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ASSAY OF DILTIAZEM AND DEACETYLDILTIAZEM BY CAPILLARY GAS CHROMATOGRAPHY

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SUMMARY

A highly sensitive gas chromatographic method for the analysis of diltiazem and deacetyldiltiazem in plasma or serum is reported. After silylation with bis(trimethylsilyl)trifluoroacetamide, separation was obtained on a cross-linked fused-silica column and detection was by electron-capture. The minimum measurable concentrations were 3 and 1 ng/ml for diltiazem and deacetyldiltiazem, respectively. Intra- and inter-day coefficients of variation were less or equal to 6.0 and 8.0%, respectively, for both compounds. The method was used to study the kinetics of a single oral dose of 60 mg of diltiazem hydrochloride in a patient with renal failure.

INTRODUCTION

Diltiazem, *d-cis*-(+)-3-(acetyloxy)-5-[2-(dimethylamino)ethyl]-2,3-dihydro-2-(4-methoxyphenyl)-1,5-benzothiazepine-4-(5*H*)-one (I, Fig. 1) is a calcium channel blocker mainly used in the treatment of variant angina [1,2]. Studies of the relationship between the serum concentrations of the drug and its effect have indicated that improvement of the symptoms of angina are obtained when serum concentrations are at least 100 ng/ml [3,4]. Diltiazem is extensively metabolized in humans by hydrolysis of the ester function, by O-demethylation and by N-oxidation of the side-chain. At least six metabolites have been identified in urine, where they are excreted mainly as glucuronide and/or sulphate conjugates [5,6].

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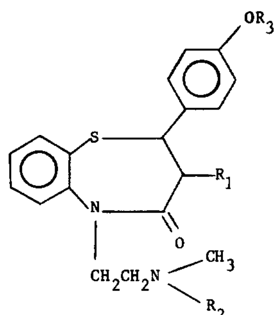

I	$R_1 = OCCH_3$ (C=O)	$R_2 = CH_3$	$R_3 = CH_3$
II	$R_1 = OH$	$R_2 = CH_3$	$R_3 = CH_3$
III	$R_1 = OCCH_3$ (C=O)	$R_2 = H$	$R_3 = CH_3$
IV	$R_1 = OH$	$R_2 = H$	$R_3 = CH_3$
V	$R_1 = OH$	$R_2 = CH_3$	$R_4 = H$

Fig. 1. Chemical structures of I and of metabolites II, III, IV and V.

Though diltiazem has been available for some years, very little information on its clinical pharmacokinetics is available. This may be due, in part at least, to difficulty in analysing the drug in biological fluids. Published methods for the analysis of I in human plasma are based on either gas chromatography (GC) on packed columns using either nitrogen [7,8] or electron-capture detection (ECD) [9,10] or high-performance liquid chromatography (HPLC) using ultraviolet detection [11–13].

We were interested in studying the kinetics of diltiazem and one of its metabolites, deacetyldiltiazem (II, Fig. 1) in patients with renal failure. The analytical method we needed had to be both highly selective, owing to the presence of higher concentrations of some endogenous compounds in the serum of these patients, and sensitive, since most of these patients are anaemic and, therefore, the volume of blood withdrawn at each sampling had to be minimal.

In our hands, the GC methods that have been published showed a number of peaks due to endogenous compounds, which interfered with either or both I and II. Furthermore, some of these methods [7,8] required more than 1 ml of serum in order that concentrations below 10 ng/ml could be measured. Similarly, the column liquid chromatographic methods that have been reported had to be abandoned owing either to lack of sensitivity [11,12] or to lack of equipment on our part [13]. We therefore developed a GC method for the analysis of I and II in serum or plasma, which involved separation on a capillary column and ECD. The proposed method also provides the basis for analysis of three other metabolites besides deacetyldiltiazem, namely N-monodemethyldiltiazem (III, Fig. 1), N-

monodemethyldeacetyldiltiazem (IV, Fig. 1) and O-demethyldeacetyldiltiazem (V, Fig. 1).

EXPERIMENTAL

Materials

Diltiazem and deacetyldiltiazem (as the hydrochloride salts) and metabolites III–V were kindly donated by Nordic Labs. (Montreal, Canada). Prazepam was purchased from the USP Reference Department (Rockville, MD, U.S.A.) and bis(trimethylsilyl)trifluoroacetamide (BSTFA) was obtained from Sigma (St. Louis, MA, U.S.A.). All other solvents and chemicals were obtained from the usual commercial sources and were used as received, except for *n*-pentane which was distilled before use.

Apparatus and chromatographic conditions

A Hewlett-Packard Model 5890 gas chromatograph equipped with a split injector, a ^{63}Ni electron-capture detector and a 30 m $\times$ 0.25 mm I.D. cross-linked fused-silica (5% phenyl polymethylsiloxane) capillary column was used (Durabond 5, Chromatographic Specialities, Brockville, Canada). The oven was operated isothermally at 265°C, and the detector and injection port temperatures were set at 300°C and 280°C, respectively. Helium was used as the carrier gas at a flow-rate of 1.0 ml/min and the make-up gas was argon–methane (95:5) at a flow-rate of 37 ml/min. A 50:1 split ratio was established, and the column head pressure was set at 13 lb./sq.in. Peak heights and areas were measured by a Hewlett-Packard 3390 A integrator.

Standard solutions

A standard solution of I was prepared by dissolving 5 mg of the hydrochloride salt in 1000 ml of water. A 2-ml aliquot of this solution was then diluted to 100 ml to give a final solution of 100 ng/ml. A 100 ng/ml solution of deacetyldiltiazem hydrochloride was prepared in a similar fashion. A standard methanolic solution of I was prepared by dissolving 2.5 mg of the hydrochloride salt in 500 ml of methanol. A 10-ml aliquot of this solution was then diluted to 100 ml to give a working solution of 500 ng/ml. A 500 ng/ml solution of II in dimethyl sulphoxide was similarly prepared. Prazepam (5 mg) was dissolved in 10 ml of methanol and the solution was made up to 1000 ml with water. A 1-ml aliquot of this solution was then diluted to 100 ml with water to give a working solution of 50 ng/ml prazepam.

Extraction and derivatization procedures

The pH of 1-ml aliquots of human plasma or serum was adjusted to 8 with Sorensen phosphate buffer (0.5 *M*). To each aliquot were added 1 ml of the prazepam solution (50 ng/ml) and 2 g of sodium chloride. The aqueous mixtures were mixed thoroughly on a Vortex for 30 s and the pH of the solutions was readjusted to 8 with a few drops of 0.75 *M* sodium hydroxide. Then 5 ml of *n*-pentane containing 3% 2-propanol were added to the aqueous solutions and the

mixtures were shaken for 10 min. After centrifugation, the organic phase was transferred to an evaporation tube, the extraction procedure was repeated once more, and the organic extracts were pooled and evaporated to dryness in a water-bath at 45 °C. The residue was then resuspended in 50 μl of acetonitrile, and 5 μl of BSTFA were added. The solution was allowed to stand at room temperature for 10 min and then evaporated to dryness in a stream of nitrogen. The residue was resuspended in 25 μl of *n*-hexane, and a 2-μl aliquot was injected into the gas chromatograph.

Calibration curves

Different aliquots of the standard solutions of I and II were added to 1.0 ml of blank plasma followed by 0.2 ml of the prazepam solution (50 ng/ml). The ranges of concentrations of I and II were 5–150 and 5–55 ng/ml, respectively. All samples were extracted, derivatized and analysed as above. Calibration curves based on the peak-height ratios of either I or II to the internal standard (I.S.) were constructed using five different concentrations in triplicate. The data were subjected to linear regression analysis to give the appropriate calibration factor.

pH Extraction profile of I and II

Aliquots of 1 ml of the diltiazem aqueous solution (100 ng/ml) were added to each of a series of extraction tubes. The pH of the solutions was adjusted to 5.0, 6.0, 7.0, 7.4, 8.0 and 9.0 with either phosphate or carbonate–bicarbonate buffers. Samples were prepared in triplicate for each pH. The resulting solutions were extracted, derivatized and analysed as already described, except that the I.S. was replaced by an external standard, which was added in ethereal solution prior to evaporation of the organic solutions. The peak-height ratios of I to the external standard obtained after analysis of each solution were compared. The pH extraction profile of 50 ng of II over the pH range 6.2–9.0 was similarly determined.

Absolute recovery of I

Aliquots of 1 ml of the working solution of I (100 ng/ml) were extracted in triplicate and analysed as already described, except that an external standard was used. The peak-height ratios (I/external standard) obtained were compared with those obtained after direct analysis of the same amount of I in methanol in the presence of the external standard.

Stability of I and II in frozen human plasma

To each of six tubes were added 1 ml of human plasma, 0.5 ml of the diltiazem working solution and 0.25 ml of the deacetyldiltiazem working solution. The contents of three tubes were extracted and analysed as described above after addition of the internal standard whereas the other three tubes were frozen at −80 °C. Then 39 days later, the frozen samples were analysed for their content of I and II and the values obtained were compared with those measured on the day of preparation.

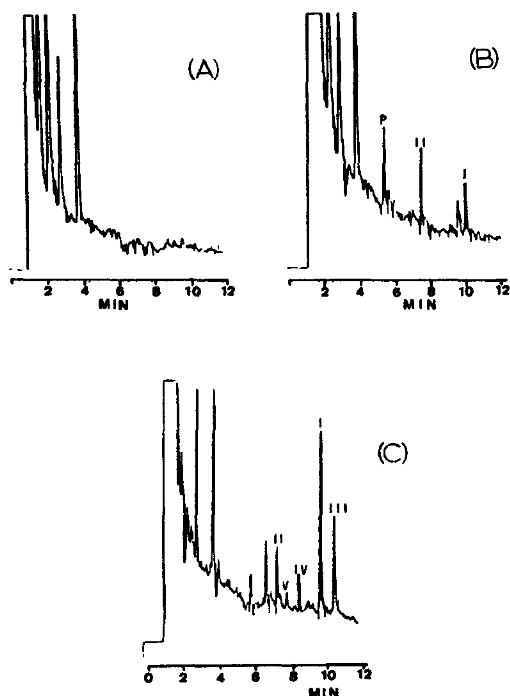

Fig. 2. Chromatograms of BSTFA-treated extracts of 1-ml aliquots of (A) control serum, (B) serum spiked with 90 ng of I, 45 ng of II and 10 ng of the internal standard (prazepam, P), (C) serum spiked with I, II, III, IV and V. Retention times: I, 9.9 min; II, 7.5 min; III, 10.5 min; IV, 8.4 min; V, 7.8 min; P, 5.5 min.

Clinical application of the assay

Diltiazem hydrochloride was administered as a 60-mg oral dose (Cardizem®) to a male patient with chronic renal failure (age 53 years; body weight 75.5 kg) treated by continuous ambulatory peritoneal dialysis. Blood samples were withdrawn before and at 0.5, 1.0, 1.5, 2.0, 4.0, 6.0, 8.0 and 10.0 h post-dose. The serum was separated by centrifugation and frozen at −80°C till it was analysed for its contents of I and II using the method described herein.

RESULTS AND DISCUSSION

Typical chromatograms of BSTFA-treated extracts of a control sample of plasma obtained from a patient with chronic renal failure and of the same fluid spiked with I, II and the I.S., prazepam (P), are shown in Fig. 2A and B. A complete baseline separation was obtained between I and II, and no interference from endogenous compounds was observed.

Selectivity towards metabolites and other drugs

Recently, Goebel and Kölle [13] reported the presence of peaks due to metabolites III and IV in chromatograms of extracts of serum of patients receiving multiple doses (three 60-mg doses per day) of diltiazem. We therefore used the

chromatographic conditions described here to check whether these metabolites interfered with I and II. As can be seen in Fig. 2C, which is a chromatogram of a BSTFA-treated extract of control serum spiked with compounds I–V, each compound gave a single peak that was completely separated from the others, thus excluding any mutual interference. Furthermore, all compounds chromatographed within 10.5 min, indicating that the present method can also be used for the simultaneous analysis of compounds I–V.

The selectivity of the present assay was also checked against a number of other drugs, including β-blockers, various benzodiazepines and others that are likely to be co-administered with diltiazem. A complete list of compounds checked and their retention times is given in Table I.

Sensitivity and reproducibility

The minimum concentrations of I and II measurable by this method were 3 and 1 ng/ml, respectively. Calibration curves were linear over the range 5–150 ng/ml for I and 5–55 ng/ml for II. Correlation coefficients were always greater than 0.99. Accuracy as well as intra- and inter-day variations in the analysis of diltiazem and deacetyldiltiazem in plasma were determined using different concentrations of each compound (Table II). For I, the coefficients of variation (C.V.) for intra- and inter-day analysis were ≤ 6.0 and $\leq 7.4\%$, respectively. The C.V. for II were comparable: ≤ 5.9 and $\leq 8.0\%$ for intra- and inter-day analysis, respectively.

Extractability

The effect of different pHs of the aqueous medium on the extractability of I and II is shown in Fig. 3. The highest amount of diltiazem extracted, as indicated by the peak-height ratio of I to the external standard, was at pH 8; however, statistical analysis using the Duncan multiple-range test indicated that only the levels extracted at pH 5 and 6 were statistically different from those obtained at higher pH. Similarly for II, only the peak-height ratios obtained after extraction of II at pH 6.2 were statistically different from the values obtained over the pH range 7–9. These results indicate that both I and II can be extracted efficiently over a pH range of 7–9. In the present study, pH 8 was used since, at this pH, even though not statistically significant the highest amounts of both I and II were extracted.

The pK_a of diltiazem is 7.7 [14], therefore it would be expected that as the pH of the aqueous solution is increased, more of the compound would be extracted into the organic phase. However, an alkaline pH also favours hydrolysis of diltiazem, which explains why the peak-height ratios measured at pH 9 were not higher than those at lower pH.

The extractability of I with n-pentane–3% 2-propanol in the presence of 2 g of sodium chloride at pH 8 was determined by comparing the peak-height ratios of I to the external standard obtained after extracting 100 ng of the compound to the peak-height ratios obtained after direct analysis of the same amount of I in

TABLE I

RETENTION TIMES OF SOME DRUGS THAT MAY BE COADMINISTERED WITH DILTIAZEM

Drug	Retention time (min)
Diazepam	4.0
Loxapine	5.2
Medazepam	2.9
Oxazepam	3.4
Flurazepam	6.7
Oxprenolol	1.5
Metoprolol	1.3
Propranolol	2.0
Butoxamine	2.5

TABLE II

INTER- AND INTRA-DAY VARIATIONS IN THE MEASUREMENT OF DILTIAZEM AND DEACETYLDILTIAZEM

Compound	Amount added (ng/ml)	Amount recovered (ng/ml)	n	Recovery (%)	Coefficient of variation (%)
Intra-day variations					
Diltiazem	9.19	8.91±0.25	4	96.97±2.75	2.83
	45.96	47.46±2.83	4	103.26±6.15	5.96
	82.73	82.81±2.91	5	100.12±2.65	2.65
Deacetyldiltiazem	22.77	22.82±1.34	4	100.22±5.89	5.87
	40.97	40.90±2.12	5	99.83±5.18	5.18
Inter-day variations					
Diltiazem	9.19	9.17±0.57	8	99.78±6.22	6.24
	45.96	48.66±3.55	12	105.88±7.72	7.30
	82.73	85.41±6.35	9	103.24±7.68	7.44
Deacetyldiltiazem	22.77	23.73±1.90	11	104.22±8.34	8.01
	40.97	43.00±2.74	10	104.96±6.37	6.37

methanol. The peak-height ratio for I was 0.89±0.02 after extraction and 1.17±0.08 after direct analysis. These results indicate that "salting out" of diltiazem and with n-pentane–3% 2-propanol results in the recovery of 75.6% of the drug. This value falls in the range of 70–85% reported by others [7,10,13] for the extraction of I using n-hexane [7], methyl *tert.*-butyl ether [13] or diethyl ether–ethyl acetate [10]. During preliminary work in the development of the present method, diethyl ether was used as an extracting solvent primarily due to the ease of evaporation of this solvent. However, it had to be abandoned because of the

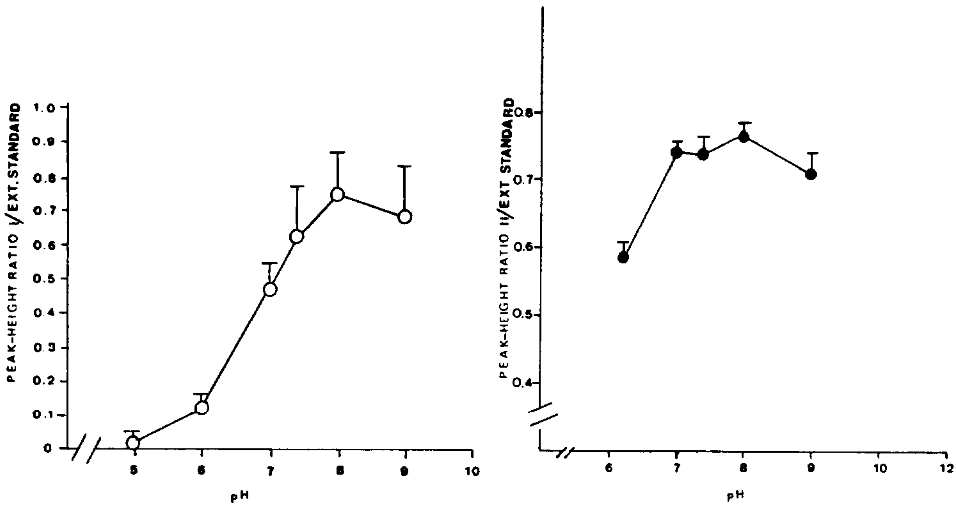

Fig. 3. pH Extraction profiles of diltiazem (○) and of deacetyldiltiazem (●).

presence of several extraneous peaks in the biological fluids from patients with renal failure.

Derivatization

In common with Rovei et al. [7] and Clozel et al. [10], we were unable completely to separate II from I, even though various chromatographic conditions were tried. Therefore, we analysed II as its trimethylsilyl (TMS) derivative after reaction with BSTFA in acetonitrile. In this case, the retention time of the derivative was 7.5 min, compared with 9.85 min for underivatized II. In order to reduce the size of the solvent front, we only used 5 μl of silylating agent. This volume was chosen after a study of the peak height of the TMS derivative versus the volume of BSTFA added (1–5 μl) showed that after the addition of just 1 μl of BSTFA, the peak height had reached a maximum and that the peak due to underivatized II had disappeared completely. When dry acetonitrile was used as the reaction medium instead of either ethyl acetate or benzene, the reaction time was

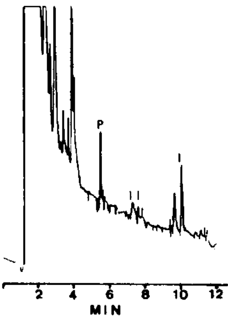

Fig. 4. Chromatogram of a BSTFA-treated extract of serum obtained from a patient with renal failure 6 h after administration of a 60-mg oral dose of diltiazem.

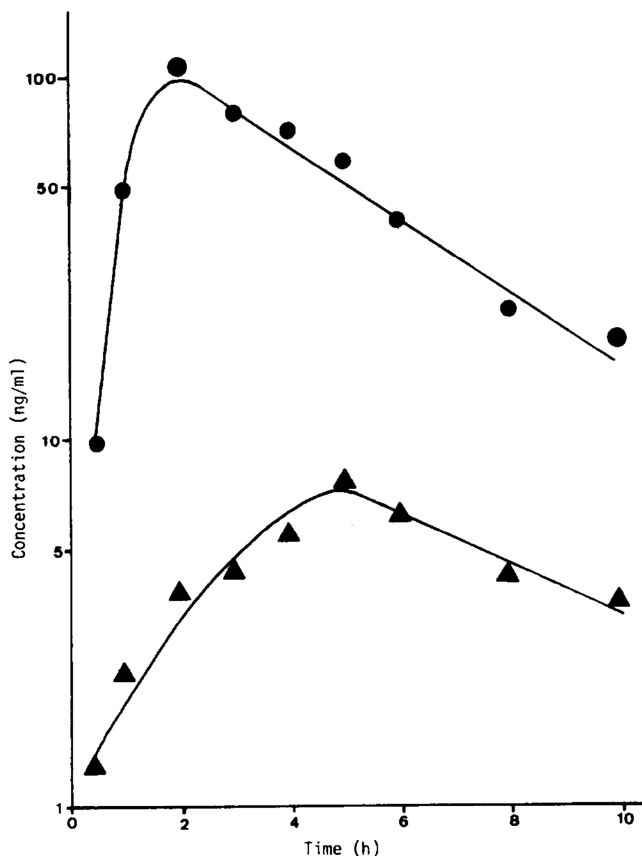

Fig. 5. Serum concentration-time profiles of I (●) and II (▲) obtained in a patient with renal failure after administration of a 60-mg oral dose of I in tablet form (Cardizem).

reduced to 10 min at room temperature compared with 1 h at 60–70°C proposed by others [7,10].

Stability of I and II in frozen human plasma

The stability of I and II in plasma was determined by comparing the amount of each compound measured immediately after adding 50 ng of I and 25 ng of II to 1-ml aliquots of plasma with the amounts remaining after 39 days of storage of the plasma samples at −80°C. The measured amounts of I and II were 48.57 ± 4.94 and 26.42 ± 1.22 ng, respectively, in the freshly prepared samples, and 45.13 ± 2.83 and 25.20 ± 1.63 ng, respectively, in the stored samples, resulting in no significant difference between the two days of analysis. Furthermore, no extraneous peaks were observed. Therefore, both diltiazem and its metabolite, deacetyldiltiazem, are stable for over one month in plasma when stored at −80°C. Rovei et al. [7] and Verghese et al. [11] used GC and HPLC methods, respectively, for the analysis of I and II, and they both reported that chromatograms of serum obtained from patients treated with diltiazem and stored for three to four

weeks showed a peak that had a similar retention time to that of deacetyldiltiazem, making analysis of this metabolite difficult.

Clinical application of the assay

The analytical method described herein was applied to study the profile of I and II in a patient with renal failure after administration of a 60-mg oral dose of diltiazem hydrochloride. Fig. 4 shows a BSTFA-treated extract of serum obtained from the patient 6 h post-dose. The levels of I and II in this sample were 40.5 and 6.4 ng/ml, respectively. No peaks due to metabolites III, IV and V were detected. The serum concentration–time profiles of I and II are shown in Fig. 5. The measured maximal concentration of I was 110 ng/ml, and it was attained 2 h post-dose. Measurable levels of II were observed up to at least 10 h post-dose; however, they were very low, with the highest concentration being 7.6 ng/ml. The elimination half-life of I in this patient was found to be 2.97 h.

In conclusion, a method for the analysis of diltiazem and deacetyldiltiazem in serum is proposed. It is highly selective and sensitive and more rapid than other published GC methods for the analysis of I and II. Furthermore, it can also be used to analyse three other metabolites of diltiazem if required.

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