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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF DILTIAZEM AND ITS METABOLITE IN PLASMA

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SUMMARY

A rapid, selective and reproducible high-performance liquid chromatographic method for the analysis in plasma of the calcium channel blocking agent, diltiazem, and one of its metabolites, deacetyldiltiazem is described. The method involves extraction with the methyl *tert.*-butyl ether of the drugs and the internal standard (verapamil), back-extraction into sulphuric acid and reversed-phase chromatography with UV detection. Over a concentration range of 10—1000 ng/ml the average coefficient of variation for diltiazem was 5.4% and for deacetyldiltiazem was 8.3%.

INTRODUCTION

Diltiazem (Fig. 1) is a newer member of the calcium channel blocking agents which is currently undergoing intensive investigation. To date some pharmacokinetic data have been obtained [1—3] using the gas chromatographic method of Rovei et al. [4]. This method which uses a nitrogen detector is quite time consuming as it involves several extraction steps and requires silylation for the quantitation of the parent drug and its metabolite, deacetyldiltiazem. We have therefore developed a simpler high-performance liquid chromatographic (HPLC) method for the measurement of diltiazem and deacetyldiltiazem in plasma that is suitable for the analysis of clinical samples.

EXPERIMENTAL

Reagents and materials

Diltiazem, *d*-3-acetoxy-*cis*-2,3-dihydro-5-[2-(dimethylamino)ethyl]-2-(*p*-methoxyphenyl-1,5-benzothiazepin-4(5H)-one, hydrochloride and its deacetyl metabolite (II) were supplied by Marion Laboratories (Kansas City, MO,

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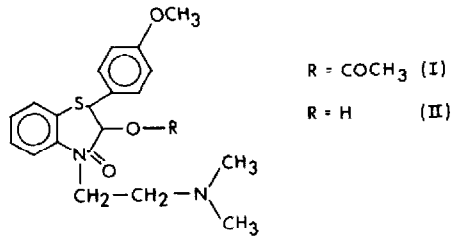

Fig. 1. Structural formulae of diltiazem (I) and deacetyldiltiazem (II).

U.S.A.). Verapamil, α-[3-[[2-(3,4-dimethoxyphenyl)ethyl]-methylamino]propyl]-3,4-dimethoxy-(1-methylethyl)-benzeneacetonitrile, hydrochloride, was used as an internal standard and supplied by Searle Labs. (Chicago, IL, U.S.A.). All analytical standards were of pharmaceutical grade (> 99% purity) and stock solutions in methanol were stored at —20°C. Ammonium dihydrogen phosphate and triethylamine were purchased from Aldrich (Milwaukee, WI, U.S.A.). Methanol and methyl *tert.*-butyl ether were of HPLC grade and obtained from Fisher Scientific (Fairlawn, NJ, U.S.A.). All other reagents were of analytical grade.

Sample preparation

Plasma (1 ml) is placed in a 15-ml capacity culture tube fitted with a PTFE-lined screw cap and the internal standard, 50 μl of verapamil (750 ng per 50 μl) is added. The samples are extracted with 5 ml of methyl *tert.*-butyl ether by shaking for 10 min on a Labquake Shaker at 80—100 strokes/min. Following centrifugation at 1000 *g* for 5 min, the tubes are immersed in a dry ice—acetone mixture and the upper organic phase transferred to a glass tube with an elongated cone bottom of approximately 100-μl volume; then 80 μl of 0.05 mol/l sulphuric acid are added and the mixture agitated in a Vortex mixer for 45 sec. After brief centrifugation, a 50-μl sample of the aqueous phase is injected onto the column.

Chromatography

The HPLC solvent delivery system is a Constametric III pump (Laboratory Data Control, Riviera Beach, FL, U.S.A.). The analytical column is a prepacked (25 cm × 4.6 mm I.D.) stainless-steel column containing Zorbax™ CN (6 μm) polar bonded-phase packing used in the reversed-phase mode (DuPont, Wilmington, DE, U.S.A.). A six-port rotary valve injector (Model 7125, Rheodyne, Berkeley, CA, U.S.A.) with a 50-μl sample loop is used for sample injection. A cyano guard column cartridge (Brownlee Labs., Santa Clara, CA, U.S.A.) is used between the injector and the analytical column which effectively minimizes the accumulation of particulate matter on the analytical column. The mobile phase is a mixture of methanol—0.05 *M* ammonium dihydrogen phosphate—triethylamine (45:55:0.25, v/v), the pH being adjusted to 5.0 with 1.0 *M* phosphoric acid. The solvent flow-rate is 1.5 ml/min with a column inlet pressure of 103.4 bars. The eluate is monitored continuously for absorbance at 237 nm using a variable-wavelength Spectro-Monitor III (Laboratory Data Control) and the detector output is displayed on a Linear Instruments Model 858 dual-pen recorder (Irvine, CA, U.S.A.).

Calibration

Calibration curves were constructed by transferring the respective stock solutions of diltiazem and deacetyldiltiazem in methanol (10—1000 μl) to culture tubes in amounts to give final concentrations of 10, 25, 50, 100, 250, 500, 800 and 1000 ng/ml. These standards were evaporated to dryness under nitrogen at 37°C and the residue dissolved in 1.0 ml plasma before extraction and assay. The amounts of the drug and metabolite in the unknown samples were obtained through weighted linear regression analysis of the standards. The sample peaks were measured and analyzed using peak height ratios (peak height of diltiazem or the deacetyl metabolite divided by the peak height of the internal standard) using linear regression employing the reciprocal of the concentration ($1/c$) as the weighting factor [5]. A 100-fold change in concentration is used in our calibration curve due to a wide variation in plasma levels depending on the route of administration and the dosage given. To quantitate the lower concentrations with better relative accuracy it was necessary to employ $1/c$ as the weighting factor rather than unit weighting which is generally used in linear regression analysis. As an alternative to weighted linear regression, two standard curves could be used, one covering the lower range (10—500 ng/ml), and another covering the higher range (500—1000 ng/ml) of concentrations. The advantage in choosing the former method lies in its convenience, and the simplicity of having one equation to define the entire concentration range. The coefficient of variation (C.V.) was used to determine the day-to-day reproducibility of the method and was calculated using normalized peak height ratios (the peak height ratio divided by the concentration of the standard).

Application of the method

This method of analysis has been employed by us in the study of diltiazem kinetics in patients with paroxysmal supraventricular tachycardia. On separate days, patients received a 20-mg infusion of diltiazem, and a 60- or 90-mg oral dose. Samples of venous blood were drawn at various intervals after each dose for 12 h. The plasma was separated and stored at −70°C for later analysis.

RESULTS AND DISCUSSION

The present HPLC assay involves an extraction into methyl *tert.*-butyl ether, followed by back-extraction into 0.05 mol/l sulphuric acid and reversed-phase chromatography using a Zorbax CN column and UV detection. The back-extraction step into 0.05 mol/l sulphuric acid greatly decreased the chromatographic interference from plasma constituents. Initial studies showed that altering the pH of the plasma samples was unnecessary for good recovery. The absolute and relative recoveries for diltiazem and its metabolite are shown in Table I. Compared with diethyl ether, the use of methyl *tert.*-butyl ether gave much cleaner chromatograms of blank plasma samples and back-extraction into 0.05 mol/l sulphuric acid gave increased sensitivity compared with other acids such as acetic, hydrochloric and phosphoric acid. The normality of the sulphuric acid was very critical for the resolution of the peaks of interest. Below 0.05 mol/l sulphuric acid the resolution was lost, and above 0.05 mol/l sulphuric acid the column life deteriorated.

TABLE I

ANALYTICAL RECOVERIES AND EXTRACTION PRECISION

Each value represents the mean of five determinations.

Drug	Concentration (ng/ml)	Recovery* (%)			
		Relative	C.V. (%)	Absolute	C.V. (%)
Diltiazem	50	100	7.8	95	13.4
	500	100	4.2	88	11.3
Deacetyldiltiazem	50	99	9.6	93	15
	500	98	3.5	92	11.5

*The relative recovery was determined by comparing peak heights of the compounds after an extraction out of 1 ml plasma to an identical extraction out of water. To determine absolute recovery the drug was evaporated in a test tube and was extracted in the same manner as the plasma and water extraction. This extraction was compared to the plasma extraction to determine the absolute recovery. Verapamil (750 ng) was added to all tubes just prior to the back-extraction into 0.05 mol/l sulphuric acid.

The Zorbax CN column was chosen after comparison with reversed-phase (C_{18}) and adsorption (silica gel) columns. Of the microparticulate columns tested the Zorbax CN column exhibited a greater separation selectivity. The relative retention times of deacetyldiltiazem, diltiazem, and the internal standard were 2.50, 3.35 and 4.0, respectively. Fig. 2 illustrates a typical chromatogram for control human plasma and a sample from a patient who received an oral dose of diltiazem. There were no interfering peaks detected in control human plasma or clinical samples at the retention times corresponding to diltiazem, its metabolite and the internal standard. However, we found — as did Rovei et al. [4] — that an interfering, endogenous peak was detected from samples that were assayed after having been frozen for a period of 3—4 weeks. The response of the UV detector to the amount of diltiazem and deacetyldiltiazem added to human plasma was linear over the 100-fold range in drug

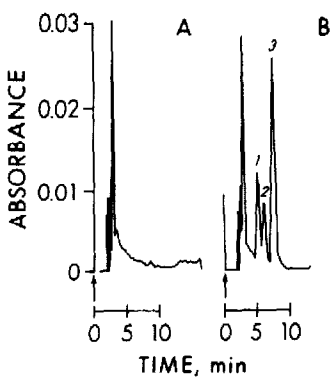

Fig. 2. Chromatograms of (A) control plasma and (B) plasma from a patient who had received diltiazem orally, showing peaks for deacetyldiltiazem (1), diltiazem (2) and the internal standard, verapamil (3) at concentrations of 13, 15 and 600 ng/ml, respectively. Mobile phase flow-rate was 1.5 ml/min and detector output displayed at a recorder chart speed of 12 cm/h; a.u.f.s. 0.01.

concentrations used (10—100 ng/ml). Correlation coefficients of 0.999 were obtained from the five calibration curves for both drug and metabolite.

The combination of low detector noise following injections of plasma extracts, high extraction efficiencies, and the high molar absorptivities at 237 nm in 0.05 mol/l sulphuric acid of diltiazem and its metabolite permit their determination in human plasma at low concentrations. The accuracy and within-run precision of the present method were determined by assaying plasma samples containing added diltiazem and deacetyldiltiazem (Table II).

TABLE II

WITHIN-RUN PRECISION AND ACCURACY

Each value represents the mean of eight determinations.

Drug	Concentration (ng/ml)		C.V. (%)
	Added	Found (±S.D.)	
Diltiazem	10	9.8 ± 1.25	12.8
	25	25.8 ± 2.85	11.1
	50	51.2 ± 3.18	6.2
	100	98.5 ± 4.31	4.3
	250	243.4 ± 4.48	1.8
	500	498.3 ± 25.19	5.1
	800	814.0 ± 38.27	4.7
	1000	989.0 ± 34.29	3.5
Deacetyldiltiazem	10	10.4 ± 0.521	5.0
	25	26.0 ± 2.32	8.9
	50	51.8 ± 4.76	9.2
	100	99.0 ± 6.35	6.4
	250	257.0 ± 16.38	6.3
	500	486.8 ± 28.60	5.8
	800	789.5 ± 39.18	4.9
	1000	1054.0 ± 58.24	5.5

The limit of quantitation is 10 ng/ml for diltiazem and deacetyldiltiazem defined as minimum signal-to-noise ratio of 4 and a C.V. of 15% or less. The lower limit of detection however, was less than 5 ng/ml. These concentrations generally yielded C.V. values greater than 15%. Therefore, 10 ng/ml is taken as a lower practical limit of quantitation. The reproducibility of the daily standard curves over a period of one month had an average C.V. of 5.4% for diltiazem and 8.3% for deacetyldiltiazem over the linearity range of the assay (Table III).

Several drugs which are often concurrently administered to cardiac patients were also examined for their possible interference with the quantification of diltiazem and its metabolite using this assay procedure (Table IV). None of the drugs tested interfered in the assay.

The application of the method in a patient who received an infusion of diltiazem is illustrated in Fig. 3. Diltiazem concentrations rose to a peak

TABLE III

STANDARD CURVE REPRODUCIBILITY OVER A PERIOD OF ONE MONTH

Drug	Standard curve No.	X of NPHR*	Standard deviation	C.V. (%)
Diltiazem	1	0.0042	0.0002	4.7
	2	0.0078	0.0003	3.7
	3	0.0049	0.0003	6.2
	4	0.0075	0.0003	3.7
	5	0.0042	0.0004	8.4
	6	0.0044	0.0003	5.9
			Mean =	5.4
Deacetyldiltiazem	1	0.0097	0.0011	11
	2	0.0115	0.0009	7.9
	3	0.0075	0.0006	7.7
	4	0.0113	0.0007	6.2
	5	0.0116	0.0013	12
	6	0.0118	0.0006	4.8
			Mean =	8.3

*NPHR = Peak-to-height ratio divided by concentration; C.V. = standard deviation divided by NPHR.

TABLE IV

RELATIVE RETENTION (K') OF DILTIAZEM, ITS MAJOR METABOLITE, OTHER CARDIOVASCULAR DRUGS AND DRUGS OFTEN ADMINISTERED CONCURRENTLY WITH DILTIAZEM

Drug	K'
Diltiazem	2.50
Deacetyldiltiazem	3.25
Verapamil	4.00
Disopyramide	1.25
Lidocaine	0.88
Procainamide	0.50
N-Acetylprocainamide	0.75
Quinidine	2.95
Pindolol	1.25
Timolol	1.00
Metoprolol	0.75
Propranolol	2.00
Atenolol	0.50
Cimetidine	0.50
Diazepam	1.50
Clonazepam	1.45
Lorazepam	1.00
Nitrazepam	1.00
Oxazepam	1.40

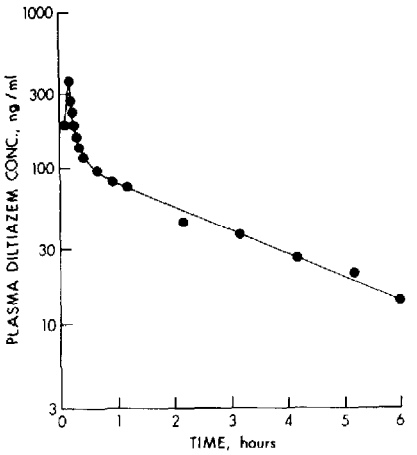

Fig. 3. Semilogarithmic plot of plasma diltiazem concentrations during and after a 10-min infusion of 20 mg of the drug.

at the end of the infusion and then declined biexponentially with a terminal half-life of 2 h consistent with the literature [2]. Concentrations of deacetyldiltiazem (not shown) rose early to a peak of 30 ng/ml and were undetectable by 30 min.

The present HPLC method described for the analysis of diltiazem and its deacetyl metabolite is accurate and reproducible within the apparent range of effective concentrations in plasma and is rapid enough that one person can analyze 15—20 samples daily in addition to the calibration curve samples. The present method is currently used in this laboratory for monitoring plasma levels during oral, intravenous, and steady state pharmacokinetic studies.

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