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AUTOMATED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC AND COLUMN-SWITCHING TECHNIQUE FOR ON-LINE CLEAN-UP AND ANALYSIS OF DILTIAZEM IN HUMAN PLASMA

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SUMMARY

An automated high-performance liquid chromatographic method for the direct injection of diltiazem plasma samples has been developed. It involves automatic injection of plasma samples (200 μl) on a C_{18} pre-column (40 μm), clean-up of the pre-column with water to remove proteins and salts and transfer of the analytes to the analytical column. During the chromatographic process, the pre-column is reset with respect to the analytical column and flushed with different solvents to remove endogenous contaminants. The analysis is performed on a C_{18} column coupled to an ultraviolet detector. The whole process (on-line clean-up and chromatography) takes ca. 12 min and is completely automated. The detection limit of the method is ca. 2 ng/ml with 200-μl aliquots of plasma sample. Very good results were obtained for the day-to-day and within-day reproducibilities (5.9 and 4.3%, respectively, in the concentration range 10–200 ng/ml in plasma).

INTRODUCTION

Diltiazem is a calcium antagonist currently used in the treatment of clinical manifestations of variant angina [1–4], and with probable efficacy as an antiarrhytmic [5] and antihypertensive drug [6]. Several methods for the measurement of diltiazem concentrations in plasma have been developed, exploiting gas chromatography [7–9] and high-performance liquid chromatography (HPLC) [10–12]. Both techniques are time-consuming when performed off-line. In gas chromatography, derivatization reactions are necessary to resolve diltiazem from the metabolite desacetyldiltiazem as well as to improve the sensitivity. In reversed-phase HPLC, acid back-purification procedures [10,11] or liquid–solid extractions [12] are sometimes required.

The method described in this paper involves direct injection of plasma samples into the chromatographic system without prior purification or separation. The

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TABLE I

STANDARD SOLUTIONS USED FOR ANALYSIS

Standard solution No.	Diltiazem concentration (ng per 20 μl)	Internal standard concentration (ng per 30 μl)
1	20	–
2	50	–
3	200	–
4	400	–
5	–	150

method consists of automatic on-line clean-up of plasma samples and HPLC with column-switching. The chromatography is performed on a C_{18} bonded-phase column coupled to UV detector.

EXPERIMENTAL

Reagents and solvents

Methanol and acetonitrile were HPLC grade, obtained from J.T. Baker (Deventer, The Netherlands); triethylamine, used for the preparation of eluent mixture, was analytical-reagent grade reagent (Carlo Erba, Milan, Italy). The water used for the preparation of buffer solutions and chromatographic eluent was HPLC grade, produced by the Milli Q-4 system (Millipore, Bedford, MA, U.S.A.). A 1-l volume of 0.05 *M* phosphate buffer (pH 2.5) was prepared mixing equal parts of 0.05 *M* potassium dihydrogenphosphate, obtained from analytical-grade salt, and 0.05 *M* phosphoric acid, obtained from 85% phosphoric acid (analytical-reagent grade, Carlo Erba); the aqueous solution was then filtered through a 0.45-μm filter (type HATF, Millipore, Molsheim, France).

Diltiazem, 3-(acetyloxy)-5-[2-(dimethylamino)ethyl]-2,3-dihydro-2-(4-methoxyphenyl)-1,5-benzothiazepin-4(5*H*)-one hydrochloride, desacetyldiltiazem, 3-(hydroxy)-5-[2-(dimethylamino)ethyl]-2,3-dihydro-2-(4-methoxyphenyl)-1,5-benzothiazepin-4(5*H*)-one hydrochloride, and the internal standard, 3-(acetyloxy)-5-[2-(dimethylamino)ethyl]-2,3-dihydro-2-(4-propoxyphenyl)-1,5-benzothiazepin-4(5*H*)-one hydrochloride, were of pharmaceutical grade and provided by the L.E.R.S. Chemistry Department (Paris, France).

Standard solutions

Stock solutions of diltiazem, its metabolite and the internal standard were prepared in methanol at a concentration of 1 mg/ml. Standard solutions were prepared from stock solutions by suitable dilutions with water and used for the preparation of plasma standards (see Table I). Stock solutions were stable for at least one month if stored at 0–5 °C, aqueous standard solutions were stable for at least one week if maintained at 0–5 °C.

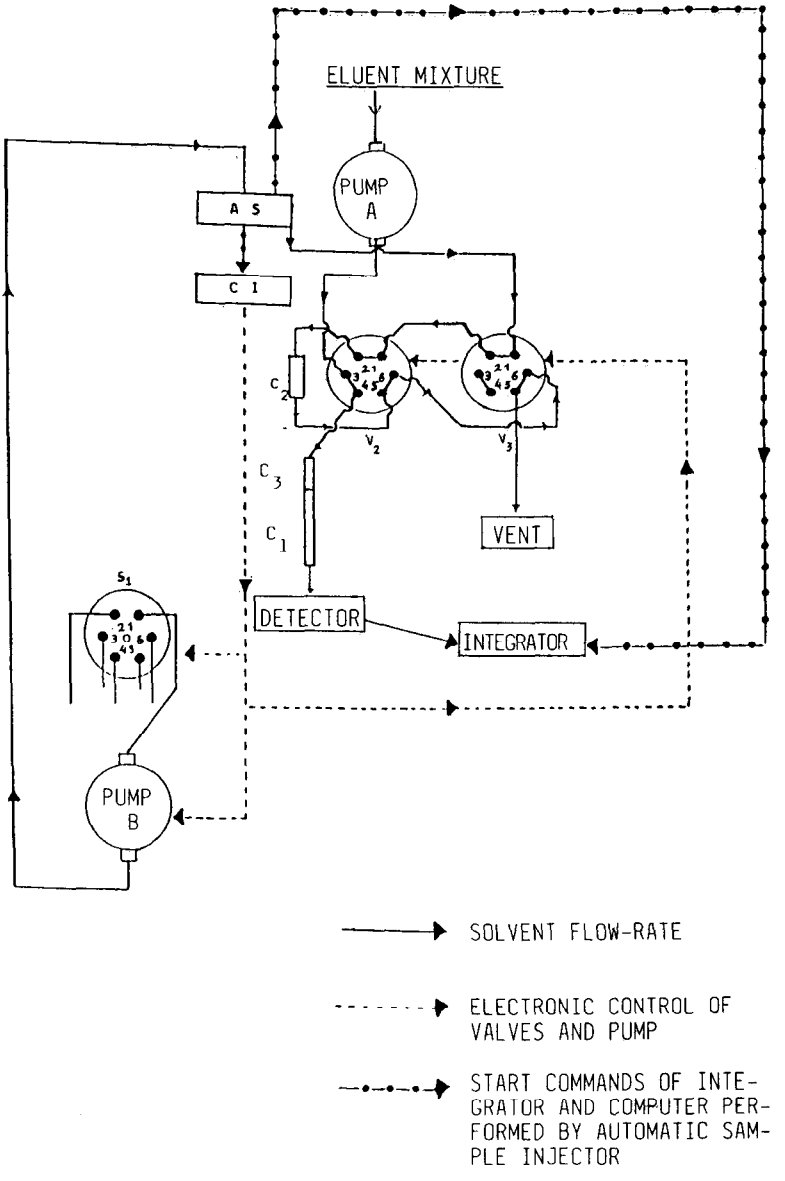

Fig. 1. Scheme of chromatographic apparatus used for automatic on-line clean-up of samples and HPLC with column-switching.

Chromatographic system

The chromatographic system consisted of a Model 414 T constant-flow pump (Kontron, Zürich, Switzerland), a Model 4025 spectrophotometric LC detector (Pye Unicam, Cambridge, U.K.) operated at a wavelength of 238 nm, a Sedex 100 automatic sample injector (Sedere, Vitry-sur-Seine, France) with an automatic valve and a loop capacity of 200 μl (the syringe was provided with a large-

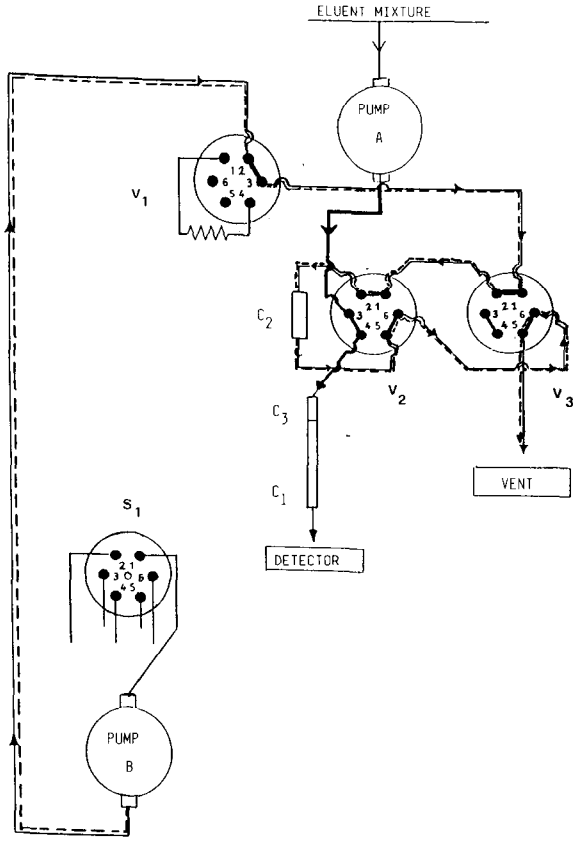

Fig. 2. Equilibrium preceding sample injection.

bore needle for sampling plasma fluid), an analytical column (25×0.46 cm I.D.) filled with 5-μm Supelcosil® LC-18-DB and maintained at ambient temperature, and a guard column (2×0.46 cm I.D.) filled with the same packing (Supelco, Bellefonte, PA, U.S.A.).

The mobile phase was acetonitrile–0.05 *M* phosphate buffer (pH 2.5) (65:35) containing 0.25% (v/v) triethylamine. The flow-rate was 1 ml/min. The detector was coupled to an SP 4270 chromatographic integrator (Spectra-Physics, San Jose, CA, U.S.A.) for the determination of peak height. The calculations were performed according to the internal standard method using a multi-point linear calibration.

This basic HPLC system was extended with a pre-column for on-line clean-up (7.5 cm×0.21 cm I.D.), filled with C_{18} CP™ Elut, 40 μm (Chrompack, Middelburg, The Netherlands), a compact switching apparatus, Model Tracer MCS-670 (Kontron) with five six-way high-pressure valves (Rheodyne, Cotati, CA, U.S.A.) for switching procedures and two six-way low-pressure valves for solvent selection, and a pump fit to the clean-up of the biological sample processed. It was not necessary to use all the available valves (high and low pressure) to run

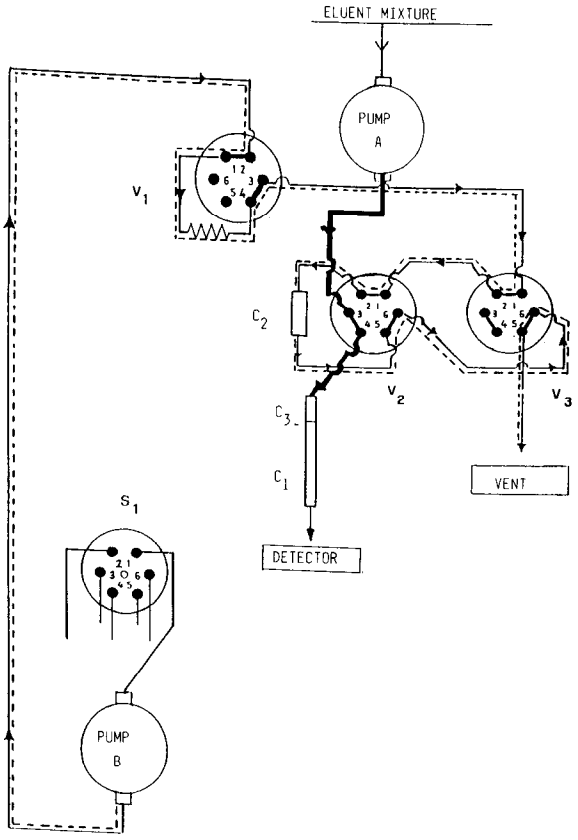

Fig. 3. Loading and clean-up of the sample on the pre-column.

the proposed method. The automation of the switching apparatus (valves and pumps) was managed by a computer or a chromatographic programmer (Model 2000, Kontron).

Operating conditions for on-line clean-up and column-switching

Fig. 1 shows the whole hardware system. The automatic sample injector (AS) controls the chromatographic process: after sampling and injecting the biological fluid it gives starting commands to the integrator and to the computer (CI) or programmer which, according to the defined program, will control and activate valves and pump B (C_2 is the pre-column, C_3 is the guard column, C_1 is the analytical column).

The main steps of the process are schematically represented in Figs. 2–5. In the equilibrium phase, preceding the injection of the plasma sample, pump B, connected to the selector (S_1), pumps water finally vented through port 5-6 of valve V_3 after flushing and equilibrating the pre-column. Meanwhile, pump A flushes and equilibrates the analytical column with the eluent mixture. Then 200 μl of plasma sample are injected into valve V_1 (sampling valve of the automatic sample injector) and transferred to the pre-column, which is then flushed with water for 4 min (at a flow-rate of ca. 1.8 ml/min) to remove proteins and salts.

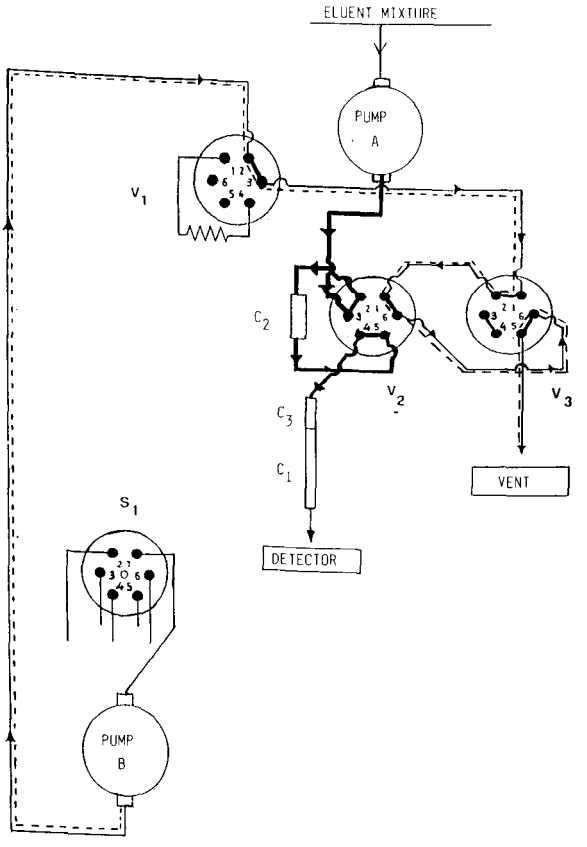

Fig. 4. V_2 switching and elution of sample from the pre-column to the top of analytical column.

After valve V_2 is switched, the analytical column and the pre-column are on the same path and are flushed by the mobile phase, which elutes the analytes from the pre-column to the top of the analytical column. After ca. 1.5 min V_2 is switched again, and while the compounds of interest are chromatographed on the analytical column, the pre-column is back-flushed by the following sequence of solvents: acetonitrile–water (1:1); acetonitrile; methanol–water (1:1); water. (This operation is performed by switching the selector S_1 to different positions and activating the valve V_3 for back-flush). At the end of the clean-up procedure the pre-column is ready for re-use.

Procedure for plasma sample

A 30-μl volume of internal standard solution (Table I, solution No. 5) was transferred to a screw-capped test-tube (Sovirel 13 with PTFE-lined caps), and 1 ml of plasma sample was added and mixed well. A separate set of standards was prepared by transferring 20-μl aliquots of standard solutions (Table I, solution Nos. 1–4) and 30 μl of internal standard solution into separate screw-capped test-tubes: 1 ml of control (pre-dose) plasma was added and mixed well. All the samples were centrifuged at 4000 *g* for 5 min at 5 °C in a refrigerated centrifuge, Model

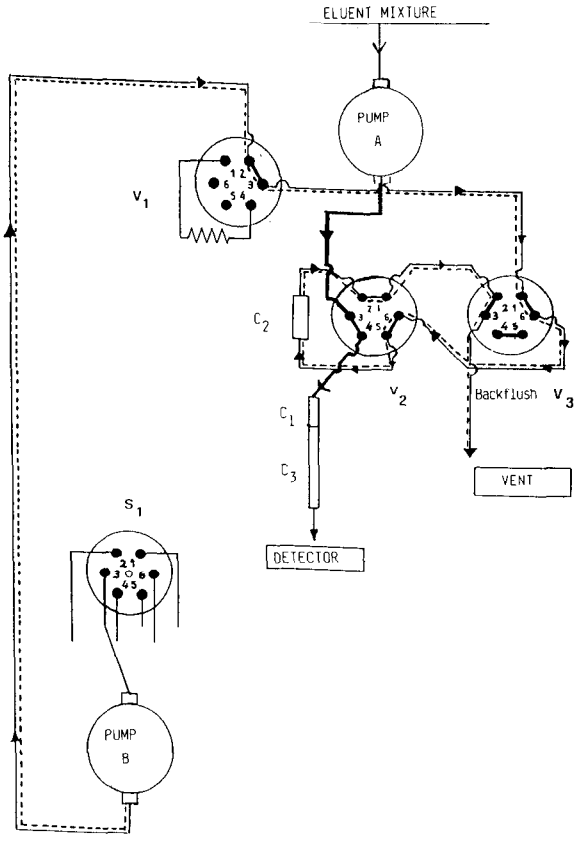

Fig. 5. Chromatography of the sample on the analytical column and back-flush of the pre-column with different solvents.

K 110 (Jouan, Saint-Nazaire, France); 200 μl of the clear supernatant were chromatographed.

Fig. 6 contrasts the traditional method (liquid–liquid extraction procedure) with the new method involving on-line purification and column-switching.

RESULTS

Linearity and detection limit

A linear correlation was found between the peak-height ratio of diltiazem and the internal standard versus diltiazem concentration in the range 10–1000 ng/ml in plasma. Linear least-squares regression performed on the peak-height ratio versus concentration gave the following equation: $y=251.7x-10.4$ ($r=0.9998$, $n=5$). The detection limit of the method was ca. 2 ng/ml of plasma for diltiazem with a signal-to-noise ratio of ca. 3:1.

Statistical validation of the method

Intra-assay precision studies were performed on control plasma spiked with different amounts of diltiazem, processed as described above (Fig. 7B and C).

Liquid—liquid extraction

To 1 ml of plasma add: – internal standard
– 1 ml of phosphate buffer pH 9 (1 *M*)
– 7 ml of *n*-hexane

Shake on a "tumble extractor", 10 min at 40 rpm

Centrifuge (5 °C) at 1500 *g* for 5 min

Transfer the supernatant into conical tubes

Evaporate the organic phase at 45°C under pure nitrogen

Dissolve the evaporation residues in a suitable volume of HPLC eluent

Inject the purified sample into HPLC column

On-line clean-up and column-switching

Inject 200 µl of plasma sample containing the internal standard into HPLC column and activate the following program (on the Programmer 2000, Kontron)

Time (min)	Function	Value	Explanations
0.01	Hold		Pump B is activated and
0.02	Flow	2	pumps water at 2 ml/min
4.00	V_2	On	Valve V_2 is switched on thus allowing pre-column and analytical column to be put in series
5.50	V_2	Off	Valve V_2 is switched off, pre-column and column put on different paths
6.00	V_3	On	Valve V_3 is switched on for back-flush
6.10	S_1	2	Selector S_1 is switched in position 2: acetonitrile—water (1:1)
8.00	S_1	3	In position 3: acetonitrile
10.00	S_1	4	In position 4: acetonitrile—water (1:1)
10.50	S_1	5	In position 5: methanol—water (1:1)
10.70	S_1	1	In position 1: water
11.00	V_3	Off	End of back-flush
12.00			End

Fig. 6. Comparison of the liquid–liquid extraction and the proposed method for determining diltiazem in human plasma.

Intra-assay precision (within-day) was obtained by replicate analysis of plasma samples on the same day (Table II). Inter-assay precision (day-to-day) was determined analysing the standard samples on several days over three weeks (Table III). The results demonstrate that the overall recovery of the drug is very high (100%) in the concentration range 10–400 ng/ml in plasma.

Selectivity and application of the method

Several blank plasma samples from different subjects were tested, and no endogenous chromatographic interference was found at the retention times of the compounds of interest (Fig. 7A). The method was applied to the determination of diltiazem in the plasma of subjects treated orally with the drug in single- and multiple-dose administration. The plasma concentration–time course of diltiazem in a healthy subject is shown in Fig. 8 (after an oral dose of 120 mg of drug), and a typical chromatogram is shown in Fig. 7D.

DISCUSSION

The proposed automated HPLC technique provides a very rapid determination of diltiazem in human plasma without prior purification of the sample. We con-

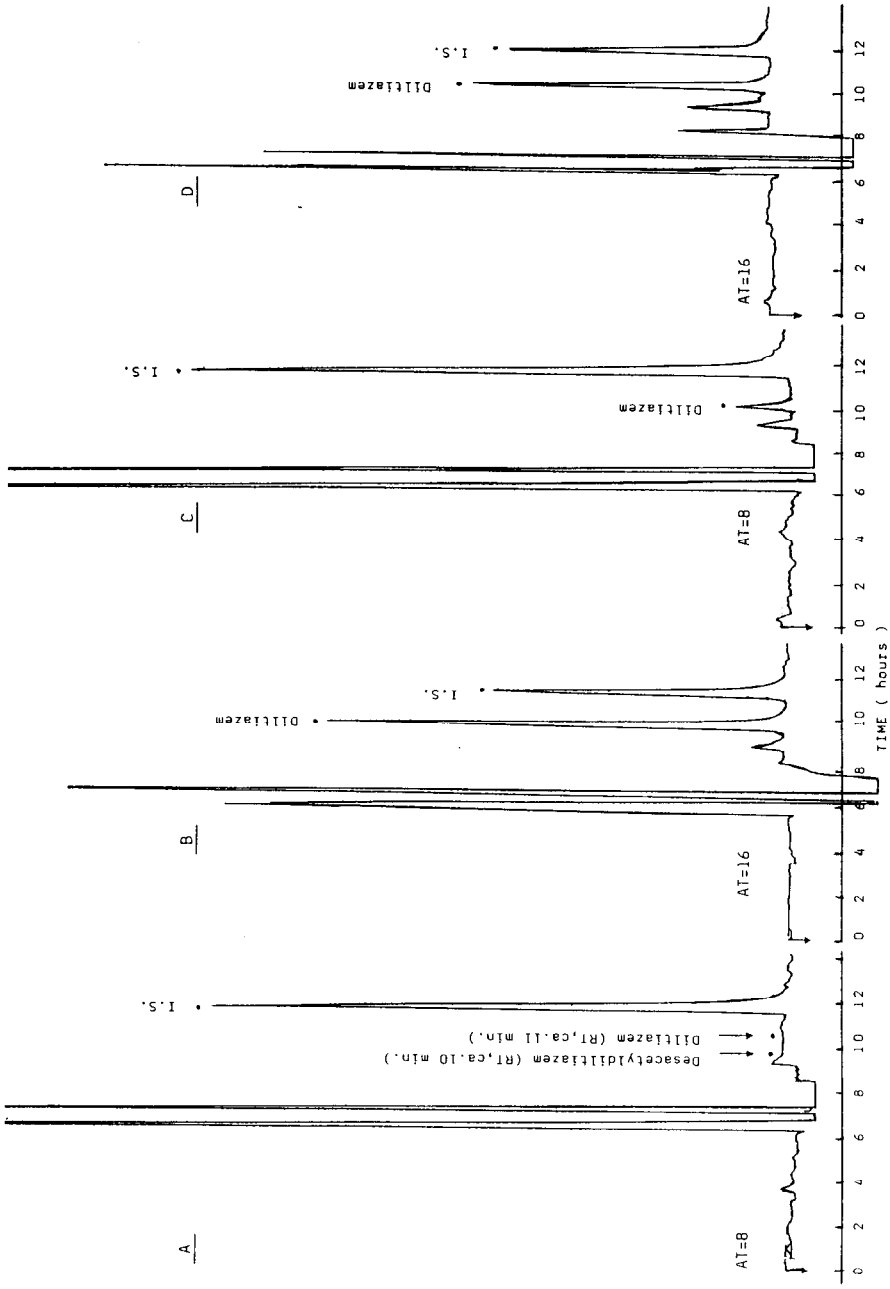

Fig. 7. (A) Chromatogram of pre-dose plasma containing the internal standard (I.S.). (B) Chromatogram of authentic standards recovered from pre-dose plasma spiked with diltiazem; nominal concentration, 200 ng/ml. (C) Chromatogram of authentic standards recovered from pre-dose plasma spiked with diltiazem; nominal concentration, 10 ng/ml. (D) Chromatogram of plasma sample from a subject 6 h after oral administration of 120 mg of diltiazem (multiple administration).

TABLE II

INTRA-ASSAY REPRODUCIBILITY FOR PLASMA SAMPLES SPIKED WITH DILTIAZEM

Amount added (ng/ml)	*n*	Amount found (mean ± S.D.) (ng/ml)	Recovery (%)	Coefficient of variation (%)
10	5	10.1 ± 0.9	101.4	8.6
20	5	18.6 ± 1.4	93.2	7.5
50	4	52.9 ± 2.2	105.9	4.1
200	5	200.4 ± 7.0	100.3	3.4
Mean			100.2	5.9

TABLE III

INTER-ASSAY REPRODUCIBILITY FOR PLASMA SAMPLES SPIKED WITH DILTIAZEM

Amount added (ng/ml)	*n*	Amount found (mean ± S.D.) (ng/ml)	Recovery (%)	Coefficient of variation (%)
10	4	10.1 ± 0.8	100.7	7.9
20	5	20.5 ± 1.1	102.4	5.4
50	5	50.6 ± 2.8	101.2	5.5
200	4	200.1 ± 1.1	100.0	0.5
400	3	400.6 ± 8.6	101.4	2.1
Mean			101.1	4.3

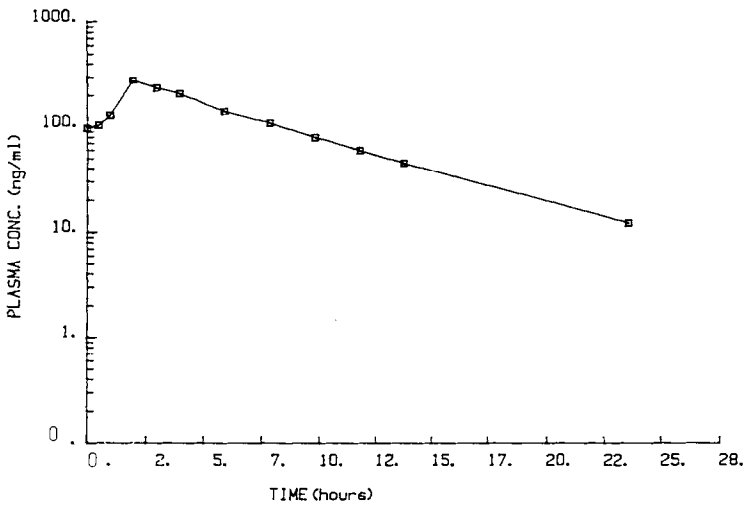

Fig. 8. Plasma concentration–time course of diltiazem in a healthy volunteer after oraly administration of a tablet containing 120 mg of drug (multiple-dose administration).

sider that so rapid and specific a method could represent an important tool in kinetic studies as well as for the control and adjustment of the dosage regimen during clinical studies. On the other hand, we do not consider it essential to extend the method to the determination of desacetyldiltiazem yet, at least.

Owing to the extensive clean-up procedure, the pre-column has a long life, replacement being necessary after about 100 plasma injections. However, pre-columns are easily dry-filled in the laboratory, each requiring only ca. 250 mg of packing material. The pre-column packing can be loaded with numerous plasma samples, up to 150, without showing a significant increase in back-pressure. A fresh pre-column, if flushed with water as described, has a back-pressure of less than 10 bar, which increases to 50 bar after ca. 150 analyses; even under these conditions the capacity of the pre-column is not affected.

The whole chromatographic process lasts ca. 12 min: it is important to keep in mind that the analytical chromatography starts after 4 min (during this time the pre-column is not connected to the analytical column). As far as the stability of diltiazem in plasma is concerned, no significant variation in drug content was found in plasma samples maintained under laboratory conditions (for ca.10 h) in comparison with similar samples maintained at 0–5 °C (for ca. 24 h).

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