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High-performance liquid chromatographic assay of dilazep in plasma after solid-phase extraction

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Abstract

A new, simple and accurate high-performance liquid chromatography method is presented for measuring dilazep in plasma, using a reversed-phase technique and UV absorption at 267 nm. Dilazep and papaverine (the internal standard) added to plasma were successfully isolated using a solid-phase extraction procedure (CN cartridges). The method was linear between 2.5–12.5 μg ml^{-1}. Over the tested concentration range the intra-day coefficient of variation for replicate analyses of plasma ranged from 2.38 to 5.27% (the day-to-day CV ranged from 2.52 to 7.99%). The detection limit for the analysis of dilazep in plasma was 50 ng with 20 μl injection. © 1997 Elsevier Science B.V.

Keywords: Reversed-phase chromatography; Dilazep; Plasma; Solid-phase extraction

1. Introduction

Dilazep,3,3′-(perhydro-1,4-diazepine-1,4-diyl)-dipropyl-bis-(3,4,5-trimethoxybenzoate), has been shown to dilate coronary arterioles by blocking an active saturable reuptake process that transports adenosine into some cells including heart cells and erythrocytes. Dilazep produces dilation by acting directly on the smooth muscle of the coronary vessels [1].

Only a few methods for quantitation of dilazep have been reported hitherto, including colorimetric [2] and liquid chromatographic (HPLC) [3–5] determinations. Sadana et al. [2] described a method based on formation of ion-pair complexes with bromocresol green, bromophenol blue, methyl orange and bromothymol blue in acidic media, extraction with chloroform and measurement of absorbance at 420 nm. This method was applied for quantitation of dilazep in pharmaceutical preparations. Sane et al. have worked out the determination of dilazep in pharmaceuticals by HPLC, using CN [3] and C18 [4] columns and UV detection at 254 nm. Only one HPLC method has been reported for the determination of dilazep in plasma [5], using a C18 column, with spectrophotometric (at 254 or 280 nm) and spectrofluorimetric (excitation 256 nm, emission at 356 nm) detection. The authors did not employ an internal standard, thereby reducing the precision and accuracy of the assay. These methods included relatively complex and time-consuming sample preparation (extraction and back-extraction with

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ethyl acetate) giving a mean recovery of approximately 98%. Therefore, a rapid, simple and selective HPLC procedure for determination of dilazep in plasma has been developed.

2. Experimental

2.1. Reagents

Dilazep dihydrochloride was kindly supplied by Asta Pharma (Germany), papaverine hydrochloride (the internal standard) was from Pharmaceutical Works 'Polfa' (Poland). Phosphate buffers: potassium dihydrogen phosphate (pH 4.5; 0.067 M) adjusted to pH 3.2 with phosphoric acid and disodium hydrogen phosphate (pH 8.3; 0.067 M), (pH tolerance $\pm$ 0.05); methanol and acetonitrile LiChrosolv® for chromatography (Merck, Germany) were used. Water was double distilled in our laboratory. Heparinized human whole blood was from the District Blood Centre in Lublin; blood samples were centrifuged and the plasma thus obtained was stored at -18°C. Stock solutions (1.0 mg ml^{-1}) of dilazep and papaverine were prepared by dissolving the appropriate amounts of substances in methanol. These solutions were stable for at least 4 months at 4°C. Working methanolic dilutions of 0.1 mg ml^{-1} and 0.05 mg ml^{-1} for dilazep and 0.1 mg ml^{-1} for papaverine were prepared from stock solutions.

2.2. Instrumentation

For solid-phase extraction cyanobonded silica (CN) minicolumns of 3 ml capacity and a vacuum manifold column processor SPE Model G-12 from J.T. Baker, (USA) were used. A liquid chromatograph type 302 from Techma-Robot Warsaw (Poland), a variable wavelength UV detector type LCD-2040, a line recorder type TZ-4620 and a computing integrator type CI-100A (only for measuring retention times) (all from Laboratorni Pristroje Praha, Czech Republic), a 250 × 4 mm steel column packed with LiChrosorb® RP-18, dp = 10 μm (Merck, Germany) were used.

2.3. Chromatographic conditions

All experiments were carried out at ambient temperature. Sample volumes of 20 μl were injected onto the analytical column with a manual HPLC injector (Laboratorni Pristroje Praha, Czech Republic). The mobile phase was acetonitrile–phosphate buffer (pH 3.2) (9:1, v/v) with a flow rate of 1 ml min^{-1}. Detection was by UV absorption at 267 nm (the absorbance maximum for dilazep), detector output range was 0.04 AUFS, and recorder chart speed was 0.6 cm min^{-1}.

3. Sample preparation and SPE procedure

To test-tubes containing 0.5 ml plasma 0.1–0.5 ml working solution of dilazep (0.05 mg ml^{-1}), 0.4 ml working solution of papaverine (0.1 mg ml^{-1}) and methanol to an equal volume 2.0 ml were added. The mixtures were centrifuged for 5 min at approximately 1100 × *g*.

After inserting into the extraction module, the minicolumns were conditioned by twice applying 2 ml of methanol, followed by two 2 ml washes with phosphate buffer pH 8.3 under reduced pressure. To each column 1 ml of phosphate buffer pH 8.3 and 1.0 ml of the supernatant from each

Table 1
Drugs tested for potential assay interference

Compound	Retention time (min)
Captopril	Not detected
Chlordiazepoxide	3.66
Chlorprotixene	12.58
Dilazep	9.75
Diltiazem	8.15
Enalapril	1.66
Flecainide	6.50
Hydrochlorothiazide	1.83
Nitrazepam	2.28
Nitrendipine	2.75
Papaverine (internal standard)	5.07
Prazosine	6.81
Procainamide	6.12
Propranolol	6.93
Theophylline	2.01

Table 2
Precision, reproducibility and accuracy of analysis of dilazep in plasma

Amount added, μg ml^{-1}	Intra-day analysis		Day-to-day analysis	
	Amount found (mean ± S.D.)	Coefficient of variation (%)	Amount found (mean ± S.D.)	Coefficient of variation (%)
2.50	2.55 ± 0.13	5.27	2.53 ± 0.20	7.99
5.0	4.94 ± 0.20	3.98	5.02 ± 0.19	3.77
7.50	7.56 ± 0.33	4.42	7.13 ± 0.39	5.51
10.0	10.19 ± 0.24	2.38	9.91 ± 0.25	2.52
12.50	12.48 ± 0.36	2.85	12.34 ± 0.41	3.36

$n = 5$, At each level of addition.

sample were injected, and allowed to pass through the columns by gravity. Afterwards, the samples were washed twice with 2 ml water and then 1 ml methanol–water (1:1, v/v). The drugs of interest were eluted with two 1 ml volumes methanol. The methanolic extracts were evaporated under a stream of nitrogen at room temperature. Each residue was reconstituted with 1.0 ml methanol and then 20 μl volumes were injected into the analytical column.

4. Linearity tests

Linear and reproducible relationships between dilazep/internal standard peak height ratios were obtained over the tested concentration range 2.5–12.5 μg ml^{-1}. From the solution containing 0.1 mg ml^{-1} dilazep, 0.25–1.25 ml volumes were pipetted, 0.2 ml of papaverine solution (1.0 mg ml^{-1}) was then added and made up with methanol to an equal volume 10.0 ml. 20 μl of each sample was injected into the column. All measurements were repeated three times at each concentration. The calibration graph based on the peak height ratios of dilazep to that of the internal standard was constructed and used to calculate the results.

5. Results and discussion

Over the concentration range 2.5–12.5 μg ml^{-1} the relationship between the peak height ratios of dilazep to the internal standard and the concentration of the drug was linear. The regression equation for standard solutions was $y = 0.0672x - 0.004$ (S.E. of slope 0.0009, S.E. of intercept 0.0077, correlation coefficient 0.9997), the regression equation for plasma samples was $y = 0.0668x - 0.001$ (S.E. of slope 0.001, S.E. of intercept 0.0083, correlation coefficient 0.9996), where y = peak height ratio of dilazep to that of the internal standard and x = concentration of dilazep in μg ml^{-1}.

Some other drugs were tested for possible interference with the described dilazep assay. The working dilutions of 0.01–0.1 mg ml^{-1} of the tested drugs were prepared and 20 μl volumes were injected into the column. Their retention times under the same chromatographic conditions and UV detection wavelength as used for the dilazep determination are given in Table 1.

In a preliminary study different pH conditions and various organic solvents for liquid-liquid extraction as well as C18 and CN cartridges for solid-phase extraction procedure were used. Extraction with CN minicolumns, involving minimal

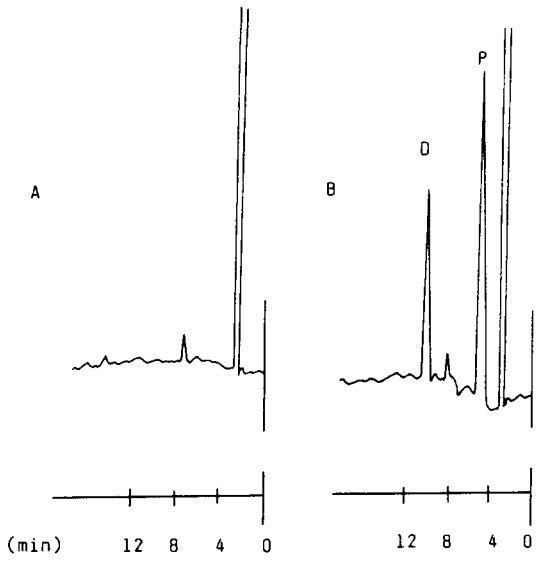

Fig. 1. Typical chromatograms of extracted plasma samples. (A), drug-free plasma; (B), plasma sample containing 7.5 μg ml^{-1} of dilazep (D), and 20.0 μg ml^{-1} of papaverine (P) (the internal standard).

handling of the sample, saving time, glassware and reagents, was stated to be faster and more reliable. It gave the best reproducibility and fairly good recovery for both substances, dilazep and the internal standard. Absolute recovery was determined by comparing the average peak heights for five extracted plasma samples at each standard concentration of dilazep and the internal standard with those for unextracted samples of identical content of both substances. The mean absolute recovery ($\pm$S.D.) over the tested range was $96.40 \pm 1.52\%$ and $97.56 \pm 0.75\%$ for dilazep and the internal standard, respectively. The precision and reproducibility of plasma dilazep analysis were determined at five concentrations of analyte by repeating the procedure five times for each concentration. The intra-day and day-to-day variability and accuracy data are shown in Table 2.

Fig. 1 illustrates examples of the chromatograms obtained from extracted pooled blank plasma and of plasma sample containing dilazep and the internal standard added from standard solutions. As shown, the peaks representing both substances are symmetrical and well removed from the solvent front and interfering peaks from the biological material.

As mentioned above, the literature relating to dilazep determination is rather scarce. Therefore, the present simple, rapid and reliable method should facilitate the analytical investigation of dilazep. The results, with regard to sensitivity and specificity, show that this method of determination of dilazep in plasma may be successfully used for both therapeutic drug monitoring and toxicological analysis.

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