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Determination of diltiazem hydrochloride in human serum by high-performance liquid chromatography

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ABSTRACT

A simple and sensitive reversed-phase high-performance liquid chromatographic method for the determination of diltiazem in human serum has been developed. The method involves a one-step deproteinization of serum for sample clean-up using acetonitrile. A LiChrosorb RP-8 column (30 cm × 4.1 mm I.D.) was eluted isocratically with acetonitrile–0.01 *M* dibasic sodium phosphate (40:60, v/v) containing 0.01% triethanolamine. Diltiazem was monitored at 237 nm and 0.1 a.u.f.s. The completion time for assay was less than 15 min, and the lower limit of quantitation was 10 ng/ml for a 100-μl injection volume. Using this method, the pharmacokinetic parameters were calculated from a serum concentration *versus* time profile of diltiazem.

INTRODUCTION

Diltiazem hydrochloride, *cis*-(+)-3-acetoxy-5-(2-dimethylaminoethyl)-2,3-dihydro-2-(4-methoxypenyl)-1,5-benzothiazepin-4(5*H*)-one hydrochloride (Fig. 1), is a calcium channel blocker currently used in the treatment of clinical manifestations of variant angina [1–5], and is also effective in the treatment of hypertension [6] and cardiac arrhythmias [7]. Diltiazem undergoes presystemic metabolism after an oral dose, which results in only 40% bioavailability [8]. After oral administration, peak plasma diltiazem concentrations are achieved within 1.5 h for the fast-release formulation and within 3–4 h for the sustained-release formulation. About 90% of the administered dose is absorbed, but extensive first-pass metabolism limits the absolute bioavailability to 30–40%. The absolute bioavailability is dose-related, but does not depend on the formulation [9].

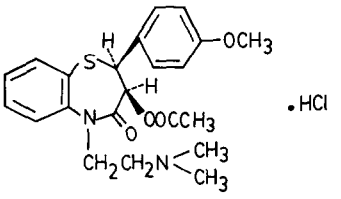

Fig. 1. Structure of diltiazem hydrochloride.

Several methods for the determination of diltiazem in biological samples using gas chromatography and nitrogen-sensitive and electron-capture detection [10,11] have been reported. However, these methods require time-consuming extraction procedure with an internal standard, and silylation of the metabolites for detection. Thin-layer chromatography has been used to analyse the drug in human biological samples [2]. High-performance liquid chromatography (HPLC) has also been used to determine diltiazem in human biological samples [11–17]. Solid-phase extraction and liquid–liquid extraction methods are also used to clean the sample for the determination of diltiazem hydrochloride [18,19].

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These methods are not efficient, as the analytical recovery was 82–86% for solid-phase extraction and 67–82% for liquid–liquid extraction. Other methods involving liquid–liquid extraction entail increased sample handling during clean-up [20–22]. These methods have no distinct advantage, as the recovery of diltiazem hydrochloride ranges between 70 and 85%, and a coefficient of variation of *ca.* 10% is observed. For the determination of diltiazem hydrochloride along with its metabolites, liquid–liquid extraction is used where an additional clean-up step is involved, because of background interferences observed during chromatographic analysis [23,24]. Another method, involving automated HPLC with column-switching for online clean-up and analysis of diltiazem and its metabolites in human plasma, has been reported [25].

This paper describes a simple and rapid sample preparation method. The extraction process using organic solvents, and the evaporation of solvents and derivatization steps have been eliminated from the sample preparation procedure. The sensitivity, reproducibility and selectivity of this method are adequate to monitor diltiazem concentrations in serum or plasma down to 2.5 ng/ml. The pharmacokinetics of the drug in healthy subjects were followed after a single 90-mg sustained-release oral dose. Pharmacokinetic parameters, such as the elimination rate constant (k_{el}), the elimination half-life ($t_{1/2}$), the time required to reach maximum concentration (t_{max}), the maximum concentration of diltiazem in serum (C_{max}) and the area under the curve (AUC), were calculated from time profile of two diltiazem formulations in a cross-over study.

EXPERIMENTAL

Equipment

The chromatographic system consisted of a Waters 510 dual-pump solvent-delivery system (Waters, Milford, MA, USA) equipped with a 100-μl loop and a Rheodyne 7125 sample injector (Rheodyne, Cotati, CA, USA). A reversed-phase LiChrosorb RP-8 column (300 mm × 4.1 mm I.D., 10 μm particle size) was used. A Waters 490 programmable multi-wavelength UV–visible detector was used. The chromatograms were recorded on a Waters 745 data module integrator. A 3010 W variable pipette was used for all quantitative sampling. Deionized water was obtained from a Milli-Q system (Millipore, Milford, MA, USA). The samples were centrifuged in a Remi RBC centrifuge (Remi Motors, Bombay, India). A Model AE163 analytical balance (Mettler Instruments, Highstown, NJ, USA) was used for weighing reagents. A vortex mixer (Kumar Industries, Bombay, India) was used. A Waters guard column (23 mm × 3.6 mm I.D.) dry-packed with 10-μm C_8 particles was used throughout the experiment.

Materials

Diltiazem hydrochloride (Cheminor Drug, Bombay, India) was assayed as per USP XXII before use. Acetonitrile, triethanolamine (HPLC grade), dibasic sodium phosphate, orthophosphoric acid and sodium hydroxide were obtained from E. Merck (Bombay, India). The borosilicate glass culture tubes and glass pipettes were obtained from Borosil glasses (Bombay, India). Fresh serum was collected from healthy human volunteers for the development of the method.

Chromatographic conditions

The mobile phase consisted of 40% acetonitrile in 0.01 *M* dibasic sodium phosphate and 0.1% (v/v) triethanolamine dissolved in ultrapure deionized water. The pH of the mobile phase was adjusted to 3.0 ± 0.1 with 85% (w/v) orthophosphoric acid. The mobile phase was filtered through a 0.45-μm Millipore filter before use and delivered at a flow-rate of 1.2 ml/min. Diltiazem was monitored by a UV–visible absorbance detector at a wavelength of 237 nm and 0.1 a.u.f.s.

Sample collection and storage

Blood samples were collected in dry borosilicate tubes. Each sample was centrifuged after coagulation of blood, and serum was separated. Serum was then stored at −20°C until analysis so as to prevent metabolic changes.

Preparation of stock solution

A stock solution of diltiazem hydrochloride was prepared by dissolving 300 mg in 50 ml of water. This was further diluted suitably to get a stock solution containing 4800 ng/ml diltiazem hydrochloride. The diltiazem hydrochloride solution was diluted in serum to construct the calibration curves. The retention time of diltiazem was determined by injecting an aliquot of the standard solution into the HPLC system.

Isolation of diltiazem from serum before chromatography

The frozen serum was thawed at 25 ± 2°C (room temperature), and 1.0 ml was pipetted into a clean, disposable, borosilicate, graduated centrifuge tube. An aliquot of the standard solution of diltiazem hydrochloride was added to 1.0 ml of serum and was mixed on a vortex-mixer for 30 s. A 200-μl aliquot of 1 *M* sodium hydroxide solution was added to serum containing diltiazem hydrochloride and again vortex-mixed for 30 s. Acetonitrile was added to make the volume to 5 ml, and the solution was mixed on a vortex-mixer for 2 min and then centrifuged at 2200 *g* for 5 min at room temperature. The supernatant was decanted into a fresh borosilicate culture tube. A 100-μl volume of this solution was injected into the HPLC system.

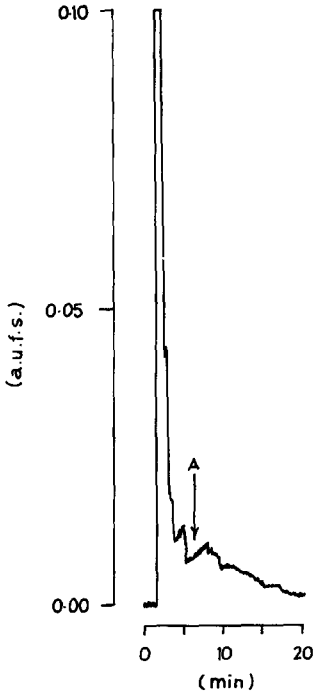

Fig. 2. Representative chromatogram of blank serum at detection wavelength of 237 nm and 0.1 a.u.f.s. A = retention time of diltiazem.

Construction of the calibration curve

Nine solutions of diltiazem hydrochloride were prepared by adding enough stock solution to obtain concentrations of *ca.* 10, 25, 50, 100, 150, 200, 300, 400 and 500 ng/ml. These solutions were prepared in serum collected from human volunteers and in water. Under the experimental conditions, the calibration curve was linear up to 500 ng/ml.

RESULTS AND DISCUSSION

Fig. 2 shows a typical chromatogram of serum with no diltiazem, and Fig. 3 shows a chromato-

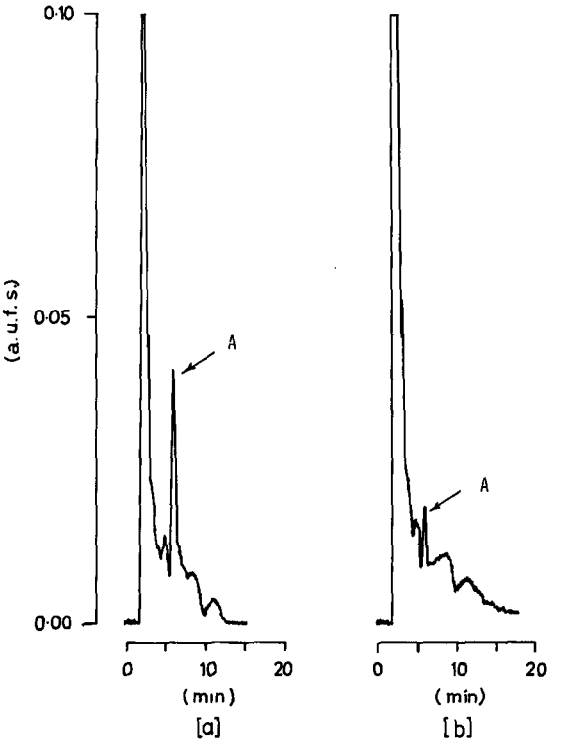

Fig. 3. Chromatograms of serum from a subject dosed with 90 mg of diltiazem hydrochloride, (a) 2.5 h and (b) 12.0 h after administration. The concentrations detected are 150 and 10 ng/ml, respectively. Detection was at 237 nm and 0.1 a.u.f.s. Peak A = diltiazem.

TABLE I

CALIBRATION CURVE OF DILTIAZEM HYDROCHLORIDE (n = 3)

In the linearity curve $y = mx + c$, x = concentration of diltiazem hydrochloride (ng/ml) and y = chromatographic peak area.

Fluid	Slope	Correlation coefficient	y-Intercept
Water	0.98	0.99	−2.18
Serum	0.96	0.99	1.15

gram of the serum of a subject who had ingested 90 mg of diltiazem hydrochloride sustained-release tablets. The chromatogram shows that the parent drug is well separated from the endogenous peaks of serum. There is no degradation of diltiazem hydrochloride during sample preparation, as the peak area is unchanged with respect to the standard peak.

The calibration curves were made in water and serum. The experiment was repeated three times, using serum obtained from different volunteers. The parameters of the two calibration curves are given in Table I. Statistical calculations of two curves at a 95% confidence level showed that the slopes of all curves were identical.

The addition of sodium hydroxide solution and acetonitrile in serum during the isolation of diltiazem before chromatography completely removes the proteins and endogenous substances. This sample clean-up procedure resulted in a clean chromatogram with no interfering peaks. The same column was later used for pharmacokinetic studies and, after at least 400 sample injections, its performance remained unchanged.

The reproducibility and accuracy of the method were determined by repetitive analysis of serum spiked with standard diltiazem hydrochloride solution. The data obtained for same-day and day-to-day analysis of the serum sample stored at −20°C are given in Tables II and III. It appears from the results that the relative standard deviation (R.S.D.) for same-day analysis

TABLE II

SAME-DAY REPRODUCIBILITY OF DILTIAZEM HYDROCHLORIDE IN SERUM

Concentration (ng/ml)	Concentration determined (mean ± S.D., n = 3) (ng/ml)	Relative standard deviation (%)	Deviation from theory (%)
10	9.97 ± 0.08	0.80	−0.30
50	48.54 ± 0.75	1.54	−2.92
100	96.81 ± 1.60	1.65	−3.19
200	213.03 ± 6.50	3.05	+6.52

TABLE III

DAY-TO-DAY REPRODUCIBILITY OF DILTIAZEM HYDROCHLORIDE IN SERUM

Concentration (ng/ml)	Concentration determined (mean ± S.D., n = 3) (ng/ml)	Relative standard deviation (%)	Deviation from theory (%)
10	10.06 ± 0.07	0.69	+0.60
50	51.28 ± 0.60	1.17	−2.56
100	95.13 ± 2.45	2.58	−4.87
200	204.09 ± 2.00	0.98	+2.05

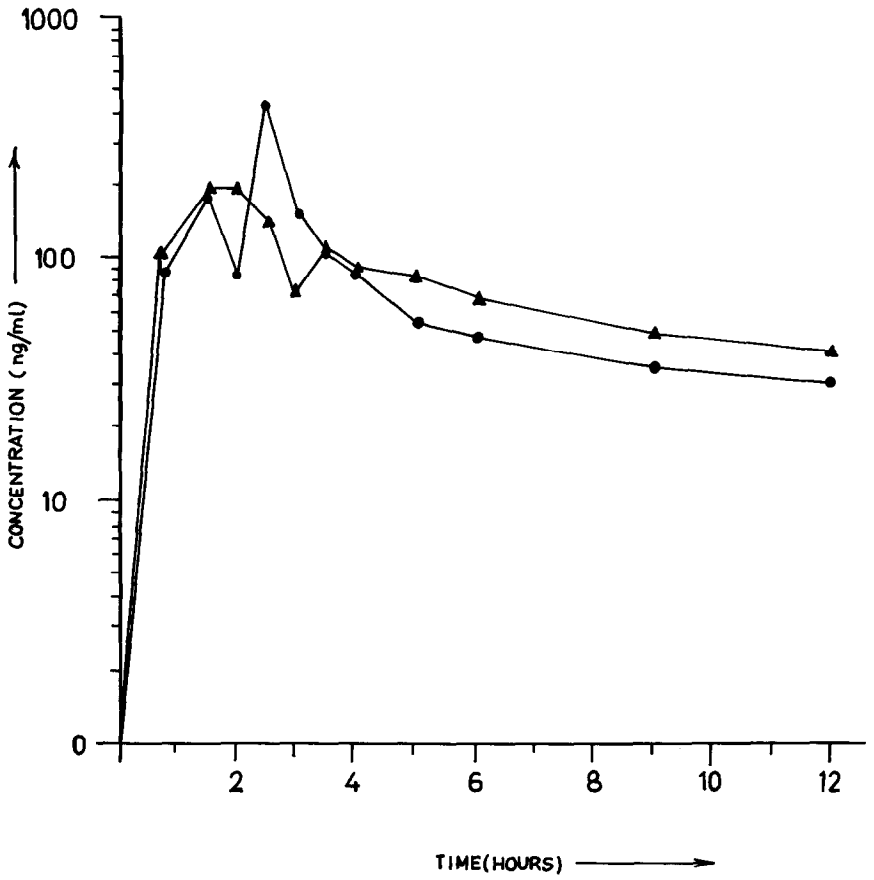

Fig. 4. Serum concentration *versus* time curve following oral administration of 90 mg of diltiazem hydrochloride: (●) formulation A; (■) formulation B.

and day-to-day analysis is within the 6% limit given by US Pharmacopeia for assay validity. There is no significant effect of the concentration of the drug in the samples. The mean recovery (n = 6) for the described method was 97.32 ± 2.67% for diltiazem hydrochloride. The lower limit of detection was 2.5 ng/ml, but was not quantifiable accurately.

TABLE IV

PHARMACOKINETIC PARAMETERS OF DILTIAZEM FOLLOWING A SINGLE 90-mg DOSE OF DILTIAZEM HYDROCHLORIDE SUSTAINED-RELEASE FORMULATIONS A AND B

Pharmacokinetic parameter	Formulation A (mean ± S.D., n = 12)	Formulation B (mean ± S.D., n = 12)
C_{max} (ng/ml)	487.87 ± 212.60	262.26 ± 139.06
T_{max} (h)	2.25 ± 0.43	1.80 ± 0.35
$T_{1/2}$ (h)	4.51 ± 2.37	6.42 ± 3.60
k_{el} (h^{-1})	0.11 ± 0.01	0.09 ± 0.01
AUC_{0-inf} (ng · h/ml)	1129.61 ± 718.53	1264.90 ± 639.28

The use of triethanolamine in the mobile phase increases the selectivity for diltiazem and improves the elution characteristics to produce sharp peaks. The assay procedure was used in pharmacokinetic studies with eight normal healthy volunteers on dosage regimens of 90 mg of diltiazem hydrochloride sustained-release formulations A and B. Fig. 4 shows average serum concentration–time curves following a morning 90-mg oral dose of diltiazem hydrochloride. The pharmacokinetic parameters used to measure the rate and extent of absorption and the elimination of diltiazem were estimated (Table IV). AUC_{0-inf} values were calculated by the trapezoidal method, and the average was found to be 1129.61 ng h/ml for formulation A and 1264.90 ng · h/ml for formulation B.

CONCLUSIONS

The reversed-phase HPLC method described in this paper can be used to determine accurately concentrations of diltiazem hydrochloride from 10 to 500 ng/ml in human serum. The sample preparation method is simple, and the chromatographic conditions are uncomplicated. The sensitivity, reproducibility and accuracy of the method make it suitable for routine pharmacokinetic studies of diltiazem hydrochloride.

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