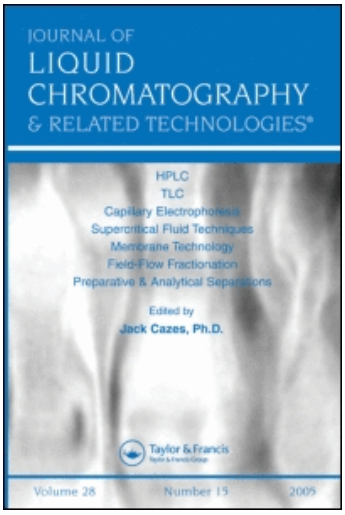

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Automated High-Performance Liquid Chromatographic Technique for Determining Diltiazem and its Three Main Metabolites in Serum

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AUTOMATED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC TECHNIQUE FOR DETERMINING DILTIAZEM AND ITS THREE MAIN METABOLITES IN SERUM

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ABSTRACT

A sensitive and automatic method for the analysis of diltiazem and its three main metabolites in acidified serum is described using solid-liquid extraction on disposable extraction cartridges coupled to injection via a loop-column in an HPLC System. This method avoids the in-vitro degradation of diltiazem and its main metabolite MA (N-monodemethyl diltiazem) into M1 (desacetyl diltiazem) and M2 (desacetyl-N-demethyl diltiazem) and eliminates the numerous manipulations involved in liquid-liquid extraction.

INTRODUCTION

Diltiazem, cis-(+)-3-acetyloxy-5-(2-dimethylaminoethyl)-2,3-dihydro-2-(4-methoxyphenyl)-1,5-benzo thiazepin-4-(5H)-one hydrochloride, is a calcium inhibitor commonly used in the treatment of angina pectoris, arrhythmia and hypertension (1).
Numerous gas chromatography methods (2-5) and high-performance

liquid chromatography techniques (6-22) have been described for the analysis of diltiazem in biological media. The most recent (12-22) permit the quantification of the main metabolites of diltiazem (DTZ), which is highly metabolized by deacetylation and N-demethylation giving rise to:

* N-monodemethyldiltiazem(MA),main metabolite,
* Desacetyldiltiazem (DAD or M1)
* Desacetyl-N-monodemethyl diltiazem (M2)

As a result of the important problems related to the instability of diltiazem and its metabolites with regard to temperature, pH and the type of medium (13,15,16,17,20), we have developed an HPLC assay with solid-liquid extraction of acidified serum followed by immediate injection via a loop-column (24) using an automatic injector. The assay is rapid, reproducible and sensitive. It avoids the in-vitro degradation of MA and DTZ into M2 and M1, respectively, and eliminates the numerous manipulations involved in liquid-liquid extraction.

MATERIALS AND METHODS

Equipment

The HPLC system comprises an LC6A pump (Shimadzu-Touzart Matignon), an ASPEC system (Automatic Sample Preparation with Extraction Columns, Gilson-France), a 5 µm cyanopropyl-silane precolumn (Merck) in place of the injection loop, a photodiode array detector (Spectro-Monitor 5000, LDC-Analytical) and a CR4A recorder-integrator-calculator (Shimadzu, Touzart et Matignon).
The analytical column is a Nucleosil C18, 5 µm, 250 mm x 4.6 ID, preceded by a Nucleosil C18, 5 µm, 1 cm, precolumn and a prefilter (Société Française Chromato-Colonne, Neuilly Plaisance, France).
Extraction is performed using 100 mg cyanopropyl Bond Elut extraction columns (Prolabo, France). Serum samples and eluates are collected into disposable polypropylene tubes (Gilson-France).

Chromatographic Conditions

The mobile phase is composed of 0.005 M heptane sulfonic acid-acetonitrile-methanol-glacial acetic acid-triethylamine (60:35:5:5.5:0.75), with a flow rate of 1.5 ml/min.
All the analyses are carried out in isocratic mode at room temperature. The detector is set at 240 nm.

Blood Sampling and Storage Conditions

Venous blood is collected into dry tubes without an activating gel (Venoject, Terumo, France). Serum is isolated by centrifugation at 4°C, 30 min after collection (samples are stored at 4°C until centrifugation). The sera are immediately stored at -20°C for a maximum of one month until assay.

Reagents

Pure diltiazem base, desacetyl-N-monodemethyl diltiazem fumarate (M2), desacetyl diltiazem base (DAD or M1) and N-monodemethyl diltiazem fumarate (MA) were supplied by Synthelabo (LERS, France).
The internal standard UP 312-04 (diphenyl-2,2-cyclopropane carboxylique acid β dimethylaminoethyl ester hydrochloride) was supplied by UPSA (Rueil Malmaison, France).
Potassium dihydrogen phosphate (Merck), disodium hydrogen phosphate dihydrate (Merck), pure formic acid (Prolabo) and heptane sulfonic acid (Kodak) were of analytical quality.
Methanol (Carlo Erba), acetonitrile (Merck), glacial acetic acid (Prolabo) and triethylamine (Prolabo) were HPLC quality.
Water was milliQ quality (Millipore).

Standard Solutions

Stock standard solutions were prepared by dissolving diltiazem base in methanol to a concentration of 1 mg/ml; stock solutions at 0.5 mg/ml were prepared similarly for N-monodemethyl diltiazem (MA), desacetyl diltiazem (M1) and desacetyl-N-demethyl diltiazem (M2).
An aqueous stock solution of internal standard was prepared at 0.2 mg/ml.
Solutions for calibration were prepared by diluting the stock solutions, first in water, then in serum, to achieve the following concentrations: 5, 10, 20, 40, 80, 100 ng/ml for diltiazem and 2.5, 5, 10, 20, 40, 50 ng/ml for the three metabolites. Aliquots (1.25ml) were stored at -20°C.

Sample Pretreatment

Serum samples are pretreated with formic acid, as follows: 6 µl of formic acid is added to 1.2 ml of test serum in disposable polypropylene tubes; after vortexing, 120 µl of internal standard (4 µg/ml) is added to each sample.

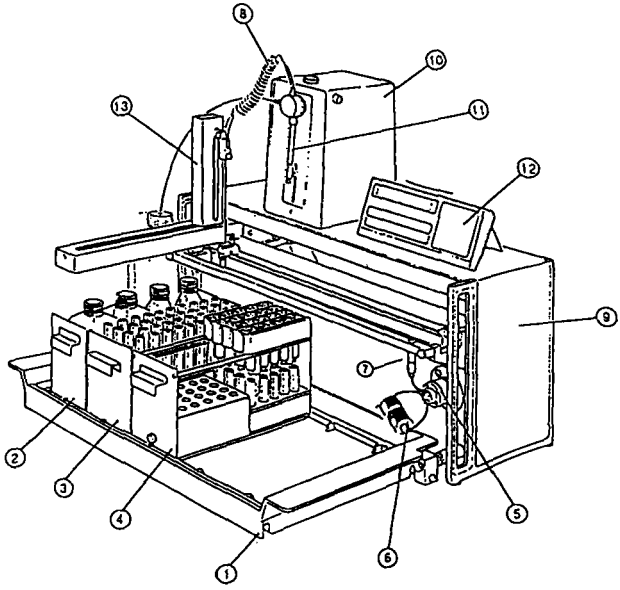

Figure 1. ASPEC (Automatic Sample Preparation with Extraction Columns)
(1) Polypropylene tray, (2) Solvent rack,
(3) Sample rack, (4) SPE rack,
(5) Rheodyne injection valve,
(6) Loop-column, (7) Loop filler port,
(8) Coiled tubing,
(9) Sample Processor and injector module,
(10) Model 401 Dilutor, (11) 10ml syringe
(12) Keypad controller, (13) Vertical arm

Extraction Procedure

The ASPEC system (Fig.1) performs all the steps from the extraction of the acidified sera on the cyanopropyl Bond Elut column to the injection into the loop-column.
First, the extraction columns are pretreated with 1 ml of methanol then 1 ml of 0.5 M phosphate buffer, pH 7.8. After depositing 1 ml of pretreated sample, the extraction column is washed with 1 ml of buffer, pH 7.8, followed by 3 ml of water.
The elution is carried out using 600 µl of methanol. The methanolic eluate is then diluted with 2.4 ml of water before

injecting 2/3 into the loop-column prewashed with 1 ml of water. After washing with 1 ml of water, the contents of the loop-column are injected into the chromatographic system.

RESULTS AND DISCUSSION

Chromatographic Separation

In our chromatographic conditions, derived from the assay technique described by Montanat et al.(14), the metabolites (M2, M1, MA), diltiazem and the internal standard gave well-separated symmetrical peaks at 7.9, 9.1, 13.3, 15.7 and 18 min, respectively (Fig. 2). The serum blanks did not give interfering peaks due to endogenous compounds at the retention times corresponding to diltiazem and its metabolites. In contrast, several times we observed a parasitic peak near that of M1 when the water used for the dilution of the eluate and rinsing was not freshly prepared using a sterilizing 0.22 um pore-size filter.

Sample collection and Pretreatment : Stability

Diltiazem and its metabolites keep best at acid pH (HCl 0,01 N) when plasma or serum samples remain at room temperature for several hours (16). The influence of two acids on the stability of diltiazem was tested: acetic and formic acids were added at 50 ul per 10 ml of serum. Figure 3 shows the degradation of diltiazem into M1 (expressed as the increase in the M1/DTZ peak ratio) when a spiked control serum was not acidified (pH 8). The ratio remained stable when the spiked serum was acidified with acetic acid (pH 4.7) or formic acid (pH 3.8) for at least 18 hours at 20°C. We chose formic acid because, contrary to acetic acid, it does not render the serum opalescent. In addition, in order to avoid the formation of a solid gel due to the gradual precipitation of fibrin in acidified plasma samples, it is necessary to use serum. Moreover, blood was collected in tubes without an activator. After six hours'storage of blood samples in tubes containing a fibrinolysis activator there was a 30-40% loss of M2, M1, MA, DTZ and IS relative to identical samples stored in the same conditions in the absence of activator. This may be explained by the binding of basic molecules to the filter-activator, as is the case of Tricyclic Antidepressant (23).

In the sample collection conditions used, there was no degradation of diltiazem or its metabolites. Yet, frozen storage (-20°C) of diltiazem calibrators or quality controls must be realized in serum because adsorption of diltiazem (about 15%)

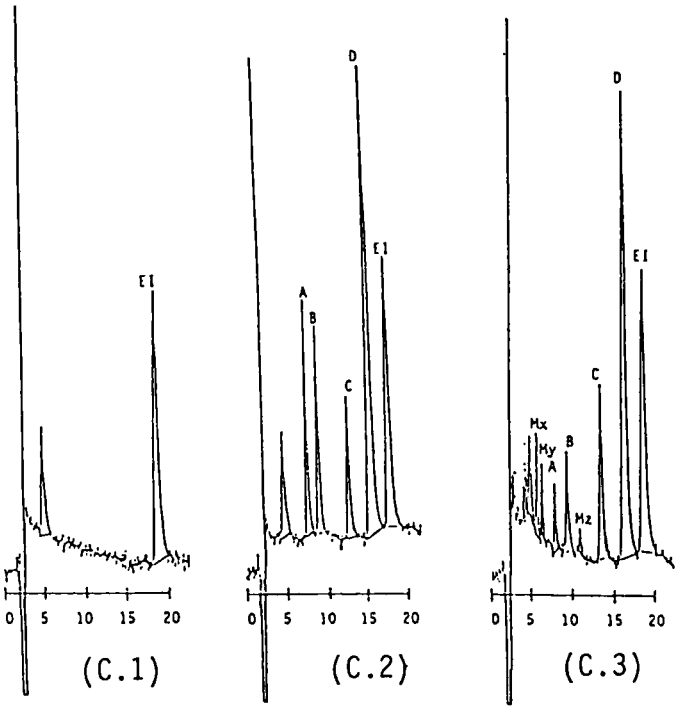

Figure 2. Chromatograms of :
(C.1) Blank serum
(C.2) Human standard spiked at :
(A) 20ng/ml of M2 , (B) 20ng/ml of M1,
(C) 20ng/ml of MA , (D) 100ng/ml of DTZ.
(C.3) a sample from a volonteer subject
after a single 120mg oral dose of DTZ :
(A) 5ng/ml of M2 , (B) 9ng/ml of M1,
(C) 24ng/ml of MA , (D) 97ng/ml of DTZ,
(Mx,My,Mz) Metabolites non identified.

occured when aqueous diltiazem solutions were stored in polystyrene tubes.

Solid-Liquid Extraction of Acidified Samples

The solid-liquid extraction procedure, combined with the injection via a loop-column presents a number of advantages relative to classic liquid-liquid extraction followed by

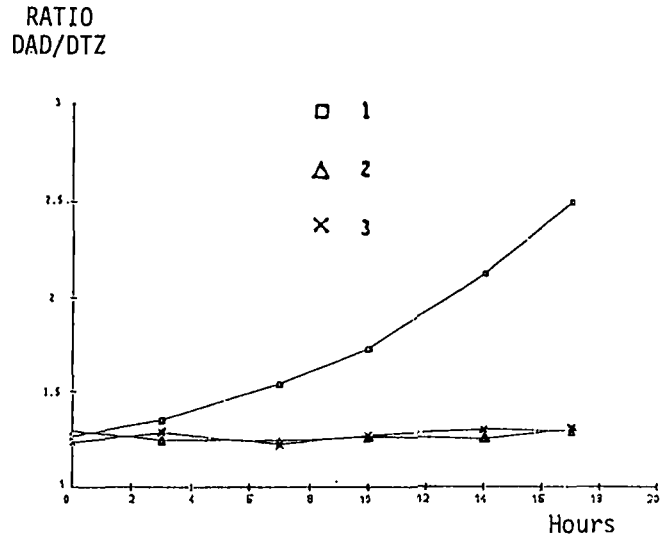

Figure 3. pH influence on the stability of DTZ.
(1) Spiked control serum (pH 8)
(2) Serum with Acetic acid (pH 4,7)
(3) Serum with Formic acid (pH 3,8).

injection via the traditional loop used by many authors.
First, it minimizes interference associated, on the one hand, with glassware (only disposable polypropylene tubes are used) and, on the other hand, with biological components: the molecules assayed in the serum sample are bound successively to two supports -- the extraction column and the loop column -- which are thoroughly washed with buffer and water following the deposit.
Second, it dispenses with the eluate evaporation step which can cause the degradation of DTZ into M1 and MA into M2.
Finally, it can be fully automated and used sequentially.
All these factors contribute to a greater stability of the compounds assayed.
Figure 1 illustrates the main elements of the ASPEC (Automatic-Sample-Preparation with Extraction-Columns) which performs the various steps from extraction to injection onto the analytical column.
The replacement of the classic injection loop used in the original technique (24) by a small cyanopropyl column (1 cm X 10 µm) allows the extraction step to be followed immediately by the

TABLE 1

Precision and accuracy of the method

Within day (n=12)

	Amount added ng/ml	Amount found ng/ml	CV %
M2	25	24,1	3,9
	50	49,9	3,1
M1	25	21,9	4,4
	50	44,5	3,8
MA	25	26,5	4,1
	50	53,7	3,0
DTZ	100	100,9	1,5
	150	150,3	4,3

Day-to-day (n=28)

	Amount added ng/ml	Amount found ng/ml	CV %
M2	15	15,0	5,0
	25	25,4	5,9
	50	52,1	5,8
M1	15	14,5	6,3
	25	25,5	5,1
	50	51,6	4,4
MA	15	15,1	5,9
	25	25,3	4,6
	50	51,7	3,7
DTZ	30	29,2	4,3
	60	59,2	4,0
	125	117	3,4
	250	240	3,2

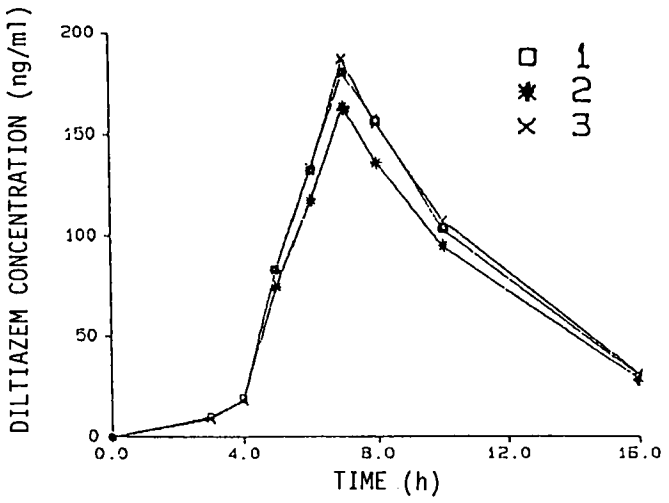

Figure 4. Diltiazem concentrations of several samples from a volonteer assaying at a 4-month interval. Results after extraction of :
(1) calibration and samples freshly frozen.
(2) Fresh calibration and same samples frozen for more than 4 months.
(3) calibration and samples frozen at the same time, 4 months ago.

injection, cutting out the evaporation step. The methanolic eluate collected after extraction on the Bond Elut column is first diluted with water to bring the proportion of methanol down to the maximum tolerated level (20%) in order to permit the binding of the assay molecules to the loop column. As diltiazem and its metabolites are very unstable in methanol-water , injection must take place immediately; this is the case in the automated sequential mode.

Characteristics of the Method

Within-run variability did not exceed 4,5%. Between-run variability for diltiazem and its metabolites are given in table 1. In the conditions described above, the response was linear

between 0 and 300 ng/l for diltiazem, and 0 and 150 ng/l for its metabolites.
The concentration below which the coefficient of variation exceeded 20% was 2.5 ng/ml for diltiazem and 1.5 ng/ml for its metabolites.
The reliability of the technique was assessed in a pharmacokinetic study over a 5-week period. No drift was observed either in the slopes of the calibration lines for the four compounds assayed, or in the values of the quality controls inserted in each run.
We studied the feasibility of assaying samples stored frozen for more than four months by assaying a sample at a 4-month interval. The concentration were calculated both using the calibration samples frozen at the same time as the sample and the freshly-prepared calibration samples. The results (Fig.4) showed good agreement between the values only when they were read on the calibration curve prepared from standards stored with the samples.
This rapid, sensitive and reproducible technique for assaying diltiazem and its metabolites avoids the problems of in-vitro degradation by the use of an automated sequential mode.

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