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# Simple and sensitive high-performance liquid chromatographic method for the determination of diltiazem and six of its metabolites in human plasma

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ABSTRACT

A sensitive, specific and reproducible high-performance liquid chromatographic technique is described for the simultaneous determination in human plasma of diltiazem (DZ) and six of its primary and secondary metabolites which are products of N- and O-demethylation, deacetylation and N-oxidation. The method involves addition of excess $KHCO_3$ to 1 ml of plasma, followed by extraction with 4 ml of ethyl acetate. The organic layer was extracted with 0.01 *M* HCl and the aqueous layer was dried under nitrogen and then reconstituted with 0.002 *M* HCl. DZ and its metabolites were free from interference and were baseline-separated. Calibration curves were linear in the concentration range studied (5–500 ng/ml for all the species). The lower limit of quantification of the assay was 5 ng/ml for DZ and the metabolites. Inter-day and intra-day coefficients of variation were less than 10%. The applicability of this procedure is shown by evaluating the kinetics of DZ and its metabolites in three patients receiving chronic DZ therapy. N-Demethyldiltiazem, deacetyldiltiazem and N-demethyldeacetyldiltiazem were found to be the major metabolites, as previously described. Deacetyldiltiazem N-oxide was found in two of the patients. The other two known but unreported metabolites in human, O-demethyldeacetyldiltiazem and N,O-didemethyldeacetyldiltiazem, were found in the plasma of all three patients.

## INTRODUCTION

Diltiazem (DZ) is a calcium channel antagonist, which is effective in the treatment of angina pectoris [1,2], hypertension [3,4] and cardiac arrhythmias [5,6]. DZ undergoes extensive first-pass metabolism and is mainly metabolised by the liver [2,7]. The major phase I metabolic pathways are N-dealkylation and deacetylation in human [2,8,9]. The primary product of demethylation and deacetylation are N-demethyldiltiazem (MA) and deacetyldiltiazem (M1), respectively. These two metabolites undergo further metabolism to N-demethyldeacetyldiltiazem (M2), O-demethyldeacetyldiltiazem (M4), and N,O-didemethyldeacetyldiltiazem (M6) and the pathways

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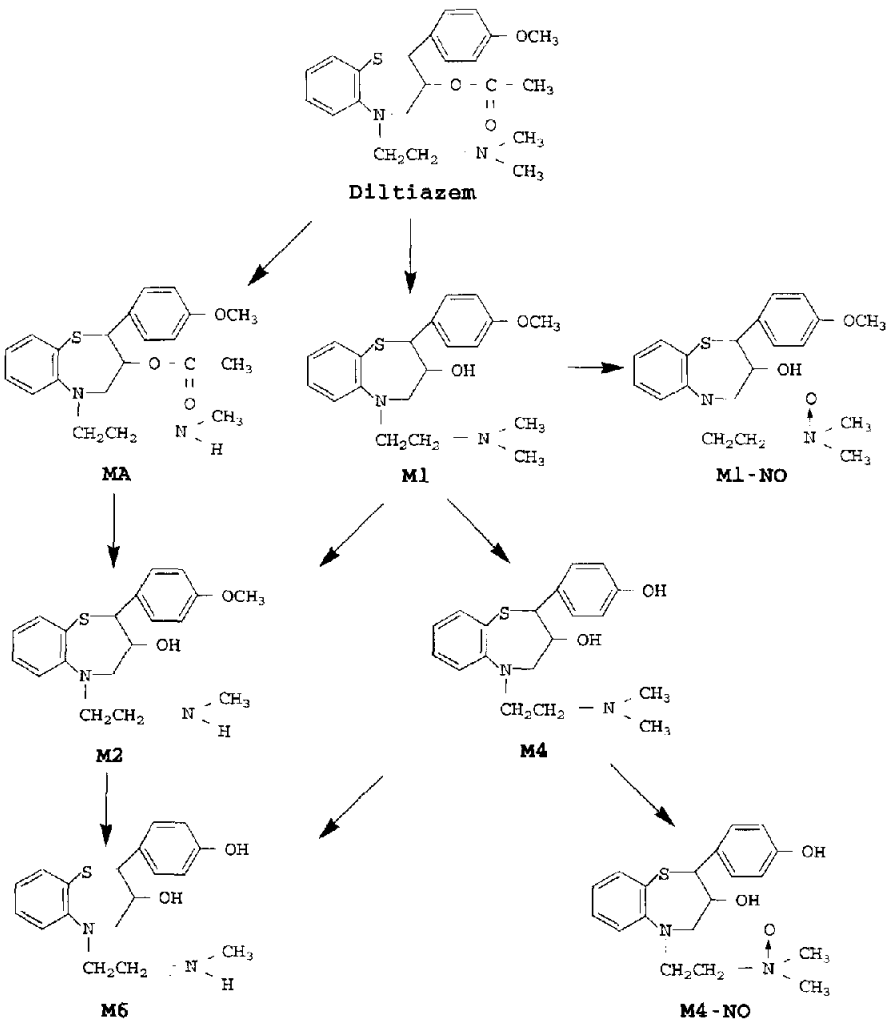


Fig. 1. Proposed metabolic pathways of diltiazem in humans.

are shown in Fig. 1. The formation of deacetyldiltiazem N-oxide (M1-NO) or O-demethyldeacetyldiltiazem N-oxide (M4-NO) by N-oxidation may also occur [8,10]. The metabolites of DZ also contribute to the pharmacological activities of diltiazem [11,12]. The primary metabolites MA and M1 have been shown to have coronary vasodilating potencies amounting to 50% [13] and 20% [14] of that of DZ.

The are several high-performance liquid chromatographic (HPLC) [15–19] and gas chromatographic (GC) methods [20,21] reported in the literature to quantify DZ and its metabolites in plasma. These methods were used to evaluate pharmacokinetics of DZ in human. Unfortunately they do not permit the quantification of all the known metabolites of DZ and they also lack the sensitivity required to study their *in vivo* kinetics. Recently, Yeung *et al.* [22] developed an HPLC assay to quantify DZ and six of its metabolites in plasma of human but this assay is labour-intensive and a chromatogram of the blank plasma is not completely free of endogenous interfering peaks. Also the metabolites are not baseline-re-

solved. Using 2 ml of plasma, the reported sensitivity of the method is 10 ng/ml with large assay variation (up to 60%) which is not suitable for our pharmacokinetic study. We have developed a simple and sensitive HPLC assay that permits quantification of DZ and six of its metabolites. This method was used to measure DZ and its metabolites in patients receving DZ chronically.

## EXPERIMENTAL

### *Chemicals*

The hydrochloride salt of DZ and its metabolites M1, M2, M4, M6, M1-NO and the fumarate salt of MA were kindly supplied by Merrell Dow Nordic Research (Laval, Canada). The internal standard, benzylamphetamine hydrochloride, was synthesized in our laboratory. Ethyl acetate and acetonitrile were HPLC grade and were purchased from BDH (Toronto, Canada). All other chemicals were of analytical grade and were obtained from BDH.

### *Instruments*

The HPLC system consisted of a Model 710B automatic injector, a Model 501 pump and a Model 441 UV detector (Waters, Mississauga, Canada). The detector was set at 214 nm. Chromatographic separation was achieved on a Waters 4 $\mu$m $C_{18}$ reversed-phase Novapak cartridge column (10 cm × 8 mm I.D.). The mobile phase was comprised of an aqueous solution of 0.045% $H_3PO_4$ and 0.1% triethylamine–acetonitrile (65:35, v/v) and was pumped at a flow-rate of 2 ml/min.

### *Standard solutions*

Stock solutions of DZ and its metabolites were prepared separately in distilled water to a final concentration of 100 $\mu$g/ml base equivalent. The concentration of the internal standard, benzylamphetamine, was 10 $\mu$g/ml. Drug-free human plasma samples (supplied by Red Cross) were spiked with solutions of DZ and its metabolites. Serial dilution with plasma gave concentrations of 5, 10, 20, 40, 100, 200 and 500 ng/ml for DZ and its metabolites.

### *Sample preparation*

At the time of analysis, plasma samples were equilibrated to room temperature. To 1 ml of plasma containing DZ and its metabolites in a glass test tube were added 50 $\mu$l of internal standard solution and excess $KHCO_3$ ($\sim$ 1 g). When effervescence stopped, the mixture was vortex-mixed with 4 ml of ethyl acetate for 15 min on a vortex shaker (IKA-VIBRAX-VXR, Terrochem, setting at 1200). After centrifugation (1000 *g*) for 10 min, the aqueous layer was frozen in a dry ice–acetone bath. The organic layer was decanted into another test tube and the aqueous layer was discarded. To the separated organic layer, 0.2 ml of 0.01 *M* HCl was added. The mixture was vortex-mixed for 10 min and centrifuged for 10 min at 1000 *g*. The aqueous layer was again frozen (dry ice–acetone bath), then separated and dried under nitrogen. The dried residue was dissolved in 0.2 ml of 0.002 *M* HCl. Aliquots of 0.10 to 0.15 ml were injected onto the HPLC system.

### *Assay validation*

The accuracy and precision of the assay were validated using quality control (QC) samples prepared by an authorized person in the laboratory. The concentration of these QC samples covered the range of calibration curve. Inter-day variations of QC samples were evaluated on three different days. Each sample was analyzed in duplicate on all three occasions. For the intra-day variation study, four additional samples at each of the concentrations were analyzed on the third experimental day.

### *Patient study*

Three patients scheduled for coronary artery bypass grafting were studied. Informed consent was obtained. The patients were not suffering from liver or renal disease. They were receiving oral diltiazem (Cardizem tablets, Merrell Dow Nordic Research), 60 mg q.i.d. for at least three months. On the day of study, an indwelling catheter was inserted into an antecubital vein prior to the routine dose of DZ. Blood samples were collected at 0, 0.25, 0.50, 0.75, 1.00, 1.50 and 2.00 h and then at every hour up to 8 h. Subsequent

dosing of DZ was withheld until after the 8-h sample. The 8-h sampling period was to characterize properly the half-life ($t_{1/2}$) of DZ and its metabolites. Each blood sample was immediately centrifuged (1500 *g*) and plasma harvested and stored at −20°C until analysis. Samples were analyzed within eight weeks after collection.

*Data analysis*

Standard curves (5–500 ng/ml for DZ and its metabolites) were constructed by plotting the peak-area ratio of DZ or its metabolites to the internal standard *versus* spiked plasma concentrations. Each spiked plasma standard was analyzed in triplicate. Linear regression analysis was used to determine the best fit line through each set of standards.

Plasma concentration *versus* time data were analyzed using the computer program LAGRAN [23]. Terminal half-life ($t_{1/2}$) was calculated from the terminal disposition rate constant $\beta$, using the relationship $t_{1/2} = 0.693/\beta$. Terminal disposition rate constant, $\beta$, was determined by using linear regression of the terminal log linear portion of the concentration–time profiles. Maximum concentration ($C_{max}$) and time to achieve this value ($t_{max}$) were obtained directly from the data. Data are presented as mean ± S.D.

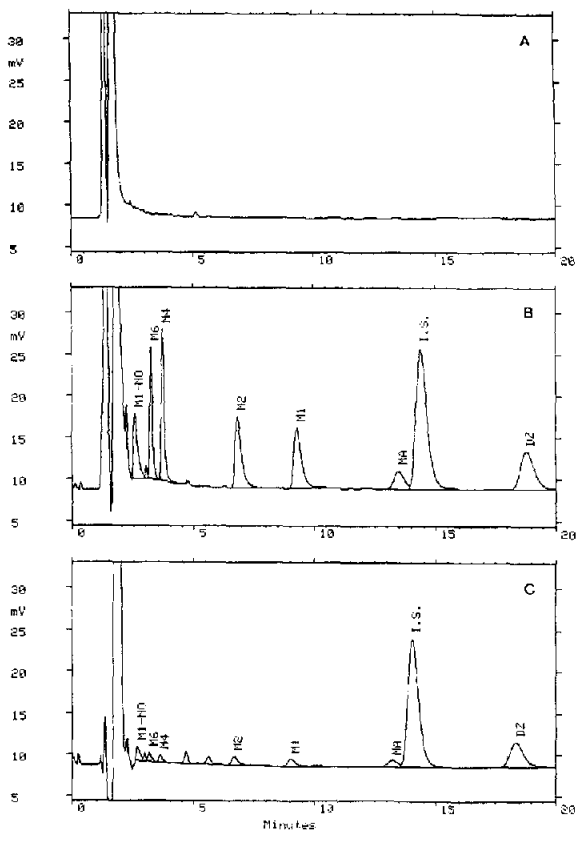


Fig. 2. Chromatogram of (A) drug-free plasma, (B) plasma spiked with 200 ng/ml diltiazem and 100 ng/ml metabolites and (C) a plasma sample from a patient receiving 60 mg diltiazem q.i.d.

## RESULTS

Chromatograms obtained from a blank plasma sample and plasma sample spiked with DZ (200 ng/ml) and its metabolites (100 ng/ml) are shown in Fig. 2A and B. These chromatograms indicate that the drug and metabolite peaks are well resolved and endogenous interference was absent.

The method was applied to study the pharmacokinetics of diltiazem and its metabolites in three patients. Fig. 2C is a chromatogram of a plasma sample collected from one patient. All six metabolites were found in this patient. In addition to the six peaks of known composition, two peaks at 4.7 and 5.7 min, which contained unknown components that did not interfere with the analysis, were also detected in all three patients studied. These peaks may represent unknown DZ metabolites or may be contributed by concomitantly administered drugs.

Calibration curves prepared from standard solutions were linear ($r^2 > 0.99$) in the concentration ranges studied (5–500 ng/ml for both DZ and its metabolites). Results of the validation study are presented in Table I. The coefficient of variation, an indicator of the precision, was less than 10% for DZ and metabolites for both inter-day and intra-day assay. The estimated concentration expressed as the percentage of the spiked concentration revealed the accuracy of the method which ranged from 93 to 105%. Using our extraction procedure, recoveries of DZ and its metabolites were more than 75% with the excep-

TABLE I

ACCURACY AND PRECISION OF THE ASSAY FOR DILTIAZEM AND ITS METABOLITES ($n$ = 6)

| Compound | Concentration added (ng/ml) | Concentration found (mean ± S.D.) (ng/ml) | |
|---|---|---|---|
| | | Intra-day | Inter-day |
| DZ | 500 | 503.4 ± 20.8 | 505.7 ± 19.6 |
| | 200 | 194.6 ± 6.31 | 195.8 ± 8.81 |
| | 10 | 10.03 ± 0.55 | 10.10 ± 0.84 |
| MA | 250 | 233.3 ± 4.61 | 237.9 ± 9.30 |
| | 100 | 103.2 ± 4.65 | 97.97 ± 5.18 |
| | 5 | 4.92 ± 0.28 | 5.18 ± 0.39 |
| M1 | 250 | 251.2 ± 6.71 | 246.0 ± 10.5 |
| | 100 | 96.10 ± 3.90 | 100.0 ± 4.23 |
| | 5 | 4.69 ± 0.46 | 5.00 ± 0.52 |
| M2 | 250 | 246.6 ± 6.50 | 242.6 ± 7.78 |
| | 100 | 96.22 ± 4.02 | 99.70 ± 3.31 |
| | 5 | 4.98 ± 0.32 | 4.96 ± 0.26 |
| M4 | 250 | 251.4 ± 13.1 | 244.1 ± 15.0 |
| | 100 | 98.82 ± 5.61 | 100.4 ± 2.73 |
| | 5 | 5.08 ± 0.72 | 5.24 ± 0.28 |
| M6 | 250 | 246.3 ± 2.93 | 245.4 ± 11.6 |
| | 100 | 104.1 ± 5.55 | 100.5 ± 4.88 |
| | 5 | 5.15 ± 0.38 | 4.97 ± 0.27 |
| M1-NO | 250 | 256.4 ± 11.5 | 247.1 ± 12.7 |
| | 100 | 93.57 ± 6.49 | 99.68 ± 4.74 |
| | 5 | 4.98 ± 0.46 | 4.95 ± 0.52 |

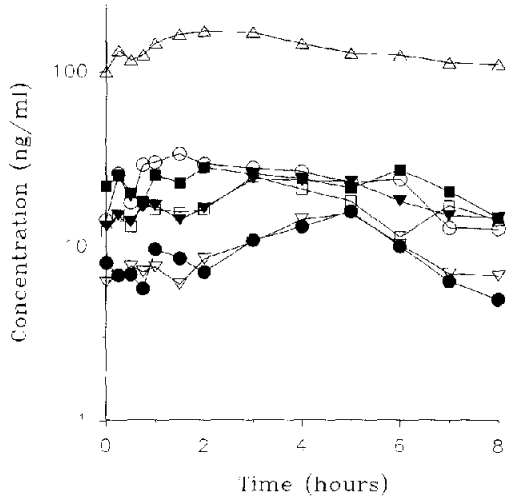


Fig. 3. Plasma concentration–time profile of diltiazem and its metabolites in a patient receiving 60 mg q.i.d. diltiazem. ○ = M1-NO; ● = M6; ▽ = M4; ▼ = M2; □ = M1; ■ = MA; △ = DZ.

tion of M1-NO, which was 41.9 ± 5.95 and 27.8 ± 2.0% at concentrations 200 and 10 ng/ml, respectively. A similar finding for M1-NO (~30%) was reported by Yeung *et al.* [22] who attributed the poor recovery of M1-NO to adsorption onto glass or other surfaces and high aqueous solubility. We found that a mixture of methylene chloride and ethyl acetate in the ratio of 1:3 or 1:4 increased the extraction efficiency of M1-NO, M6 and M4 slightly but decreased the extraction efficiency of M1, MA and DZ and thus the mixture of methylene chloride and ethyl acetate has no advantage over ethyl acetate alone.

The plasma concentration *versus* time profiles of diltiazem and its metabolites in one patient are provided in Fig. 3 and the pharmacokinetic parameters are presented in Table II. The pharmacokinetic data presented here are consistent with

TABLE II

PHARMACOKINETIC PARAMETERS OF DILTIAZEM AND ITS METABOLITES IN THREE PATIENTS RECEIVING 60 mg OF DILTIAZEM q.i.d.

The parameters are expressed as mean ± standard deviation.

| Compound | $C_{max}$ (ng/ml) | $T_{max}$ (h) | $t_{1/2}$ (h) | $AUC_{0-6}$ (ng/ml · h) |
|---|---|---|---|---|
| DZ | 177.5 ± 22.3 | 2.7 ± 0.6 | 4.2 ± 2.6 | 852.1 ± 123.0 |
| MA | 32.9 ± 5.2 | 3.7 ± 1.5 | 6.7 ± 3.0 | 155.3 ± 29.0 |
| M1 | 32.5 ± 9.4 | 3.0 ± 0.0 | 6.1 ± 1.7 | 135.2 ± 25.8 |
| M2 | 29.8 ± 13.3 | 3.3 ± 0.6 | 3.7 ± 2.4 | 142.8 ± 52.3 |
| M4 | 9.9 ± 5.1 | 3.3 ± 1.5 | 3.8 ± 1.5 | 45.9 ± 21.6 |
| M6 | 14.7 ± 3.9 | 4.0 ± 1.7 | 3.0 ± 2.1 | 61.6 ± 6.9 |
| M1-NO[a] | 20.6 | 1.3 | 5.3 | 93.5 |

[a] M1-NO was detectable in the plasma of two patients.

literature values [8,22,24–26]. MA, M1 and M2 were the major metabolites present in the patients examined by us. Their levels were less than that of DZ. M1-NO was not detectable in one of these patients. The calculated oral clearance ($D_o$/$AUC_{0-6}$) of 70.4 l/h (1.17 l/min) in those patients was within the reported limits [8,24].

## DISCUSSION

When compared to the analytical procedure described by Yeung *et al.* [22], which is the only reported method that permits quantification of DZ and its six metabolites, the method now described is relatively simple and rapid. The method described by Yeung *et al.* [22] requires two-step extraction followed by drying of 10 ml organic phase and after reconstitution, washing twice with methyl *tert.*-butyl ether and hexane. Sample treatment in our assay involves only one extraction and a back-extraction. The calibration curve for DZ can be extended up to 1000 ng/ml and a linear response is still obtained. Using the extraction procedure reported here, the quantifiable limit in plasma is 5 ng/ml for DZ and its metabolites and the detection limit for the metabolites is $<2$ ng/ml. Plasma volume ranging from 0.5 to 1.0 ml is found to be adequate in quantifying low DZ and metabolite concentrations using our method. The assay procedure described by Yeung *et al.* [22] requires a larger volume of plasma, is less sensitive and has a larger inter-assay variability.

The addition of $KHCO_3$ was to render the plasma alkaline (pH $\sim$8.3–8.5) for better extraction as a lower pH resulted in poor recoveries and a higher pH would cause hydrolysis of DZ and MA [16,27,28]. Optimal extraction with no degradation is achieved at pH $<8.5$ [27,28]. Hoglund and Nilsson [16] found that saturating the plasma with sodium chloride increased the extraction efficiency of MA, M1, M2 and M4 with a hexane–2-propanol (95:5) mixture. The use of $KHCO_3$ in our method provides a plasma pH optimum for extraction and may have increased the extraction efficiency of the ethyl acetate. Under this extraction condition DZ and its metabolites were stable. Drying the back-extracted aqueous phase gives a clearer chromatogram.

Several compounds were investigated for use as internal standard. Since DZ and its metabolites differ widely in their physicochemical properties, the selection of an ideal internal standard is difficult. Benzylamphetamine, which is not used as a drug, was chosen as internal standard because of its clear separation from DZ and its metabolites, its appropriate retention time (earlier than DZ, 14.3 min), symmetrical peak shape

and good extraction recovery. The likelihood of analytical interferences from other drugs that could be administered during diltiazem therapy was examined. Lidocaine and its metabolites monoethylglycinexylidide and glycinexylidide, diphenhydramine, metoprolol, bupivacaine, all of which may be present in patient plasma along with DZ and its metabolites, did not interfere with the assay.

The $C_{18}$ Novapak cartridge column could be utilized for at least six months or for the analysis of more than 500 samples without any deterioration in its performance.

MA and M1 are often identified as the major metabolites of DZ but M2 should also be considered as a major metabolite of DZ. Its $C_{max}$ and AUC values were comparable to those of MA and M1 in these three patients studied. Yeung *et al.* [22], in a single 90-mg oral DZ study, made a similar observation. Also, they could not detect M1-NO in two out of four volunteers. M1-NO was found in two patients in our study. The other two metabolites M6 and M4, whose pharmacokinetic data have not been reported in any other studies, were also detected and quantified in these patients. It is possible that these metabolites have accumulated after chronic oral diltiazem and the sensitivity of the present assay has allowed their quantification.

This HPLC method demonstrated adequate performance with respect to precision, accuracy and specificity. It is linear and reproducible within the plasma concentration range observed in patients. The method is rapid, sensitive and is applicable for the determination of the pharmacokinetics of diltiazem in man. Currently this method has been applied to pharmacokinetic studies in patients undergoing coronary artery bypass surgery and it is also used in our laboratory to study the time-dependent pharmacokinetics and drug interactions of diltiazem in the chronically instrumented dog.

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