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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF DILTIAZEM AND FOUR OF ITS METABOLITES IN PLASMA

APPLICATION TO PHARMACOKINETICS

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SUMMARY

A selective and very sensitive ion-pairing reversed-phase high-performance liquid chromatographic method has been developed for the simultaneous determination of diltiazem and four of its metabolites in plasma. The structurally related compound propionyl deacetyldiltiazem was used as the internal standard and bromide was employed as the pairing ion. Plasma samples were extracted with methyl-*tert.*-butyl ether followed by back-extraction into 0.01 *M* hydrochloric acid and evaporation to dryness. High-performance liquid chromatographic analysis was carried out using C_{18}-bonded silica and methanol—ammonium bromide—acetonitrile—triethylamine as the mobile phase. Using UV detection at 237 nm, the lower limit of detection in plasma was 0.1—0.2 ng/ml; calibration curves were linear between 1 and 800 ng/ml.

The present assay procedure has been applied to monitoring plasma levels in intravenous and oral pharmacokinetic studies in several animal species and humans. The applicability of the new method could be confirmed by comparing plasma levels obtained by high-performance liquid chromatography with those obtained by gas chromatography.

INTRODUCTION

Diltiazem, *cis*-(+)-3-(acetyloxy)-5-[2-(dimethylamino)ethyl]-2,3-dihydro-2-(4-methoxyphenyl)-1,5-benzothiazepin-4-(5H)-one hydrochloride (I, Fig. 1), a member of the class of calcium-channel blocking agents, has been shown to be a potent coronary vasodilator [1, 2] used in the treatment of variant angina [3–5].

Several methods have been published for the determination of diltiazem in

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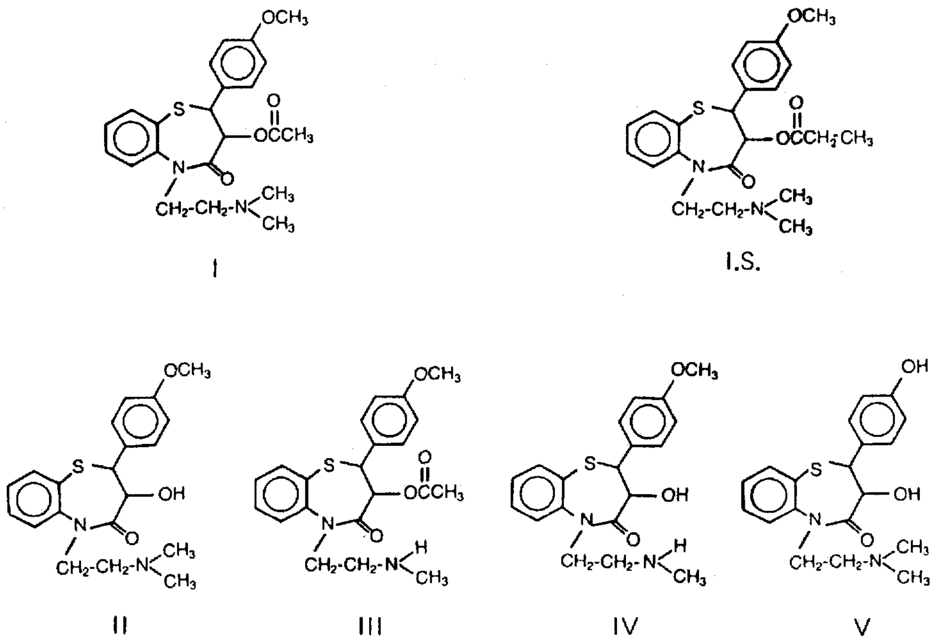

Fig. 1. Chemical structures of diltiazem (I), four of its metabolites (II—V) and the internal standard propionyl deacetyldiltiazem.

plasma, involving either gas chromatography (GC) using a nitrogen-sensitive [6, 7] or an electron-capture detector [8, 9], or high-performance liquid chromatography (HPLC) comprising UV detection [10, 11]. Most of these chromatographic procedures also include the quantitation of deacetyldiltiazem (II, Fig. 1), generally considered to be the principal metabolite of diltiazem, but they do not permit the separation and determination of additional metabolites present in plasma, particularly N-monodemethyldiltiazem (III, Fig. 1) which was recently found to be the major metabolite in humans [12]. Moreover, most techniques, especially those based on HPLC using UV detection, lack sensitivity. Therefore, we developed a very sensitive and selective HPLC method suitable for the quantitation of the generally low diltiazem plasma levels present during the terminal elimination phase in (single-dose) pharmacokinetic investigations. In addition, a simultaneous determination of four major metabolites of diltiazem was attained.

EXPERIMENTAL

Materials

Methanol (Merck, Darmstadt, F.R.G.) and acetonitrile (Rathburn Chemicals, U.K.) were of HPLC grade. Methyl-*tert.*-butyl ether (for synthesis; Merck-Schuchardt, F.R.G.) was distilled prior to use; all other solvents and chemicals were of analytical-reagent grade (Merck or Fluka, Switzerland). For aqueous solutions, water was purified by reverse osmosis and additionally passed through a water purification system for adsorption of organic substances (Millipore, Neu Isenburg, F.R.G.).

Diltiazem · HCl, deacetyldiltiazem · HCl and propionyl deacetyldiltiazem oxalate (internal standard) were obtained from the Chemical Department, Gödecke Research Institute, Freiburg, F.R.G. The N- and O-demethyl metabolites (III—V) were supplied by Tanabe Seiyaku (Japan). The structures of these compounds are given in Fig. 1.

Instrumentation

The following system was used for HPLC: a Perkin-Elmer Series 10 pump fitted with a six-port rotary valve (Model 7125, Rheodyne, Cotati, CA, U.S.A.) and a 50-µl sample loop; a Kratos Spectroflow 773 UV detector (Kratos, Karlsruhe, F.R.G.) which was operated at 237 nm; an LCI-100 laboratory computing integrator (Perkin-Elmer, Überlingen, F.R.G.). Prepacked stainless-steel columns (12.5 cm × 4.6 mm I.D.) containing C_{18}-bonded 5-µm silica (Spherisorb ODS-II) were purchased from Bischoff (Leonberg, F.R.G.).

Chromatography

The mobile phase consisted of methanol—0.04 *M* ammonium bromide in water—acetonitrile (40:36:24) and 0.06 ml of triethylamine to give a pH of ca. 8.5. The solvent mixture was degassed by ultrasonication immediately before use. All separations were performed isocratically at a flow-rate of 1.2 ml/min at ambient temperature.

Standard solutions

Stock solutions of I—IV were prepared by dissolving weighed quantities of the salts corresponding to 2 mg of the free base in 100 ml of water. Appropriate volumes of the aqueous stock solutions were diluted to give concentrations within the range 0.02—8 µg/ml.

These solutions were used as plasma standards by adding 50 µl to 1 ml of blank plasma, covering the concentration range 1—400 ng/ml for diltiazem and 1—100 ng/ml for the metabolites, respectively. A solution of the internal standard (I.S., Fig. 1) was prepared by diluting the stock solution (2 mg of free base per 100 ml) to give a final concentration of 1 µg/ml. When stored at 4°C, these solutions did not show any degradation for at least two months.

Extraction procedure

A 1-ml aliquot of plasma and 100 µl of the internal standard solution are placed in a 10-ml centrifuge tube, and the mixture is extracted with 5 ml of methyl-*tert*.-butyl ether by mixing for 20 min on a mechanical shaker. After centrifugation at 1200 *g* for 10 min, the organic phase is transferred to another centrifuge tube and back-extracted with 1.5 ml of 0.01 *M* hydrochloric acid. Following centrifugation, the organic phase is aspirated and discarded. The remaining aqueous phase is transferred to a tapered flask and evaporated to dryness on a rotary evaporator at 30°C. In order to ensure the complete removal of hydrochloric acid vapours, the evaporated flasks are flushed with a gentle stream of nitrogen. The residue is dissolved in 100 µl of the mobile phase and 20—50 µl aliquots are injected into the liquid chromatograph.

Data analysis

Calibration curves were obtained by assaying four to five blank plasma

samples spiked with diltiazem and metabolites covering the expected concentration range. Peak-height ratios were plotted against concentrations, and linear regression analysis was performed. The resulting slope was used to calculate the amount of diltiazem and its metabolites in unknown samples.

The plasma level—time data were evaluated by means of a non-linear least-squares regression procedure using the TOPFIT program package [13] on a desk computer (Wang 2200). The relevant pharmacokinetic parameters were calculated according to the procedures described in ref. 14.

RESULTS

Characteristics of the method

Selectivity and specificity. Fig. 2 shows typical chromatograms of an extract of blank human plasma (a), plasma spiked with compounds I–V and the internal standard (I.S.) (b) and two plasma samples of a volunteer who received diltiazem orally: 8 h after the last dose of a repeated administration of 3 × 60 mg (c); 16 h after the last administration (d). The identity of the metabolites found in plasma with the authentic substances II—IV was demonstrated by comparing the retention times and was confirmed by gas chromatography—mass spectrometry. Comparing chromatogram b with c and d, an additional peak was observed (M_x), probably representing another metabolite of I which

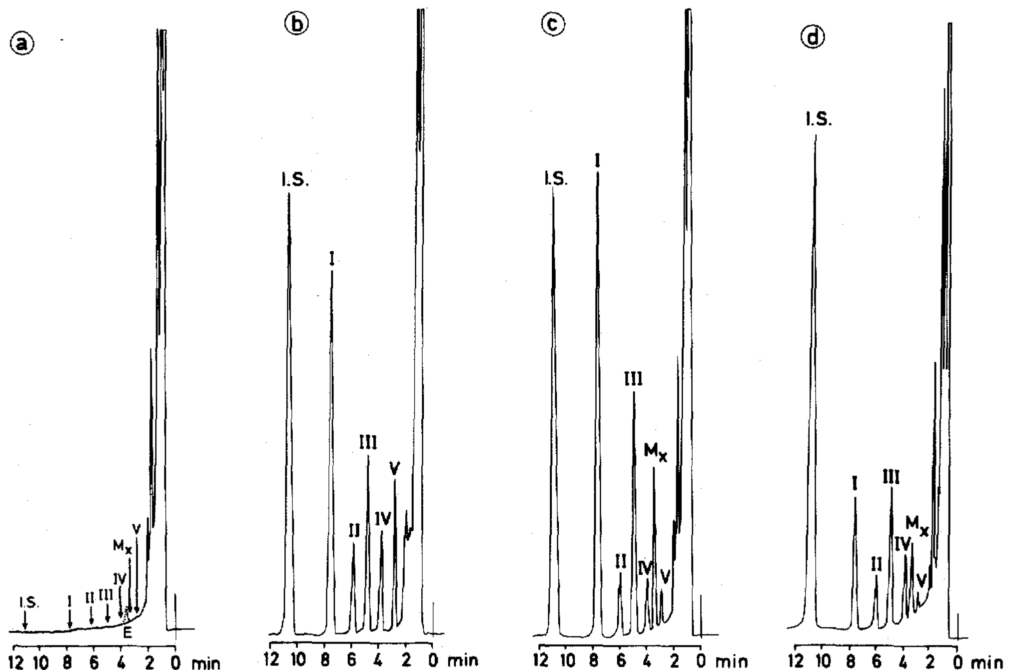

Fig. 2. Chromatograms of diltiazem and its metabolites from plasma (M_x: probably additional metabolite of I, not identified yet). (a) Blank human plasma; (b) plasma spiked with 50 ng of I, 20 ng of III, 10 ng of II, IV and V and 100 ng of I.S. (about half of the amount injected); (c) plasma of a human subject 8 h after the last dose of a repeated oral administration of 3 × 60 mg per day; (d) 16 h after the last administration.

has not been identified yet. All peaks had good shape and were well separated. There were no interfering peaks caused by endogenous substances except a small one (E, Fig. 2a) detected in some of the plasma samples and having a retention time similar to that of compound M_x. Concurrent administration of quinine or quinidine, however, may impede the quantitation of diltiazem and, in particular, of deacetyldiltiazem.

Recovery and detection limit. The recovery was determined at two levels (20 and 200 ng/ml) for all compounds by comparing the peak heights obtained by direct injection of standard aqueous solutions to those obtained after the whole extraction procedure (Table I). The recovery is ca. 80% and independent of concentration. Based on a signal-to-noise ratio of 4:1, the lower limit of detection was estimated at 0.2 ng/ml for compounds I–IV and ca. 0.1 ng/ml for V. However, since the lifetime of the stationary ODS phase is limited, the sensitivity decreases after several weeks of operation.

Linearity and reproducibility. The calibration curves were linear in the range tested, 1—800 ng/ml for the parent compound diltiazem and 1—400 ng/ml for the metabolites, with correlation coefficients $\geqslant$ 0.998. The accuracy (difference between the amount added to blank plasma and the amount found) and the reproducibility (represented by the relative standard deviation of the mean of replicate analyses) of the method were determined by processing blank plasma samples spiked with a higher and a lower amount of diltiazem and its metabolites. Six samples were run for each of the concentrations. The results are given in Table I.

Applicability of the method

Comparison with gas chromatographic data. The validity of the method was confirmed by comparing plasma levels obtained by this assay to those obtained by a GC method using electron-capture detection [8]. Blood samples were taken from different subjects who had received various doses of diltiazem orally. Plasma concentrations of diltiazem were determined first by GC and, more than six months later, by means of the HPLC technique described here. The results, illustrated in Fig. 3, show that HPLC and GC values are well corre-

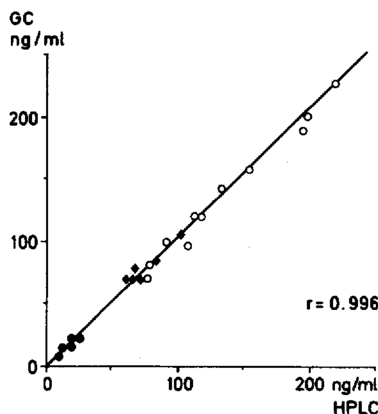

Fig. 3. Diltiazem plasma levels obtained by HPLC in comparison to those obtained by GC (slope of the straight line = 0.997; intercept not significantly different from zero). The different symbols represent different subjects who were given various doses of diltiazem.

TABLE I

RETENTION TIMES, RECOVERIES, DETECTION LIMITS, ACCURACIES, AND REPRODUCIBILITIES DERIVED FROM REPEATED ASSAYS

Compound	Retention time (min)	Recovery (%)	Detection limit (ng/ml)	Accuracy and reproducibility (n = 6)			
				Concentration added (ng/ml)	Concentration found (ng/ml)	Percentage difference from theoretical value	Coefficient of variation (%)
I	7.6	82.5	0.2	200	199.2	0.4	1.6
				10	10.1	1.0	2.3
II	6.0	81.5	0.2	100	102.2	2.2	2.1
				5	5.2	4.0	5.7
III	4.9	78.0	0.2	100	99.7	0.3	1.5
				5	5.0	0.0	2.6
IV	3.9	78.0	0.2	100	101.3	1.3	2.4
				5	5.2	4.0	4.9
V	2.9	82.0	0.1	100	100.9	0.9	2.9
				5	4.9	2.0	3.9
I.S.	10.8	82.0	—	—	—	—	—

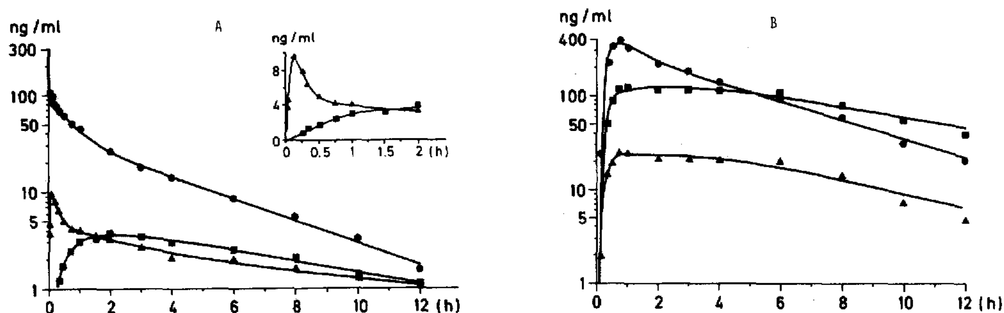

Fig. 4. Plasma level—time courses of diltiazem (●), deacetyldiltiazem (▲) and the principal metabolite N-demethyldiltiazem (■) following intravenous administration of 0.5 mg of I per kg (A) and after oral administration of 6 mg of I per kg (B) to a beagle dog.

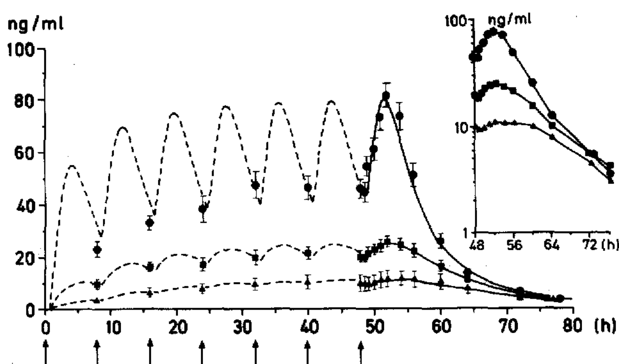

Fig. 5. Computer iteration best fits for diltiazem (●), demethyldiltiazem (■), and deacetyldiltiazem (▲) plasma levels following multiple administration of 60-mg doses of I (means ± S.E., n = 9), profiles after the last dose additionally in logarithmic scale.

lated (r = 0.996; slope = 0.997; intercept not significantly different from zero), indicating the validity of both methods and, in addition, the stability of the drug in plasma when stored at −20°C.

Application to pharmacokinetics. The new method has been applied to several pharmacokinetic studies analysing plasma samples of various animal species (dog, rabbit, guinea pig) as well as human volunteers and patients. Examples of plasma level—time courses are given in Figs. 4 and 5. Fig. 4 shows the concentration curves of diltiazem (I), deacetyldiltiazem (II) and demethyldiltiazem (III) following an intravenous dose of 0.5 mg of diltiazem per kg and after oral administration of 6 mg of diltiazem per kg as aqueous solution to a beagle dog.

Due to a considerable first-pass effect, the amount of the metabolites is significantly higher after oral administration, especially with regard to the principal metabolite III (the concentration of which exceeds that of diltiazem after about 5—6 h).

The half-lives were found to be 2.7—3 h for diltiazem and 5—6 h for metabolites II and III, respectively. [Comparison of area under the curve (AUC) values for diltiazem after intravenous and oral administration gave an absolute bioavailability of 63%.]

Plasma profiles of I, II and III (mean values ± S.E., $n = 9$), derived from a steady-state pharmacokinetic study in humans, are depicted in Fig. 5. The subjects were given seven 60-mg doses of diltiazem as tablets in 8-h intervals. Plasma samples were taken to determine pre-dose levels and the entire profiles following the last dose. Steady state was reached by the second day of treatment. As shown by Fig. 5 (logarithmic plot), the parent compound (I) is more rapidly eliminated than the metabolites. The respective mean half-lives were 6.4, 8.7 and 9 h for I, II and III, respectively.

DISCUSSION

The main problems in developing this assay were: (a) to achieve an adequate and rapid separation under isocratic conditions for a simultaneous determination of intact diltiazem and its metabolites present in plasma; (b) to suppress the interaction of the basic substances with the stationary support (especially for the hydroxylated compounds) in order to prevent peak tailing and broadening; (c) to obtain a sufficiently high sensitivity to quantitate very low plasma levels present during the terminal elimination phase in pharmacokinetic studies following single doses of diltiazem.

These problems were overcome by using ion-pair chromatography and adding organic bases to the eluent.

The best separations were achieved using bromide as the counter ion since larger organic counter ions may dominate the chromatographic behaviour of the ion pair formed, resulting in a decrease of selectivity [15]. In spite of ion-pairing, basic eluent conditions were still required to achieve good separations and well shaped peaks, especially for the deacetylated compounds. Triethylamine was chosen as the base additive because of its retention and separation properties, and since triamines exhibit the lowest attack on the silica matrix [16]. Nevertheless, salt-containing aqueous solvents may have a detrimental effect on C_{18} phases even under mild basic conditions and, in addition, crystallization may occur in the pump, injection valve and other parts of the chromatographic system. Therefore, the system was flushed with bromide and base-free eluent after analysis. Re-equilibration did not take any longer than 30 min.

When changing columns, there was usually no need to modify the composition of the mobile phase due to batch-to-batch variations of the reversed-phase material. If necessary, however, chromatographic conditions can be optimized by varying the concentration of the counter-ion and the amine additive.

Several non-polar solvents (e.g. *n*-hexane, cyclohexane, toluene, benzene and benzene—*n*-octanol) were tested as extractants; however, a more polar solvent was required to isolate the metabolites as well. Methyl-*tert.*-butyl ether was chosen because of its physical properties, particularly its stability against peroxide formation [17].

A back-extraction step was essential to minimize interfering peaks, especially when analysing in the lower nanogram range. Evaporation of the acid phase did not lead to any hydrolysis of the acetylated compounds under the conditions given. Nevertheless, it was found to be advantageous to remove residual hydrochloric fumes by a stream of nitrogen to prevent deacetylating during storage.

The method described proved to be very suitable for pharmacokinetic investigations. Its high sensitivity permits the determination of very low plasma levels of diltiazem and four of its metabolites. The selectivity of this assay enables the separation of diltiazem, deacetyldiltiazem and additional metabolites. This assay also revealed that, in confirmation of recent findings [12], N-demethyldiltiazem is the principal metabolite in plasma.

It is important to note that some authors did not mention any of these additional metabolites in spite of using the same extraction solvent (methyl-*tert*.-butyl ether) [10]. It is obvious that diltiazem peak coinciding with any of its metabolites will not only result in diltiazem plasma levels that are too high but, owing to longer half-lives of the metabolites, the measured half-life of diltiazem would appear to be prolonged, indicating unexpected accumulation following multiple oral administration.

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