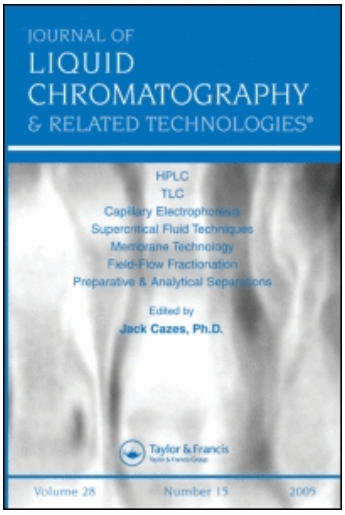

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Determination of Diltiazem and its Metabolites in Plasma by High Performance Liquid Chromatography

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To cite this Article Boulieu, R. , Bonnefous, J. L. and Ferry, S.(1990) 'Determination of Diltiazem and its Metabolites in Plasma by High Performance Liquid Chromatography', Journal of Liquid Chromatography & Related Technologies, 13: 2, 291 — 301

To link to this Article: DOI: 10.1080/01483919008049544

URL: http://dx.doi.org/10.1080/01483919008049544

JOURNAL OF LIQUID CHROMATOGRAPHY, 13(2), 291-301 (1990)

DETERMINATION OF DILTIAZEM AND ITS METABOLITES IN PLASMA BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

In this high performance liquid chromatographic method described for the analysis of diltiazem and its metabolites, the compounds of interest are separated with high efficiency and selectivity on a Nucleosil C18 column, 3 μm particle size. Stability assay shows a significant degradation of diltiazem and its metabolites in blood samples and only a significant degradation of the major metabolite, N-demethyldiltiazem in plasma when samples are kept at room temperature. This observation shows the necessity of observing rigorous conditions during collection, transport and treatment of blood samples. This method is applied to the analysis of plasma samples from patients under diltiazem therapy.

INTRODUCTION

Diltiazem is a calcium channel blocker currently used in the treatment of variant angina, hypertension and supraventricular tachyarrythmias (1). Diltiazem is extensively metabolised by deacetylation, N-demethylation, O-demethylation and conjugation (2).

Several high performance liquid chromatographic (HPLC) methods have been developed for the determination of diltiazem and its metabolites (3-8). Among these methods some described only the analysis of diltiazem and deacetyldiltiazem considered as the major metabolite and three (5, 7, 8) report the separation of others metabolites and particularly N-demethyldiltiazem (MA) which was identified to be an important metabolite (2, 9, 10) with pharmacological activity as a coronary vasodilator (2, 10).

In the present paper we describe a selective HPLC method using a Nucleosil C18 column, 3 µm particle size for the simultaneous determination of diltiazem and the most important metabolites, deacetyldiltiazem (M1), N-demethyldiltiazem (MA) and N-demethyldeacetyldiltiazem (M2). The small particle 3 µm column employed in this technique provides a higher degree of efficiency within a faster analysis time than the 10 or 5 µm column used in the techniques cited above. The method was applied to the analysis of these compounds in plasma from patients after chronic oral administration of diltiazem. Furthermore, the stability of diltiazem and its metabolites in blood and plasma samples was investigated.

MATERIALS AND METHODS

Apparatus

The liquid chromatograph consisted of a Model 510 HPLC pump equipped with a Model 481 variable-Wavelength absorbance detector, a Model 712 WISP sample processor (all from Waters, Saint Quentin -

Yvelines, France) and a D-2000 chromato integrator (Merck, Nogent-sur-Marne, France).

We used a Nucleosil C18 column (15 cm x 4.6 mm ID), 3 µm particle size preceded by a Nucleosil C18 guard-column (1.5 cm x 4.6 mm ID) (Société Française de chromato-colonne, Neuilly-Plaisance, France).

Reagents

Diltiazem (DTZ), deacetyldiltiazem (M1), N-demethyldiltiazem (MA), N-demethyldeacetyldiltiazem (M2) and propionyldeacetyl-diltiazem used as internal standard were generously supplied by the Clinical Research Department, L.E.R.S., Paris, France.
Acetonitrile, ammonium dihydrogen phosphate, boric acid, sodium chlorid and orthophosphoric acid "suprapur" were purchased from Merck, Nogent-sur-Marne, France). Triethylamine was from Fluka (Buchs, switzerland)

Chromatographic Conditions

The compounds of interest were separated using an isocratic elution mode. The mobile phase consisted of acetonitrile/ ammonium dihydrogen phosphate buffer (0.1 mol/L), 37/63 by vol., containing 0.06 % of triethylamine. The pH of the final solution was adjusted to 5.9 with orthophosphoric acid. The flow rate was 1 ml/min and the detection was performed at 237 nm.

Sample Collection and Storage

Blood samples were collected in a heparinized tube. Each sample must be centrifuged without delay at low temperature (+ 4°C) and plasma samples immediately stored at - 20°C until analysis to prevent metabolic changes.

Extraction Procedure

To 1 ml of plasma we added 50 µl of internal standard (propionyl-deacetyldiltiazem, 5 µg/ml) and 500 µl of borate buffer (0.1 mol/L, pH 9). The mixture is extracted with 5 ml of methyltertbutyl ether by mixing for 20 minutes on a mechanical shaker and then centrigufed at 1500 x g for five minutes. The organic phase is transferred to another centrifuge tube and back-extracted with 500 µl of 2.1 mmol/L orthophosphoric acid by vortex-mixing for 1 minute.

After centrifugation, we discarded the organic phase and evaporated the aqueous phase under nitrogen at 40°C. We reconstituted the residue with 100 µl of mobile phase and injected 60 µl of this solution for chromatographic analysis.

RESULTS AND DISCUSSION

Chromatographic Separation

Diltiazem and its metabolites are weak amines, thus their resolution in reversed-phase chromatography can be influenced by the pH of the mobile phase and the ion concentration of the buffer.

Figure 1 (a) shows that the pH of the mobile phase affects mainly the column capacity ratio (k') of the compounds. From this figure, diltiazem and its metabolites should be correctly resolved within a reasonable elution time at pH 5.9.

The effect of the ionic strength on the retention is represented in figure 1 (b). An increase of the ionic strength from 0.01 mol/L to 0.1 mol/L decreases significantly the retention.

The retention of diltiazem and its metabolites is also strongly affected by the concentration of organic modifier, the retention of the compounds decreases as the concentration of acetonitrile increases.

Ionic strength and acetonitrile content are defined in order to obtain the optimal separation of the compounds of interest. However, peak tailing has

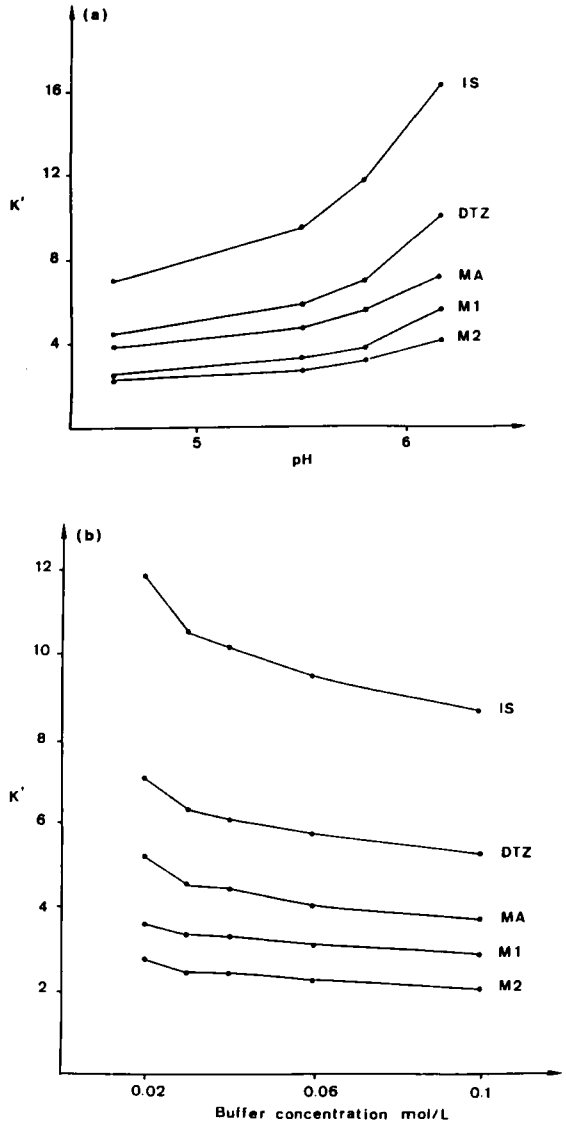

Figure 1 - Influence of the mobile phase composition on column capacity ratio (K') :
(a) effect of the pH ; mobile phase : acetonitrile / 0.01 mol/L phosphate buffer, 37/63 by vol. with 0.06 % of triethylamine
(b) Effect of the ionic strength ; mobile phase : acetonitrile / phosphate buffer, 37/63 by vol. with O.O6 % of triethylamine, pH 5.9.

been observed without improvement with regard to variation in mobile phase composition. This phenomenon, probably due to the interaction of amino groups with the residual silanols of alkyl-silica bonded phase, has been eliminated by addition of triethylamine at low concentration to the mobile phase as suggested by Horvath and collaborators (11,12).

From these data, the optimal mobile phase composition for the separation of diltiazem and its metabolites was determined. The corresponding chromatogram is shown in figure 2.

Recovery, Linearity and Reproducibility

Analytical recovery of each component was determined by addition of known concentrations of diltiazem and metabolites to plasma. The extraction and analyses were then performed as described and the peak heights compared with those obtained by direct injection of aqueous standards. Mean recoveries (%) and (SD), (n=7) at different concentrations was 67 (5.8) for M2, 74.5 (5.7) for M1, 79.5 (6.2) for MA, 81 (7.5) for Diltiazem and 90.5 (4.1) for internal standard.

The relationship between concentration and peak height of each compound was linear up to 300 ng/ml. The detection limit was 5 ng/ml for each compound when 1 ml of plasma was extracted.

Analytical precision and accuracy were assessed by repeated analysis of serum controls containing various concentrations of diltiazem and metabolites. The results are shown in table 1.

There was no interference with the compounds of interest by other drugs which are often co-administered with diltiazem : diazepam, flunitrazepam, midazolam, pancuronium bromide, quinidine, verapamil, propanolol.

Stability Assay

The stability of Diltiazem and its metabolites was investigated in blood and in plasma samples.

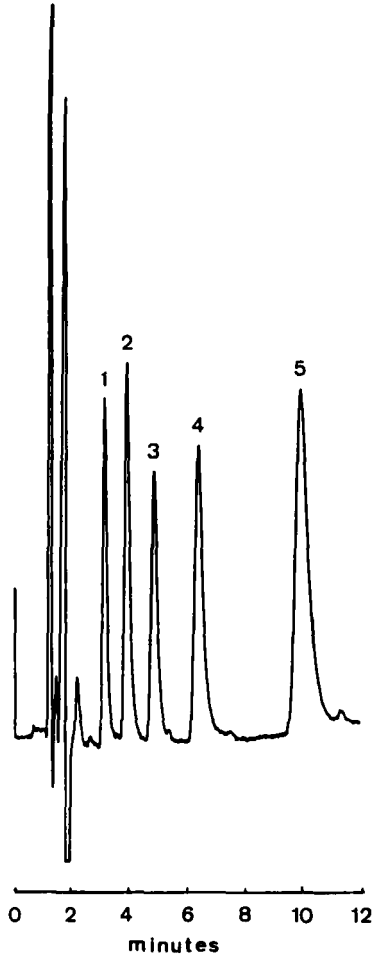

Figure 2 - Chromatographic separation of diltiazem and its metabolites. Chromatographic conditions are described in materials and methods.
Peaks : 1- N-demethyldeacetyldiltiazem (M2). 2- deacetyldiltiazem (M1). 3- N-demethyldiltiazem (MA). 4- diltiazem. 5- propionyl-deacetyl diltiazem (internal standard).

TABLE 1
Precision and accuracy

	Within-day, n = 5			day to day, n = 5		
	added concentration ng/ml	measured concentration ng/ml	CV,%	added concentration ng/ml	measured concentration ng/ml	CV,%
M2	25	20.9	4.9	25	22.2	4.9
	150	142.0	2.9	150	151.0	4.7
M1	25	25.0	7.3	25	25.6	2.8
	150	142.6	1.6	150	148.9	3.3
MA	25	25.6	3.0	25	24.3	4.2
	150	147.1	1.2	150	148.5	2.7
DTZ	25	25.4	3.4	25	24.4	5.4
	150	148.3	3.6	150	149.8	2.0

In blood samples kept at room temperature, a significant degradation of diltiazem was observed. When whole blood is centrifuged one hour after collection, 90 % of diltiazem is recovered. Under the same conditons, only 60 % of the original amount of the major metabolite N-demethyldiltiazem (MA) is recovered. This important decrease appears to be related to a slight increase of the peak with a retention time corresponding to M2.

In blood samples kept at 4°C for two hours, the degradation of diltiazem and MA is less important, 95 % of the drug and of the major metabolite MA has been recovered.

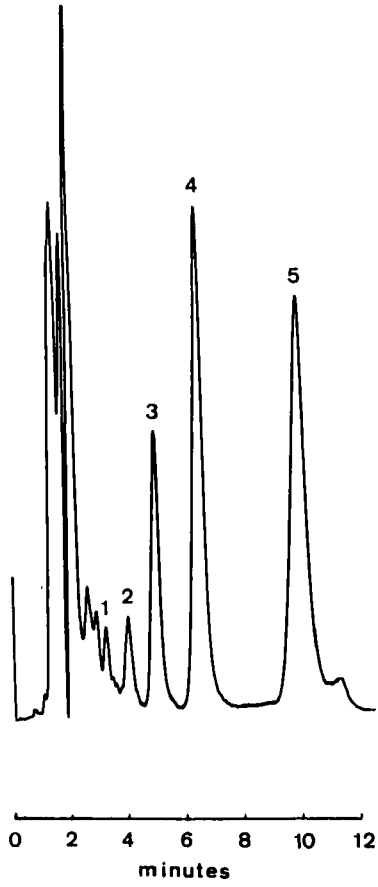

Figure 3 - Representative chromatogram of a plasma sample collected 2.5 hours after oral administration of 60 mg of diltiazem from a patient under chronic diltiazem therapy (60 mg, third daily). Injection volume : 60 ml. Peaks 1- N-demethyldeacetyldiltiazem (M2), 19 ng/ml. 2- deacetyl-diltiazem (M1), 29 ng/ml. 3- N-demethyldiltiazem (MA), 125 ng/ml. 4-diltiazem, 242 ng/ml. 5- propionyldeacetyldiltiazem (internal standard), 250 ng/ml.

In plasma , only the major metabolite MA was found unstable when samples are kept for two hours at room temperature whereas at 4°C, no degradation occurred.

These data suggest that marked degradation occur in blood and plasma samples kept at room temperature. Besides, Dubé et al (8) have recently noted a significant variation of MA in plasma kept during four days at 4°C.

Our study shows that for an accurate determination of diltiazem and its metabolites, especially the major metabolite MA, blood samples must be centrifuged immediately at low temperature and plasma samples immediately frozen at - 20°C until analysis to prevent metabolic conversion.

Applications

The present method was used to determine the concentration of diltiazem and its metabolites in plasma samples from patients under diltiazem therapy. The typical chromatogram obtained from a patient after chronic oral administration of diltiazem is shown in figure 3. MA was found as the major metabolite in plasma samples (50 % of the parent drug, diltiazem) whereas deacetyldiltiazem (M1) represents only 12 %.

Further pharmacokinetic studies will be performed in patients under cardiopulmonary bypass using this HPLC method and observing the rigorous conditions described in this paper.

ACKNOWLEDGMENTS

We thank Dr. Jean-Jacques Lehot who kindly entrusted us with the study of his patients and also the Clinical Research Department L.E.R.S., Paris, France for supplying diltiazem and related compounds.

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