were found to interfere with celiprolol, desmethyldiltiazem and diltiazem, respectively.

Clinical application

Calcium channel and beta adrenergic receptor blockers are established therapies for cardiovascular conditions including hypertension and angina pectoris. Diltiazem is a widely prescribed calcium channel blocker. Celiprolol is one of the first of a new generation beta adrenergic receptor blockers that is highly cardioselective with beta-2 adrenergic receptor agonist properties. These properties make it potentially useful for patients that need a beta blocker but who have varying degrees of pulmonary dysfunction, poor peripheral blood flow, modest insulin resistance, or are physically active [7, 8].

Samples obtained from the subject taking diltiazem and celiprolol were analysed using this procedure. The 4-h sample is shown in Fig. 2. The total analysis time is less than 25 min. Disposition of celiprolol and diltiazem and its metabolites are shown in Fig. 4. It appears that desmethyldiltiazem is the major diltiazem metabolite, and other unidentified peaks are due to other diltiazem metabolites. The celiprolol concentrations are lower than expected, indicating a degree of variability [25–28]. These results suggest that this assay is accurate and reproducible within the effective concentration range for celiprolol and diltiazem. During an 8-h work day, approximately 20 samples can be injected onto the column for subsequent detection.

Discussion

Celiprolol, diltiazem and its active metabolites are small, basic molecules that adsorb to all stationary phases used in reversed-phase chromatography. This ubiquitous adsorption results in varying degrees of non-symmetrical peaks unless silanol-suppressing reagents are added to the mobile phase. The addition of these agents to the mobile phase results in an improvement in the shape of the peak, reten-

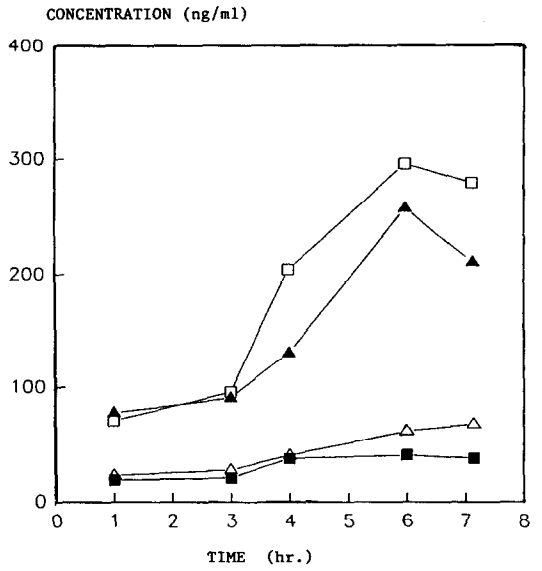

Figure 4
Plasma concentrations of diltiazem obtained from one research subject. A white male volunteer (37 years) was started on a once-a-day formulation of diltiazem (240 mg) and cardioselective beta adrenergic receptor blocker, celiprolol. Blood samples were obtained during normal working hours. For conditions see Table 1 footnote. Peaks: □ = celiprolol, ■ = deacetyldiltiazem, △ = desmethyldiltiazem, ▲ = diltiazem.

tion time, and reproducibility. Unfortunately, the actions of these agents are concentration-dependent, and this concentration varies according to the source and kind of packing of the column, which leads to irreproducibility within and between laboratories. In addition, these agents may prolong column equilibration and restoration time. The present analysis overcomes these limitations by utilizing a special, commercially-prepared short alkyl chain, deactivated reversed-phase support.

Patients with severe hypertension and/or angina may require multiple drug regimes for adequate control of blood pressure or chest pain. The combination of diltiazem and celiprolol represents a typical combination. Laboratory analysis may be required for either research or monitoring purposes. The present method can be applied to therapeutic monitoring or compliance testing where the aim is to assure that peak concentrations are within a desired range. Silanol deactivation results in an LC method that has good resolution and reproducibility. The method is relatively simple and rapid allowing the analysis of 20 samples a day.

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