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SHORT COMMUNICATION

STABILITY STUDY OF DILTIAZEM AND TWO OF ITS METABOLITES USING A HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD

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INTRODUCTION

Diltiazem is a calcium antagonist which is currently used in the treatment of angina pectoris, hypertension, and supraventricular arrhythmias.[1–4] At present, in our laboratory, diltiazem and six of its metabolites have been identified and quantified in human biological fluids either by gas chromatography[5] or high performance liquid chromatography.[6] As a first biotransformation step, diltiazem may be either demethylated to MA (N-monodemethyldiltiazem) or deacetylated to DAD (desacetyldiltiazem). These two metabolites may undergo further biotransformation to M_4 (O-demethyldesacetyldiltiazem), M_2 (N-demethyldesacetyldiltiazem) or M_6 (N,O-didemethyldesacetyldiltiazem). The formation of DAD or M_4 N-oxide intermediates may also occur. Despite the identification of these possible metabolites in humans, the pharmacokinetics of diltiazem are still poorly understood. Some authors reported that DAD was a major metabolite,[7,8] while others have failed to detect any DAD[5,9,10] in the plasma of diltiazem-treated patients. This raised some concerns as to whether DAD is a true metabolite or a degradation product. In this regard, the differences observed in the concentrations of DAD could very well be dependent on the various specimen collection and analytical procedures used. The present article summarizes the results of stability studies on diltiazem and two of its

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putative primary metabolites (DAD and MA) subjected to various stressing conditions *in vitro*.

MATERIALS AND METHODS

Pure samples of diltiazem hydrochloride and metabolites were provided by Tanabe Seigaku Ltd (Japan). Loxapine was obtained from Lederle Cyanamid Canada Inc. (Montréal, Québec, Canada) and used as the internal standard. All solvents and reagents were of analytical grade except for acetonitrile, methyl *tert*-butyl ether, phosphoric acid, and water which were HPLC grade.

The HPLC system consisted of a LDC/Milton Roy (Riviera Beach, FL, U.S.A.) constaMetric III solvent delivery system, a Waters Associates. (Milford, MA, U.S.A.) Model 441 absorbance detector with fixed wavelength at 214 nm, a Rheodyne (Cotati, CA, U.S.A.) Model 7120 injector with 50 μl sample loop and a Shimadzu (Kyoto, Japan) Model C-R3A data processor. The separation system was a Hichrom (Reading, U.K.) 10 cm × 4·9 mm stainless steel, 5 μm, C_8 Spherisorb reversed-phase column. Mobile phase, 0·005 M phosphate buffer at pH 3·0 – acetonitrile (57:43) containing 1·25 μM of dibutylamine was run at a flow rate of 1·4 ml min^{-1}. All analyses were performed at room temperature.

Stock solutions of diltiazem and metabolites were prepared in 0·01 N HCl at concentrations of 0·1 mg ml^{-1}. Stability studies in 1 N HCl were carried out on aliquots of stock solutions diluted 10 times with 1·11 N Cl. Immediately before injection into the HPLC system, these 1N solutions were buffered with 0·05 M sodium phosphate solution to yield diltiazem solutions at a concentration of 1 ng μl^{-1}. Stock solution of diltiazem was diluted in 0·01 N HCl, water and various buffers, to a final concentration of 1 ng μl^{-1}. The pH of some of these solutions was adjusted to the pH of the diluent and the stability studies initiated. The stability of a mixture of diltiazem, DAD, MA and M_2 in plasma and phosphate buffer, both at physiological pH, was also studied. The initial concentration of each compound was 25, 50 or 100 ng ml^{-1}. Calibration curves were prepared by adding 2·5, 5, 10, 15, 25, 50, 75, 100, 150, and 200 ng of diltiazem and its metabolites to 1 ml of plasma. The percentage recovery of each compound was determined.

In the extraction procedure, 2 ml of plasma containing diltiazem, DAD, and MA were transferred to a screw-capped culture tube. One hundred microlitres of the internal standard solution (0·1 mg%) were added and the tube was vortexed for 15 s. Then, 3 ml of 0·05 M phosphate buffer (pH: 7·5) were added and the mixture was briefly agitated. Following the addition of 6 ml of methyl *tert*-butyl ether, the mixture was agitated for 10 min on a reciprocating shaker and then centrifuged for 10 min at x800g. The organic layer was transferred to a conical tube where 100 μl of 0·05 N sulfuric acid were added. This solution was vortexed for 45 s and centrifuged for 4 min at x800 g. The organic phase was discarded and a volume up to 50 μl of the acidic solution was injected directly into the sample loop.

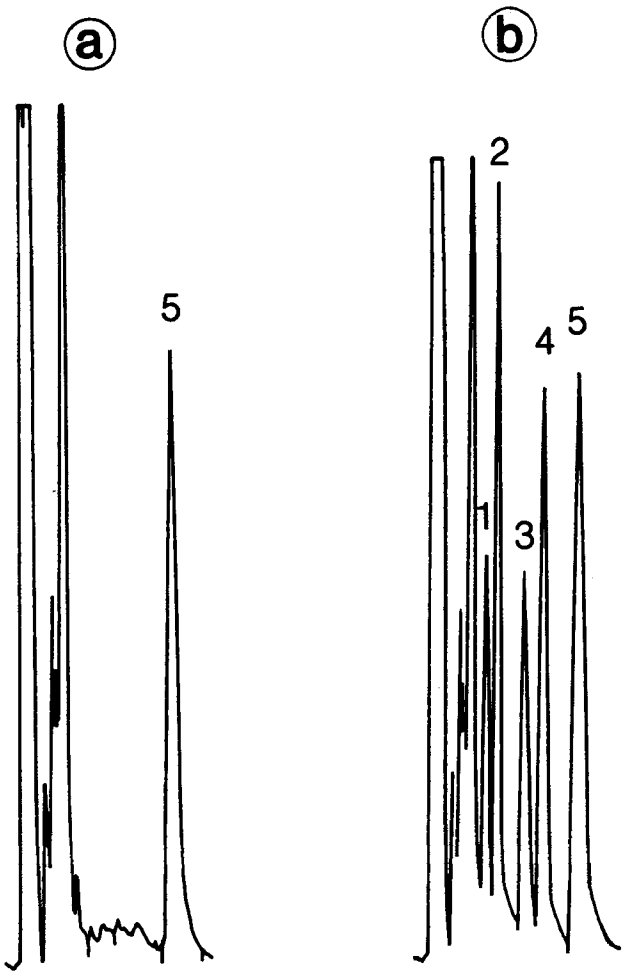

Figure 1. Typical HPLC chromatograms of extracts of human plasma spiked with ⓐ 100 ng/mL of loxapine (5); and, ⓑ 25 ng/mL of M_2 (1), 50 ng/mL of DAD (2), NA (3) ⓓ and diltiazem (4), and 100 ng/mL of loxapine (5)

RESULTS AND DISCUSSION

Under our chromatographic conditions, DAD, MA, diltiazem, and loxapine gave symmetric, well-resolved peaks at 3·4, 4·5, 5·3, and 6·9 min, respectively (Figure 1). Blank plasma samples did not show any significant interference from endogenous compounds at the retention times corresponding to that of the drug and its metabolites. The peak-height ratios (compound/internal standard) of each compound of interest were calculated for several concentrations ranging from 2·5 to 200 ng ml^{-1}. There was a very good correlation between peak-height ratio and the concentration, the correlation coefficients being 0·9988, 0·9995,

and 0·9974 for diltiazem, DAD, and MA, respectively. Recovery for diltiazem and DAD was over 90 per cent while MA was recovered to approximately 75 per cent. The limit of detection of the method for diltiazem, DAD, and MA was 1 ng ml^{-1} of plasma.

Table 1. Stability of diltiazem after 24 h exposure to various stressing conditions

Experimental conditions		% Decrease in diltiazem	% Increase in DAD†
HCl 1 N	25°	59·5	52·2
HCl 1 N	80°	100·0*	58·8
HCl 0·01 *N*	25°	0	
HCl 0·01 *N*	80°	56·8	52·6
Water	80°	3·5	2·3
Buffer pH 1·3	45°	50·0	36·5
pH 5·8	45°	<2·0	1·5
pH 7·4	45°	36·0	25·0
Plasma pH 7·4	25°	0	
Plasma pH 7·4	37°	25·0	29·0

*In less than 2 h.
† Relative to initial concentration of diltiazem.

The stability of diltiazem when exposed to various pH and temperature conditions for 24 h was investigated (Table 1). These two variables proved to be determinant in the degradation of diltiazem. In most cases where a reduction in diltiazem concentration was noticed, a corresponding increase in the concentration of DAD was observed. In very acidic conditions, the degradation of diltiazem was marked and even more so when the temperature was increased. Nearly 60 per cent of diltiazem was degraded to DAD when stored for 24 h in 1N HCl at room temperature. In similar acidic conditions but at 80°, all the diltiazem was degraded in less than 2 h. Under these latter conditions, the increase in DAD accounted for only 59 per cent of the amount of diltiazem originally present. The incubation of DAD alone in 1 N HCl at 80° resulted in a 37 per cent degradation of DAD to products which could not be detected with the present chromatographic conditions. Therefore, it is likely that diltiazem is totally degraded to DAD which is then transformed to unknown products in acidic media maintained at high temperature. In 0·01 N HCl solutions, neither diltiazem nor DAD showed signs of degradation after 24 h. However, when the temperature was increased to 80°, more than half of the diltiazem was degraded after 24 h. Less than 10 per cent of DAD was degraded when incubated alone in 0·01 N HCl at 80°. At a temperature of 45° and at pH of 1·3, 50 per cent of diltiazem was degraded after 24 h. In water and buffer, both at a pH near 6, the temperature of incubation had little effect on diltiazem stability. There was no degradation of diltiazem in plasma stored at room temperature for 24 h whereas there was a 25 per cent reduction in the concentration of diltiazem and an

increase of 29 per cent for DAD at a temperature of 37°. These results have important implications for any evaporations that might be done during the processing of samples for analysis.

Table 2. Stability of diltiazem, (D) DAD, MA, and M_2 in various media at room temperature

(a)

Medium		Time (hs) 12	24	36	48
HCl pH 2·0	D	105 ± 1·6*	105 ± 4·1	100 ± 5·6	101 ± 3·0
	MA	105 ± 2·3	107 ± 3·2	102 ± 8·4	104 ± 2·4
	DAD	105 ± 2·8	107 ± 1·5	102 ± 10	107 ± 5·4
Plasma ph 7·4	D	107 ± 5·1	102 ± 0·7	99 ± 3·1	90 ± 6·4
	MA	99 ± 6·7	79 ± 4·8	72 ± 6·7	49 ± 10·3
	DAD	113 ± 5·5	119 ± 2·1	117 ± 4·6	123 ± 5·3
Phosphate Buffer pH 7·4	D	101 ± 0·1	115 ± 3·5	93 ± 5·3	100 ± 12·3
	MA	99 ± 15	114 ± 6·4	91 ± 8·8	99 ± 11·5
	DAD	110 ± 13·6	123 ± 7·9	105 ± 12·8	119 ± 11·7

(b)

Medium		Time (hs) 0·5	1	2	4
Whole blood	D	100	101 ± 7·0†	97 ± 6·0	89 ± 7·5
	MA	100	103 ± 6·4	96 ± 5·5	88 ± 8·0
	DAD	100	105 ± 8·5	119 ± 7·2	116 ± 6·8
	M_2	100	109 ± 6·5	127 ± 8·3	133 ± 7·7

*Values represent the mean per cent ± S.D. of initial concentration of diltiazem, MA and DAD. Initially, the mixture contained 25, 50, or 100 ng ml^{-1} of each compound.
†Values represent the mean per cent ± S.D. of initial concentration from patient samples at 0·5 h.

As shown in Table 2, the stability of diltiazem, MA, and DAD was also followed over time in an acidic medium, plasma, and phosphate buffer (pH: 7·4) maintained at room temperature. Diltiazem, MA, and DAD dissolved in 0·01N HCl solutions did not undergo any degradation even if they were kept at room temperature for periods up to 48 h and exposed to daylight. However, a degradation of less than 5 per cent of diltiazem was noticed in stock solutions stored at 4° for 1 month. The amount of diltiazem present after 48 h in plasma or buffer at pH 7·4 did not differ significantly from that at time 0. After a period of 24 h, the amount of MA decreased significantly in plasma but not in buffer; only 50 per cent of the MA present initially was found after 48 h. The reduction of the MA peak was associated with an increase in the peak corresponding in retention time to M_2, suggesting the deacetylation of MA to M_2. The fact that degradation

of MA was not observed in buffer at pH 7·4, would suggest that the degradation of MA in plasma could be associated with an enzymatic process, e.g. carboxylesterase.

The stability of diltiazem, DAD, and MA in whole blood was also studied (Table 2). Blood samples from patients receiving multiple doses of diltiazem were immediately placed on ice; they were kept on ice until the stability study at 25° was initiated less than 30 min later, when they were removed and allowed to equilibrate at ambient temperature. Diltiazem, DAD, and MA were stable for 2 h, but diltiazem and MA showed signs of degradation after 4 h. This was accompanied by an increase of 16 per cent in DAD and of 33 per cent in M_2. These results indicate that the blood samples should not only be refrigerated immediately after collection but also centrifuged to separate the plasmatic fraction.

A long-term stability study was also conducted on plasma samples stored at 4° and −20° (Table 3). Diltiazem and DAD were found to be stable for up to 6 weeks at −20° while some degradation of diltiazem was observed after 7 days at 4°. It appears that MA was completely converted to M_2 after 7 days at 4°. Only 66 per cent of the original amount of MA was present after 4 weeks at −20°.

Previous studies using gas chromatography for the determination of diltiazem and derivatives of its metabolites failed to reveal the presence of DAD in the plasma from humans after a massive diltiazem overdosage[9] or in volunteers receiving a single dose of diltiazem.[5] With similar techniques, Sugihara *et al.*[10] also failed to detect the presence of DAD in human plasma samples. The HPLC analytical method described in this paper was used for the determination of pharmacokinetic parameters of diltiazem, MA, and DAD in the plasma of more than 100 volunteers given single or multiple dose treatments (G. Caillé, unpublished results). MA was found to be the principal metabolite of diltiazem, accounting for more than 30 per cent of the concentration of the parent drug. DAD was considered to be a minor metabolite since it represents only 10 per cent of the diltiazem concentration. The inter-subject variation of the C_{min} of MA was only 18 to 25 per cent while it was 79 to 103 per cent for DAD. The results of these studies suggest that some of the DAD present in plasma samples could result from the degradation of diltiazem. While others did not detect the presence of MA in plasma samples, they indicated that DAD steady-state levels represented 10 to 20 per cent of the parent drug.[7,12,13] Moreover, DAD levels as high as those of diltiazem have also been reported.[14] Such high DAD levels could be attributed to the degradation of diltiazem. Similarly, undetectable levels of MA could be due to its degradation in M_2, which is more rapid than that of diltiazem.

Does diltiazem degradation to DAD occur *in vivo* or only *in vitro* during the analytical procedures? The *in vitro* results suggest that the degradation of diltiazem to DAD could also occur *in vivo*. Conditions such as the extreme pH of the stomach or large intestine and a body temperature of 37° are factors that will enhance the degradation of diltiazem. Therefore, a significant fraction of

Table 3. Long-term stability of a mixture of diltiazem (D) and its metabolites in plasma stored at various temperatures

Storage time (days)	Amount (%) remaining*							
	D		DAD		MA		M_2	
	4°	-20°	4°	-20°	4°	-20°	4°	-20°
1	91	99	104	102	107	116	113	103
4	96	98	103	101	70	100	136	106
7	82	93	116	102	N.D.	92	197	114
14	–	90	–	93	–	94	–	119
28	–	100	–	98	–	66	–	134
42	–	102	–	104	–	65	–	149

*Initial concentration of each product was 100 ng ml^{-1}.
N.D. not detectable.

plasmatic DAD may result from the *in vivo* degradation of diltiazem and not from its metabolism. As a consequence, this could lead to erroneous interpretations not only of the pharmacokinetics but also of the metabolic capacity of individuals. It is also evident from these various studies with plasma and whole blood that the steps involved in the processing of specimens are important determinants of the stability of diltiazem and more so for MA. Therefore, it is essential that the plasma be separated by refrigerated centrifugation immediately after collecting the blood and the plasma samples stored at –20° until the time of analysis.

The results of this study indicate that diltiazem is stable for at least 6 weeks in frozen plasma, but degradation is likely to occur quite rapidly if plasma is exposed to temperatures above 25°. MA is much less stable than diltiazem and analysis of plasma samples should be performed at most within 2 weeks after collection even if the samples are stored at –20°. Moreover, if frozen plasma is stored for a long period of time, this may favor the formation of a contaminant which has a retention time similar to that of DAD.[7,11,12] There is no significant degradation of diltiazem or its metabolites if the extraction and analysis are done in the way recommended in this paper.

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