Journal of Chromatography, 615 (1993) 111–116
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6774

High-performance liquid chromatographic determination of diltiazem and two of its metabolites in plasma using a short alkyl chain silanol deactivated column

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(First received September 14th, 1992; revised manuscript received January 26th, 1993)

ABSTRACT

A simple and reproducible method for the determination of diltiazem and its two major metabolites, demethyl- and deacetyldiltiazem, is presented using a new column containing a short alkyl chain silanol deactivated support. This method involves the extraction of alkalinized plasma with a hexane–isopropanol mixture (95:5, v/v) followed by back-extraction into 5 m*M* sulfuric acid. Reversed-phase liquid chromatography is used with ultraviolet detection at 237 nm over a concentration range of 20–400 ng/ml for the compounds. Imipramine is used as the internal standard. Within-day and between-day coefficients of variation are less than 10%. The lower limits of detection are 4, 2 and 4 ng/ml for diltiazem, deacetyl- and demethyldiltiazem, respectively. Samples can be stored for up to thirty days with no significant degradation. The assay has clinical applicability.

INTRODUCTION

Diltiazem, like other calcium channel blockers, possesses pharmacologic effects that are related to its ability to inhibit the influx of calcium ions during membrane depolarization in vascular and cardiac smooth muscle [1]. It is indicated for the treatment of hypertension, angina and control of ventricular rate in patients with atrial fibrillation or flutter [2–8].

Since diltiazem represents the first calcium blocker in the benzothiazepine class, it is frequently used in clinical trials to compare newer agents within this class. Diltiazem is also extensively metabolized. In the liver diltiazem is mainly metabolized to its N-demethyl and O-deacetyl derivatives. These two metabolites are pharmacologically active [9] and undergo further biotransformation to relatively inactive metabolites, *e.g.*, O-demethyldeacetyldiltiazem, N-demethyldeacetyldiltiazem and N,O-didemethyldeacetyldiltiazem [10]. Therefore, reproducible analytical assays are needed.

Unfortunately, diltiazem and its metabolites are small basic molecules that adsorb to stationary phases used in reversed-phase liquid chromatography. This results in non-symmetrical peaks due to their hydrogen bonding and/or ion-exchange interactions with uncapped silanols in the support. Silanol suppressing (*e.g.*, diethylamine, triethylamine) or ion pairing (*e.g.*, NaBr) reagents are frequently added to the mobile phase to diminish this interaction. This predictably causes several problems [11] such as irreproducible retention times, long column equilibration and restoration times, degradation of the silica matrix of reversed-phase columns and, in preparative work, additional separation steps to clean and collect analytes in an environment free of ei-

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0378-4347/93/$06.00

ther silanol-suppressing or ion-pairing reagents. Furthermore, silanol-suppressing reagents work in a concentration-dependent manner, and reliability is variable depending upon the source and quality of packing material [11].

Previously described high-performance liquid chromatographic (HPLC) methods for diltiazem used either C_8, C_{18}, CN or silica columns with or without silanol-suppressing or ion-pairing reagents [12–29]. It is not surprising, therefore, that variability exists within and between published methods with regards to resolution, retention time and reproducibility. Thus, we present a simple and selective HPLC method that quantitates both diltiazem and two of its active metabolites. A short alkyl chain, deactivated reversed-phase support is used and is composed of 100-Å bonded silica. Silanol deactivation reduces tailing of these basic analytes and no ion-pairing or suppressing reagents are required.

EXPERIMENTAL

Reagents and chemicals

Diltiazem, deacetyldiltiazem and demethyldiltiazem were supplied by Marion Laboratories (Kansas City, MO, USA). The internal standard, imipramine · HCl, was supplied by Sigma (St. Louis, MO, USA). 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), NaOH (10 *M*), and sodium chloride. HPLC-grade solvents were used either for extraction (*e.g.*, hexane, isopropanol) or in the mobile phase (*e.g.*, methanol) and were also purchased from Sigma.

Instrumentation

The HPLC system consisted of a Spectra Physics Model S8875 autosampler (San Jose, CA, USA), a Waters Assoc. Model 510 constant-flow reciprocating pump solvent delivery system (Milford, MA, U.S.A), 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 Assoc. Model 710 data module integrator. A new 25 cm × 4.6 mm I.D. (5 μm particle size) stainless-steel reversed-phase, short alkyl chain, silanol deactivated column (SCD 100) was obtained from SynChrom (Lafayette, IN, USA) and was used at ambient room temperature.

Chromatographic conditions

The mobile phase consisted of methanol–water (50:50, v/v) and dibasic potassium phosphate (0.04 *M*). The pH was adjusted to 6.0 with 85% phosphoric acid. The solvent flow-rate was 1 ml/min which produced a column pressure of 10.3 MPa. Stock solutions of diltiazem and its two metabolites were prepared in Milli-Q-filtered water at a concentration of 2 μg/ml. Dilution of this solution was made in order to prepare the plasma standards needed to construct the calibration curves. Imipramine (10 μg/ml) was used as the internal standard. Retention times were determined by injecting an aliquot of the standard solution into the HPLC system. The chromatograms from this system is illustrated in Fig. 1.

Extraction procedure

Diltiazem and its metabolites were extracted from plasma using a simple, two-step extraction procedure. An aliquot (25 μl) of the internal standard solution (10 μg/ml) was added to 1.0 ml of plasma. Borate buffer (pH 9.0) (1 ml) and 0.5 g of NaCl were then added and the mixture was vortex-mixed for 30 s. Hexane-isopropanol (95:5, v/v) (6 ml) was then added and the tubes were vortex-mixed for 30 s and shaken for 10 min. The samples were centrifuged at 700 *g* for 10 min and the organic layer was separated and back-extracted with 200 μl sulfuric acid (5 m*M*). These tubes were vortex-mixed for 30 s, shaken for 10 min and centrifuged for an additional 5 min at 700 *g*. For HPLC analysis, a portion (40 μl) of the acidic, aqueous solution was injected.

Validation study

Plasma standards were prepared by adding aliquots of the respective stock solutions of diltiazem, deacetylditiazem and demethyldiltiazem to

culture tubes in amounts to give final concentrations in drug-free human plasma of 20.0, 40.0, 100.0, 200.0, 300.0 and 400.0 ng/ml. Peak-height ratios were then plotted as a function of the concentrations of the analyte, *e.g.*, diltiazem/internal standard.

To test accuracy and precision of this assay, 60.0 and 350 ng/ml plasma samples were prepared. The within-day (n = 10) and between-day (n = 10) variations were evaluated by repetitive analysis of these spiked plasma samples. The amounts of the drug and metabolite in these samples, as well as the unknowns, were obtained through linear regression analysis of the six plasma standards. All samples were measured and analyzed using peak-height ratios.

Lower limits of detection were determined using aqueous stock solutions, with the limit of detection 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 a concentration of 200 ng/ml. Plasma samples were prepared fresh (200 ng/ml) and comparisons of the peak-height ratios for diltiazem and its metabolites were made to the ratio from samples frozen at −20°C for a period of thirty 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 I). Concentrations were chosen to represent those commonly encountered in clinical practice.

Application of the method

Two hypertensive male volunteers were receiving diltiazem 90 mg three times a day to control elevated blood pressure. During a follow up clinic visit, clinicians decided to discontinue one of the patient's therapy. The patient agreed to take a final 90-mg dose of diltiazem and to have ten blood samples obtained over the subsequent 12 h (Fig. 2). Another patient agreed to have his blood drawn 2 h after the 90-mg morning dose of diltiazem (Fig. 1). Plasma was collected immediately and stored at −70°C until analyzed.

TABLE I

CAPACITY RATIOS (k') FOR COMPOUNDS COMMONLY COADMINISTERED

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–water (50:50, v/v) and dibasic potassium phosphate (0.04 M). The pH was adjusted to 6.0 with phosphoric acid (85%). The solvent flow-rate was 1 ml/min which produced a column pressure of 10.3 MPa.

Compound	Plasma concentration	k'
Aspirin	100 μg/ml	–
Atenolol	50 ng/ml	–
Caffeine	10 μg/ml	–
Lidocaine	5.0 μg/ml	–
Metoprolol	50 ng/ml	–
Nifedipine	25 ng/ml	–
Ibuprofen	20 μg/ml	–
Propranolol	50 ng/ml	0.97
Deacetyldiltiazem	200 ng/ml	1.27
Demethyldiltiazem	200 ng/ml	2.2
Desipramine	250 ng/ml	2.54
Diltiazem	200 ng/ml	3.0
Theophylline	10 μg/ml	3.1
Imipramine	250 ng/ml	3.77
Verapamil	240 ng/ml	3.8

RESULTS AND DISCUSSION

Fig. 1 shows typical chromatograms of, from left to right, an extracted drug-free blank plasma sample, a 300 ng/ml extracted plasma sample and the 2-h sample from the volunteer. The elution order is deacetyldiltiazem, demethyldiltiazem, diltiazem, followed by the internal standard, imipramine. There were no interfering peaks detected in the pooled blank plasma sample. However, we found additional peaks in the sample obtained from the hypertensive patient. Others have detected such endogenous peaks [13].

Analysis of ten standard curves revealed that the curves for diltiazem and its two metabolites

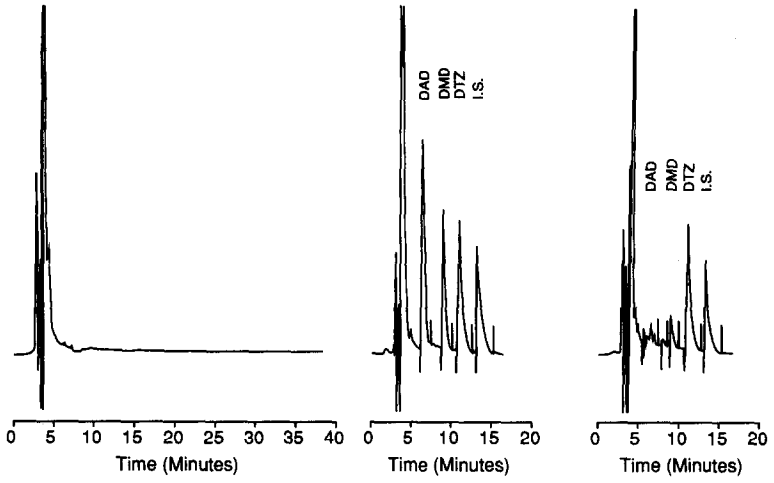

Fig. 1. Chromatograms obtained on analysis of a drug-free sample (left), a 300 ng/ml extracted plasma standard (middle) and a 2-h sample from a hypertensive patient (32.0 ng/ml deacetyldiltiazem, 74.0 ng/ml demethyldiltiazem, 307.0 ng/ml diltiazem). The column was a 25 cm × 4.6 mm I.D. stainless-steel reversed-phase, short chain silanol deactivated column (SCD 100). The mobile phase consisted of methanol–water (50:50, v/v) and dibasic potassium phosphate (0.04 *M*). The pH was adjusted to 6.0 with phosphoric acid (85%). The solvent flow-rate was 1 ml/min which produced a column pressure of 10.3 MPa. Peaks: DAD = deacetyldiltiazem; DMD = desmethyldiltiazem; DTZ = diltiazem; I.S. = internal standard, imipramine.

were linear over the concentration range 20.0–400.0 ng/ml. All correlation coefficients were >0.998. The slope values were calculated to be 0.91, 0.55 and 0.55 for deacetyldiltiazem, demethyldiltiazem and diltiazem, respectively. The *y*-intercept values were calculated to be −7.4, −1.7 and −4.4 for deacetyldiltiazem, demethyldiltiazem and diltiazem, respectively. From these equations the concentrations of analytes were calculated.

Accuracy and precision

The reproducibility of the procedure was evaluated by analyzing ten replicate samples contain-

TABLE II

ASSAY PRECISION AND REPRODUCIBILITY

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–water (50:50, v/v) and dibasic potassium phosphate (0.04 *M*). The pH was adjusted to 6.0 with phosphoric acid (85%). The solvent flow-rate was 1 ml/min which produced a column pressure of 10.3 MPa.

Compound	Concentration added (ng/ml)	Within-day		Between-day	
		Concentration found (mean ± S.D.) (ng/ml)	C.V. (%)	Concentration found (mean ± S.D.) (ng/ml)	C.V. (%)
Diltiazem	60	62.4 ± 1.6	2.6	60.8 ± 6.0	9.9
	350	337.0 ± 11.8	3.5	344.8 ± 9.8	2.8
Deacetyldiltiazem	60	63.6 ± 3.5	5.5	62.5 ± 3.3	5.3
	350	356.6 ± 10.8	3.0	343.6 ± 10.8	3.1
Demethyldiltiazem	60	58.5 ± 1.5	2.6	61.6 ± 6.0	9.8
	350	350.5 ± 10.4	3.0	354.8 ± 14.9	4.2

ing diltiazem and its two metabolites at low (60.0 ng/ml) and high (350.0 ng/ml) concentrations on the same day (n = 10) and over ten consecutive days (Table II). Coefficients of variation were less than 10% for all values.

The mean (± S.D.) extraction efficiencies of diltiazem (200.0 ng/ml), deacetyldiltiazem (200.0 ng/ml), demethyldiltiazem (200.0 ng/ml) and imipramine (250.0 ng/ml) were 71.0 ± 3.0, 58.8 ± 1.4, 75.6 ± 2.0 and 66.3% ± 4.9%, respectively. The extraction efficiencies for diltiazem and its metabolites are lower than previously reported using similar methods [21,26]. One reason for this could be the extraction buffer. Diltiazem and its metabolites have pK_a values of approximately 8.0, which is lower than that of the alkalinizing agent used in the extraction, which was 9.0. Thus, approximately 90% of these agents will be in the unionized, extractable form. Additionally, adsorption of diltiazem and its metabolites to glass probably also occurred since we did not use silanized glassware.

The limits of detection of the assay for diltiazem, deacetyldiltiazem and demethyldiltiazem were calculated to be 4.0, 2.0 and 4.0 ng/ml, respectively.

Comparison of peak-height ratios for diltiazem and its metabolites from freshly prepared plasma samples (200 ng/ml) to the ratio from samples frozen at −20°C for a period of thirty days showed modest differences. Diltiazem changed by 1.0%. This was accompanied by an increase in the deacetyldiltiazem and demethyldiltiazem, 7.0 and 2.2%, respectively. Similar results have been reported previously [21]. This indicates that samples drawn for pharmacokinetic purposes can be analyzed within thirty days.

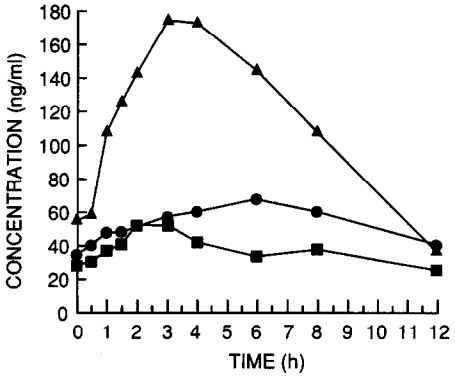

Fig. 2. Plasma concentrations of diltiazem obtained from one hypertensive patient. The subject was taking 90 mg of diltiazem three times a day, prior to having the medication discontinued. The patient agreed to take his last 90-mg dose of diltiazem the morning of the clinic visit and then have ten blood samples obtained over the subsequent 12 h. (■) Deacetyldiltiazem; (●) demethyldiltiazem; (▲) diltiazem.

No endogenous sources of interference were observed (Fig. 1). To assess the potential for chromatographic interference from drugs that are commonly coadministered with diltiazem in the clinical setting, we evaluated a number of representative compounds (Table I). Propranolol, desipramine, theophylline and verapamil were detected, if present, at therapeutic concentrations. Propranolol and desipramine might interfere with deacetyldiltiazem and demethyldiltiazem, respectively. Theophylline and verapamil will interfere with diltiazem and imipramine, respectively (Table I).

Clinical application

Plasma samples from two patients with hypertension were analyzed using this procedure (Figs. 1 and 2). These results suggest that this assay is accurate and reproducible within the effective concentration range for diltiazem. During an 8-h work day, approximately thirty samples can be injected onto the column with subsequent detection.

CONCLUSION

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 can then cause variability in peak shape which results in several predictable problems that may explain the variability in resolution, retention times and reproducibility that exists within and between published methods. The present analysis over-

comes these limitations by utilizing a special, commercially prepared short alkyl chain, deactivated reversed-phase support. Silanol deactivation results in a method that has good resolution and reproducibility. The method is relatively simple and rapid allowing the analysis of thirty samples a day. We have analyzed 800 samples on a given column without deterioration of the support.

Although several methods exist that use a column extraction procedure for diltiazem [27,28], it should be recognized that these methods involve many steps such as column activation, washing and elution steps prior to sample analysis. Additionally, in the current market place, the extraction columns are relatively expensive.

ACKNOWLEDGEMENT

This work was supported by grants from the United States of America, State Department, Peace Fellowship Program.

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