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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF DILTIAZEM AND FOUR OF ITS METABOLITES IN PLASMA: EVALUATION OF THEIR STABILITY

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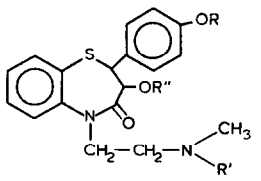
SUMMARY

A rapid, specific and reproducible high-performance liquid chromatographic method was developed for the simultaneous determination of diltiazem and four of its metabolites in plasma. The method involves extraction with methyl *tert.*-butyl ether, back-extraction into 0.017 *M* phosphoric acid followed by reversed-phase chromatography on a 3-μm particle, 15-cm ODS column with UV detection at 237 nm. Overall the recovery of each compound was reproducible and greater than 85%. Calibration curves were linear over the concentration range 10–250 ng/ml, with within-day or between-day coefficients of variation not exceeding 12%. A stability study indicates that while diltiazem is stable for at least six weeks in frozen plasma, more than 30% degradation of the major metabolite, N-monodesmethyldiltiazem, was observed after four weeks at −20°C. The assay procedure has been applied to monitoring of plasma levels in patients receiving chronic oral therapy.

INTRODUCTION

Diltiazem (Fig. 1) is a calcium channel blocker currently used in the treatment of angina pectoris, hypertension and supraventricular arrhythmia [1]. Several high-performance liquid chromatographic (HPLC) methods [2–8] have been published for the determination of diltiazem and a considered major metabolite, deacetyldiltiazem (M1). However, only a few of these procedures permit separation and determination of other metabolites [3,5–7] specially N-monodesmethyldiltiazem (MA) which was reported to be the major metabolite in human [9] and to accumulate in plasma following chronic dosing [5,6,10]. MA and M1 undergo further biotransformation to form deacetyl-N-monodesmethyldiltiazem (M2), deacetyl-O-demethyldiltiazem (M4) and deacetyl-N,O-demethyldiltiazem (M6) [9]. Only two HPLC methods [5,7] described the simultaneous analysis of diltiazem and these five metabolites in plasma but their



	R	R'	R''
Diltiazem	CH_3	CH_3	$COCH_3$
MA	CH_3	H	$COCH_3$
M1	CH_3	CH_3	H
M2	CH_3	H	H
M4	H	CH_3	H
M6	H	H	H

Fig. 1. Chemical structures of diltiazem and five of its metabolites.

specificity towards other drugs was not thoroughly investigated. Furthermore, preliminary results have indicated that MA is unstable and could be degraded to form M2.

The method described here provides a sensitive, specific and reproducible HPLC determination of diltiazem and its different metabolites in plasma for application to drug monitoring or multiple-dose pharmacokinetic studies in patients. In addition, the stability of the drug and the different metabolites in plasma was investigated by this method.

EXPERIMENTAL

Reagents and materials

Diltiazem hydrochloride and deacetyldiltiazem hydrochloride were supplied by Nordic Labs. (Montreal, Canada). The N- and O-demethyl metabolites (MA, M2, M4 and M6) were generously donated by Tanabe Seiyaku (Osaka, Japan). The structure of these compounds are given in Fig. 1. The internal standard, ethylmethyl glycinexylidide (EMGX), was obtained from Astra Lab. (Södertälje, Sweden). Methanol, acetonitrile (J.T. Baker, Phillipsburg, NJ, U.S.A.), methyl *tert.*-butyl ether (Burdick and Jackson Labs., Muskegon, MI, U.S.A.) and triethylamine (TEA) (Fisher Scientific, Fairlawn, NJ, U.S.A.) were of HPLC grade. All other solvents and chemicals were of analytical grade. All aqueous solutions were prepared using double-distilled water.

Chromatography

The chromatographic system consisted of a Perkin-Elmer Series 4 solvent delivery system, an ISS-100 autosampler also from Perkin-Elmer (Norwalk, CT, U.S.A.) and a Kratos Spectroflow 773 variable-wavelength UV detector operated at 237 nm (Kratos Analytical Instruments, Westwood, NJ, U.S.A.). The detector output was quantitated by the Perkin-Elmer Model 3600 data station with the Chrom 2 software (Perkin-Elmer) and the signal was displayed on a Perkin-Elmer Model 660 printer.

Chromatographic experiments were performed on a Spherisorb ODS 2, 3-μm, 150 mm×4.6 mm I.D. column (Chromatography Sciences, Ville St.-Laurent, Quebec, Canada). The mobile phase, prepared fresh daily, consisted of a mixture (60:40, v/v) of acetonitrile and 0.01 *M* ammonium phosphate buffer containing 0.06% TEA with pH adjusted to 3.75. All separations were performed isocratically at a flow-rate of 1 ml/min at ambient temperature (ca. 22°C) yielding a back-pressure of 16 MPa.

Standard solutions

Diltiazem. A stock solution of 1.0 mg/ml diltiazem free base was prepared in 0.01 *M* hydrochloric acid and standard solutions of 20 and 1 μg/ml in 0.01 *M* hydrochloric acid were made by serial dilutions.

Metabolites. Separate stock solutions of the metabolites were prepared in methanol at a concentration of 100 μg/ml. Sequential dilutions to 20 and 1 μg/ml were made in 0.01 *M* hydrochloric acid.

Internal standard. A 1 mg/ml aqueous solution of EMGX was prepared and further diluted with 0.01 *M* hydrochloric acid to give a working solution of 1.25 μg/ml.

A 100-μl aliquot (125 ng) was added to each 1-ml aliquot of plasma standard or specimen.

Extraction procedure

To 1 ml of plasma placed in a 15-ml capacity culture tube, fitted with PTFE-lined screw cap, were added 100 μl of the internal standard solution and 5 ml of methyl *tert.*-butyl ether as extraction solvent. The tubes were shaken for 10 min on an Evapo-mix (Buchler Instruments, Fort Lee, NJ, U.S.A.), then centrifuged at 1120 *g* for 10 min. A 4-ml aliquot of the organic phase was transferred to a screw-capped conical tube and back-extracted with 100 μl of 0.017 *M* phosphoric acid by agitating for 1 min on a Vortex mixer. After centrifugation, the organic phase was discarded and residual solvent was evaporated at 45°C under a nitrogen stream before injecting a 50-μl aliquot of the aqueous phase onto the column.

Calibration

Calibration curves were constructed by transferring aliquots of the respective standard solutions of diltiazem and its metabolites MA, M1, M2 and M4 to blank plasma to give final concentrations of 25, 50, 100, 150, 200 and 250 ng/ml. These calibration standards were extracted as described above. The concentration of diltiazem and its metabolites in unknown samples were determined by using the linear regression line (unweighted) of peak-area ratios versus concentration of calibration standards.

RESULTS AND DISCUSSION

Chromatographic performance

Chromatographic conditions were chosen to achieve adequate and rapid separation of diltiazem and the different metabolites under isocratic conditions and

minimum interferences from endogenous plasma constituents. Diltiazem and its metabolites are weakly basic amines; as expected from these types of compounds in reversed-phase chromatography, the retention behavior was strongly influenced by the pH of the mobile phase, the ionic strength and the concentration of the organic co-solvent (acetonitrile) in the eluent.

The use of a Spherisorb ODS 3-μm particle column was dictated by the need to obtain a highly efficient separation system to resolve all the peaks. The separation was, however, unsatisfactory because of broad or asymmetrical peaks regardless of manipulation of the mobile phase. Compounds with amino functional groups are known to interact strongly with the stationary support [11–13] causing peak tailing and broadening. This phenomenon can be eliminated with the addition of aliphatic amines to the mobile phase [11–13]. Kiel et al. [14] have demonstrated that short-chain tertiary amine modifiers, e.g. TEA, are very effective in improving peak symmetry and reducing retention of solutes with amino functional groups. The addition of TEA to the mobile phase had a drastic effect on the retention behavior of diltiazem and some metabolites. As the amount of TEA was increased, the peak symmetry improved but concentration above 0.1% resulted in loss of resolution. The inclusion of 0.06% TEA in the mobile phase consisting of acetonitrile and ammonium phosphate buffer gave good separation of all compounds with acceptable peak shape within 12 min. Triamines may cause degradation of the silica matrix of reversed-phase columns. However, at the low concentration of TEA used in the mobile phase, the performance of the column was maintained for at least five months of operation or for analysis of approximately 1000 samples. New columns were conditioned by injecting 100-μl aliquots of 5% TEA.

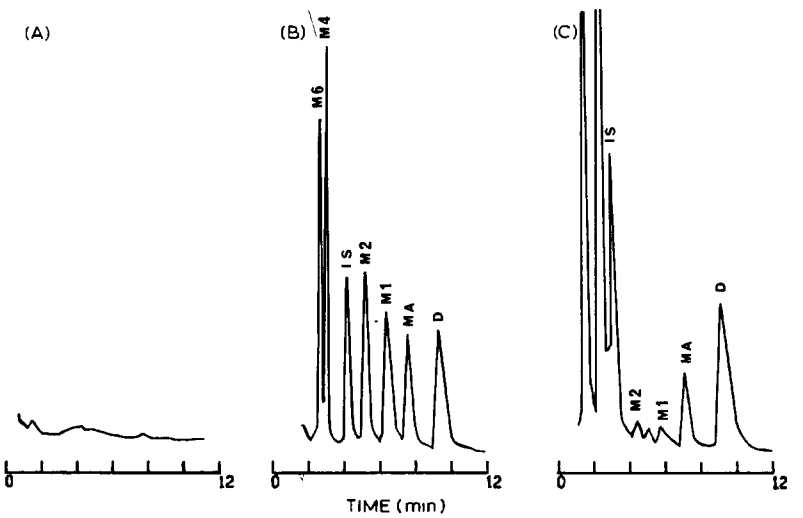


Fig. 2. Chromatograms of extracts from (A) blank human plasma, (B) plasma spiked with 100 ng/ml each of diltiazem, the five metabolites and the internal standard and (C) plasma sample from a patient receiving oral diltiazem chronically. The peak between M1 and M2 is assumed to come from metoprolol which the patient also received. (10 mV = full scale.)

Sensitivity and specificity

Fig. 2 shows typical chromatograms of an extract of blank human plasma (A), a plasma standard spiked with diltiazem and the different metabolites at 100 ng/ml and EMGX as internal standard (B) and a plasma sample of a patient receiving oral diltiazem chronically at a dose of 60 mg every 8 h (C). Blank plasma samples were free of endogenous contaminants at the retention times corresponding to the compounds (Fig. 2A). Interferences from drugs likely to be administered concurrently with diltiazem were investigated and their retention times are listed in Table I. The results indicate no interference with diltiazem or MA. However, some compounds were found to coelute with the more polar metabolites or the internal standard. Such interference is illustrated in Fig. 2C where the patient was also receiving metoprolol.

Sufficient sensitivity for the detection of diltiazem and its metabolites was achieved using UV detection at 237 nm. The limit of detection for diltiazem, MA, M1 and M2 was 2 ng/ml of plasma and 0.5 ng/ml for M4 and M6 based on a signal-to-noise ratio of 3. However, the reliable limit of quantitation was set at 10 ng/ml based on coefficients of variation less than 12%.

TABLE I

DRUGS TESTED FOR POSSIBLE INTERFERENCE

Compound	Retention time (min)
M6	3.30
M4	3.65
M2	5.70
M1	6.81
MA	7.88
Diltiazem	9.49
I.S. (EMGX)	4.73
Procainamide	3.13
Lidocaine	3.14
Naproxen	3.28
Caffeine	3.50
Metoprolol	4.31
n-Acetylprocainamide	4.40
Flurazepam	4.60
Ibuprofen	5.43
Piroxicam	5.63
Diazepam	5.94
Quinidine	6.18
Hydralazine	6.56
Theophylline	6.68
Acetylsalicylic acid	6.89
Hydrochlorothiazide	7.03
Verapamil	13.43
Cimetidine, propranolol, nifedipine	> 15
Acetaminophen, furosemide, digoxin	> 15

Linearity and reproducibility

Calibration curves of each compound were linear over the concentration range 10–250 ng/ml with correlation coefficients greater than 0.995 and minimal intercepts. The day-to-day coefficient of variation in the slope of the calibration curves was 8.5% or less for the individual compounds (Table II).

The accuracy and intra-day precision of the present method were evaluated by assaying eight replicates of plasma sample spiked with diltiazem and the metabolites. Coefficients of variation were less than 12% over the entire range of concentrations, and deviations from nominal concentrations were less than 10% (Table III).

Recovery

The extraction recovery of each compound was determined by comparing the peak area obtained by direct injection of standard aqueous solutions to those obtained after the plasma extraction procedure (Table IV). For diltiazem, M2, M4 and the internal standard, the absolute recoveries were greater than 85% and independent of the concentration. For MA and M1, the recoveries were lower between 25 and 50 ng/ml (80%), but reached 100% between 100 and 250 ng/ml.

The extraction conditions were based on obtaining a high recovery of the drug and the metabolites with minimum interferences. As observed by other investi-

TABLE II

STANDARD CURVE REPRODUCIBILITY OF DILTIAZEM AND ITS METABOLITES ($n=6$)

Compound	Mean slope	Coefficient of variation (%)	r
M4	0.0120	3.8	0.997
M2	0.0106	8.5	0.995
M1	0.0121	4.1	0.997
MA	0.0089	5.8	0.996
D	0.0115	4.6	0.996

TABLE III

INTRA-DAY PRECISION AND ACCURACY ($n=8$)

Values in parentheses are coefficients of variation (%).

Nominal concentration (ng/ml)	Concentration found (ng/ml)				
	M4	M2	M1	MA	D
25	27.3 (7.9)	27.2 (10.0)	24.8 (6.7)	23.3 (7.2)	24.1 (9.7)
50	53.9 (7.4)	48.1 (12.7)	51.3 (8.6)	48.6 (9.7)	50.5 (9.5)
100	107.9 (4.3)	103.7 (7.1)	107.3 (3.7)	102.3 (6.5)	104.8 (4.9)
200	191.4 (3.8)	193.4 (10.0)	196.6 (4.7)	192.3 (7.3)	190.1 (6.4)

TABLE IV

ABSOLUTE RECOVERIES OF DILTIAZEM, ITS METABOLITES AND THE INTERNAL STANDARD FROM SPIKED PLASMA SAMPLES ($n=10$)

Compound	Concentration (ng/ml)	Recovery (%)	Coefficient of variation (%)
M4	25	92.2	7.2
	150	94.3	8.0
M2	25	88.9	11.1
	150	86.1	9.0
M1	25	81.2	7.4
	150	104.2	5.5
MA	50	79.0	11.3
	150	97.7	4.9
D	25	105.7	11.1
	150	105.9	6.3
IS	125	106.2	12.0

TABLE V

STABILITY OF DILTIAZEM AND ITS METABOLITES IN PLASMA KEPT AT 4°C

Time (h)	Percentage of initial concentration*				
	M4	M2	M1	MA	D
1	98.1	106.3	98.3	102.5	97.6
24	104.6	112.8	104.0	106.6	90.9
96	107.4	136.3	103.0	69.6	96.3
168	105.2	196.5	116.0	N.D.**	81.6

*Plasma samples spiked with 100 ng/ml of each compound.
**N.D. = non detectable.

gators [2,5,6], diltiazem was efficiently extracted without plasma pH adjustment. Good recoveries were also achieved for the metabolites at pH 7.4. Several extraction solvents were tested. Compared to diethyl ether, ethyl acetate, chloroform or methylene chloride, methyl *tert.*-butyl ether gave better recoveries for all compounds considered as well as providing cleaner chromatograms. However, a back-extraction step into 0.017 *M* phosphoric acid was essential to minimize interfering peaks and to improve chromatographic resolution. The use of strong acids such as sulfuric acid, hydrochloric acid or even more than 0.017 *M* phosphoric acid resulted in peak splitting.

Stability

The stability of diltiazem and the metabolites was investigated from spiked plasma samples stored at 4 and −20°C (Tables V and VI). At 4°C, no significant degradation of diltiazem was observed until day 7 where only 82% of the original amount of the drug remained. The disappearance of diltiazem was accompanied

TABLE VI

STABILITY OF DILTIAZEM AND ITS METABOLITES IN FROZEN PLASMA (−20°C)

Time (weeks)	Percentage of initial concentration*				
	M4	M2	M1	MA	D
1	99.6	113.4	102.0	91.9	93.4
2	99.3	100.8	94.9	95.6	97.0
4	97.8	149.3	98.1	66.1	100.4
6	103.7	124.4	104.2	84.9	101.9

*Plasma samples spiked with 100 ng/ml of each compound.

by a corresponding increase of a peak with chromatographic retention similar to that of the deacetyldiltiazem, M1. MA was found to be very unstable at 4° with only 70% recovered after four days. Complete degradation had occurred by seven days and the increase of the peak at the retention time of M2 suggested that it was completely deacetylated. M4 did not show any significant variation over the seven-day period.

Unlike the results observed at 4°C, diltiazem was very stable in frozen plasma (−20°C) for a period of at least six weeks (Table VI). Despite freezing, MA showed significant and variable degradation from four weeks on. Two frozen aliquots of spiked plasma were analyzed each week. The amount recovered at six weeks was larger (85%) than that at four weeks (66%). This confusing result could not be explained, but in each case the decrease of MA was accompanied by a proportional increase of a peak with the chromatographic retention of M2 (Table VI). The results, however, clearly indicate that for an accurate determination of MA and M2, plasma samples should be stored frozen and analyzed within two weeks of collection. This instability of MA may explain why some investigators [2,15] have failed to detect it in plasma samples from patients. Following these results, an intensive stability study was undertaken to investigate the degradation of both diltiazem and MA under several stressing conditions. The results of that study will be reported separately [16].

Application of the method

The present HPLC method is sufficient rapid, sensitive and reproducible for the simultaneous determination of diltiazem and its metabolites within the plasma concentration range observed in patients. It has been used for monitoring of plasma levels following chronic oral diltiazem therapy. MA was found as the major metabolite in plasma samples with peak levels representing 40–90% of diltiazem while M1 only represented 10–20% of diltiazem. These results are in agreement with those of Montamat and Abernethy [10].

Several previously published HPLC or gas chromatographic methods have not established selectivity with regards to the different metabolites of diltiazem or other drugs often used in patients. It is obvious that inadequate specificity could

lead to spuriously high levels of certain metabolites or parent drug or failure to detect other metabolites.

REFERENCES

1 M. Chaffman and R.N. Brogden, Drugs, 29 (1985) 387.
2 C. Verghese, M.S. Smith, L. Aanonsen, E.L.C. Pritchett and D.G. Shand, J. Chromatogr., 272 (1983) 149.
3 J.P. Clozel, G. Caillé, Y. Taeymans, P. Théroux, P. Biron and F. Trudel, J. Pharm. Sci., 73 (1984) 771.
4 D.R. Abernethy, J.B. Schwartz and E.L. Todd, J. Chromatogr., 342 (1985) 216.
5 K.-J. Goebel and E.V. Kölle, J. Chromatogr., 345 (1985) 355.
6 S.C. Montamat, D.R. Abernethy and J.R. Mitchell, J. Chromatogr., 415 (1987) 203.
7 P. Höglund and L.-G. Nilsson, J. Chromatogr., 414 (1987) 109.
8 S.M. Johnson and S.K.W. Khalil, J. Liq. Chromatogr., 10 (1987) 673.
9 J. Sugihara, Y. Sugawara, H. Ando, S. Harigaya, A. Etoh and K. Khono, J. Pharm. Dyn., 7 (1984) 24.
10 S.C. Montamat and D.R. Abernethy, Br. J. Clin. Pharmacol., 24 (1987) 185.
11 K.E. Bij, Cs. Horváth, W.R. Melander and A. Nahum, J. Chromatogr., 203 (1981) 65.
12 A. Nahum and Cs. Horváth, J. Chromatogr., 203 (1981) 53.
13 J.S. Kiel, R.K. Abramson, S.L. Morgan and J.C. Voris, J. Liq. Chromatogr., 6 (1983) 2761.
14 J.S. Kiel, S.L. Morgan and R.K. Abramson, J. Chromatogr., 320 (1985) 313.
15 V. Rovei, R. Gomeni, M. Mitchard, J. Larribaud, Ch. Blatrix, J.J. Thebault and P.L. Morselli, Acta Cardiol., 35 (1980) 35.
16 G. Caillé, L.M. Dubé, Y. Théoret, F. Varin, N. Mousseau and I.J. McGilveray, Biopharm. Drug Dispos., 9 (1988) in press.