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## Note

# Diltiazem and desacetyldiltiazem analysis in human plasma using high-performance liquid chromatography: improved sensitivity without derivatization

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Diltiazem hydrochloride is a calcium antagonist currently used in the treatment of angina pectoris [1] and with probable efficacy as an antiarrhythmic [2] and antihypertensive [3]. Parent drug and the major metabolite, desacetyldiltiazem, have been measured in plasma and urine using thin-layer chromatography (TLC) [4], gas—liquid chromatography (GLC) with nitrogen—phosphorus detection after derivatization [5], GLC with electron-capture detection after derivatization [6], and high-performance liquid chromatography (HPLC) [7, 8]. These methods have inadequate sensitivity (TLC), or at low concentrations have limited reproducibility, particularly for desacetyldiltiazem when HPLC methods have been employed.

Here we describe a method for the sensitive, reproducible analysis of diltiazem and desacetyldiltiazem using HPLC with ultraviolet detection. This method fulfills the criteria for diltiazem pharmacokinetic studies, those of high sensitivity, selectivity, and reproducibility with short sample preparation time and low cost. Extraction is technically straightforward requiring only 1—2 h for complete sample preparation and one working day to complete analysis for a two-way crossover intravenous/oral dose pharmacokinetic study. This permits analysis of the same subject's intravenous and oral trial at the same time, eliminating variance introduced due to between-day analytical variability. Application of the method to an intravenous single-dose study of diltiazem in a normal volunteer is described.

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## EXPERIMENTAL

### *Materials*

Pure samples of diltiazem hydrochloride and desacetyldiltiazem were generously provided by Marion Labs. (Kansas City, MO, U.S.A.). Desipramine, used as an internal standard, was provided by USV Pharmaceuticals (Tuckahoe, NY, U.S.A.).

All other reagents, analytical-reagent grade or better, were purchased from commercial sources and used without further purification. Mobile phase components (phosphate buffer, acetic acid, methanol and acetonitrile) were filtered prior to mixing, then degassed after mixing.

### *Apparatus and chromatographic conditions*

The HPLC system consisted of a Glenco (Glenco Scientific, Houston, TX, U.S.A.) solvent delivery system, a Waters Assoc. (Milford, MA, U.S.A.) Model 440 ultraviolet spectrophotometer operated at 254 nm, and a Waters Assoc. Model U6K sample loop. Detector output was quantitated on a Fisher (Fair Lawn, NJ, U.S.A.) Model 5000 chart recorder. The separation system was a 30 cm × 3.9 mm stainless-steel (10 μm particle size) $C_{18}$ μBondapak reversed-phase column.

Mobile phase [0.06 *M* acetate buffer—acetonitrile—methanol (58:37:5), containing 5 m*M* heptanesulfonic acid, and glacial acetic acid was added to adjust pH of the final solution to 6.45] was run at a flow-rate of 1.8 ml/min. All analyses were performed at room temperature.

### *Stock solutions*

Standard solutions were prepared by dissolving the appropriate quantity of diltiazem hydrochloride to yield 100 mg diltiazem base in 100 ml methanol, and similarly 100 mg desacetyldiltiazem and 100 mg desipramine (internal standard) into 100 ml methanol. Sequential dilutions to 1 μg/ml were then made. These solutions were stored in the dark in glass bottles at 4°C and were stable for at least three months.

### *Preparation of samples*

Desipramine was used as the internal standard. A 150-μl volume of stock solution (1 μg/ml), containing 150 ng desipramine was added to each of a series of 14-ml (110 × 17 mm) conical polyethylene centrifuge tubes with tight sealing polyethylene caps (Sarstedt Scientific). A 1.0—2.0 ml sample of unknown plasma was added to each tube. Calibration standards for diltiazem and desacetyldiltiazem were prepared by adding 5, 10, 25, 50, 100, 200, 500 and 1000 ng of drug and metabolite to consecutive tubes. Drug-free control plasma was added to each of the calibration samples. One blank sample, taken from the subject being studied prior to drug administration was analyzed with calibration standards and each set of unknown samples.

### *Extraction procedure*

To each tube were added 4 ml of hexane—isoamyl acohol (98:2). The tubes were agitated gently by hand for 5 min, then centrifuged at 4°C for 5 min at

400 *g*. The organic layer was transferred to another 14-ml polyethylene centrifuge tube which contained 100 μl of 1.0 *M* hydrochloric acid. This mixture was again agitated gently by hand for 5 min and centrifuged at 4°C for 5 min at 400 *g*. The upper organic layer was discarded. A 20—80-μl aliquot of the lower aqueous phase was then injected directly into the sample loop.

*Clinical pharmacokinetic study*

A healthy 24-year-old female volunteer participated in a pharmacokinetic study of intravenous diltiazem after giving written informed consent. The subject received a single 50-mg dose of diltiazem hydrochloride (Synthelabo, Paris, France) equivalent to 46.0 mg diltiazem base, by infusion into an antecubital vein over a period of 10 min. Multiple venous blood samples were drawn into Venoject heparin-containing tubes over the following 24 h. Plasma was separated by centrifugation (400 *g* for 10 min at 4°C) and concentrations of diltiazem and desacetyldiltiazem were determined by the method described above.

Plasma diltiazem concentrations were analyzed by iterative weighted non-linear least-squares regression analysis [9, 10]. After correction of the dose to diltiazem base and of the derived coefficients for time of infusion [11], the following pharmacokinetic variables were determined: distribution half-life, elimination half-life, central compartment volume, total volume of distribution and total clearance.

## RESULTS

*Evaluation of the method*

Under the described chromatographic conditions, diltiazem, desacetyldiltiazem, and desipramine gave symmetric well resolved chromatographic peaks (Fig. 1). Drug-free blank plasma samples were consistently free of

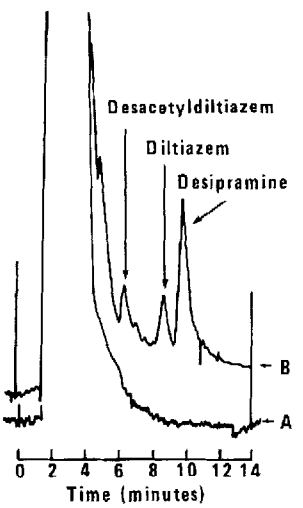


Fig. 1. HPLC profile of extract of 1 ml of plasma obtained from the subject prior to receiving diltiazem hydrochloride (A), and extract of 1 ml of plasma obtained 6 h after drug administration (B).

endogenous contaminants at the retention times corresponding to the compounds. The relation of plasma diltiazem and desacetyldiltiazem concentration to the diltiazem:desipramine and desacetyldiltiazem:desipramine peak height ratios were linear to at least 1000 ng/ml.

Analysis of ten standard curves over a three-month period indicated that the correlation coefficient is always 0.995 or greater. The day-to-day coefficient of variation in the slopes of the calibration curves was 7.9% for diltiazem and 3.7% for desacetyldiltiazem.

The sensitivity limit of the method is 2.5 ng/ml of a 2-ml extracted plasma sample for both diltiazem and desacetyldiltiazem. Within-day coefficients of variation for identical samples of diltiazem were at 5 ng/ml, 8.0% ($n$ = 6); 10 ng/ml, 8.6% ($n$ = 6); 25 ng/ml, 3.0% ($n$ = 6); 50 ng/ml, 6.0% ($n$ = 6); 100 ng/ml, 8.0% ($n$ = 6); 200 ng/ml, 3.0% ($n$ = 4); 500 ng/ml, 4.7% ($n$ = 6), and 1000 ng/ml, 3% ($n$ = 6). Similar analysis of within-day replicability for desacetyldiltiazem indicated at 5 ng/ml, 8.0% ($n$ = 6); 10 ng/ml, 7.0% ($n$ = 6); 25 ng/ml, 2.0% ($n$ = 6); 50 ng/ml, 2.6% ($n$ = 5); 100 ng/ml, 5.0% ($n$ = 6); 200 ng/ml, 9.3% ($n$ = 4); 500 ng/ml, 4.0% ($n$ = 6) and 1000 ng/ml, 2.8% ($n$ = 6). Residue analysis indicated the extraction of both diltiazem and desacetyldiltiazem is greater than 90% complete at plasma concentrations of 25, 50 and 100 ng/ml.

*Pharmacokinetic study*

Fig. 2 shows plasma dilitiazem and desacetyldiltiazem concentrations and the diltiazem pharmacokinetic function for the described subject. Derived pharmacokinetic parameters are listed in Table I.

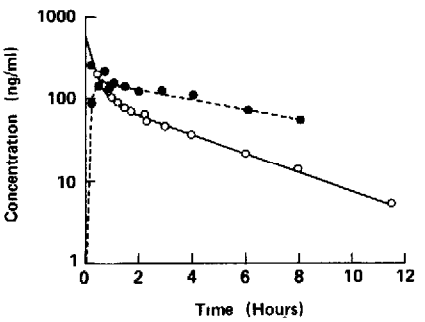


Fig. 2. Plasma diltiazem and desacetyldiltiazem concentrations and the derived diltiazem pharmacokinetic function following intravenous diltiazem hydrochloride (50 mg) administration to a healthy, 24-year-old female subject, body weight of 59.1 kg. See Table I for derived kinetic variables. (○) Diltiazem; (●) desacetyldiltiazem.

TABLE I

DERIVED DILTIAZEM PHARMACOKINETIC PARAMETERS AFTER A SINGLE 50-mg INTRAVENOUS DOSE TO A HEALTHY 24-YEAR-OLD FEMALE

| | |
|---|---|
| Distribution half-life (min) | 10.6 |
| Elimination half-life (h) | 2.62 |
| Central compartment volume (l/kg) | 1.10 |
| Volume of distribution at steady state (l/kg) | 3.89 |
| Volume of distribution by area (l/kg) | 4.73 |
| Total clearance (ml/min/kg) | 20.9 |

## DISCUSSION

A reliable, sensitive, and selective method is described for quantitation of both diltiazem and desacetyldiltiazem in human plasma by HPLC using ultraviolet detection.

A neutral pH extraction from plasma with acidic back-extraction, with direct injection of the acidic extract into the chromatographic system is the method employed. The pH of the extraction should not be permitted to exceed 8.5, since we find diltiazem to be unstable under basic conditions. When a basic solution is sequentially sampled and analyzed for drug and metabolite by the described method, the measured diltiazem concentration decreases in a time-related manner, with simultaneous increase in peak height of the chromatographic peak associated with desacetyldiltiazem. This suggests either in vitro deacetylation to desacetyldiltiazem in base or formation of a different decay product with identical chromatographic retention to that of desacetyldiltiazem in this chromatographic system. Blank human plasma samples are free of contaminants in the areas corresponding to the retention times for diltiazem, desacetyldiltiazem, and internal standard (desipramine). Other metabolites are more polar and are conjugated and rapidly filtered by the kidney, therefore do not interfere with this method in plasma [8]. This method may offer advantages over previously reported methods in that derivatization is not required, sensitivity is adequate for both drug and metabolite for single-dose pharmacokinetic studies and time required for sample preparation and analysis is short. In addition, other reported sample extraction and clean-up procedures which have been adequate for animal plasma samples [7] do not provide chromatograms which are free of interfering peaks when human plasma is extracted. Therefore we find this extraction procedure and chromatographic technique particularly useful for human pharmacokinetic studies.

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