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Direct enantiomeric separation of *cis*-(±)diltiazem in plasma by high-performance liquid chromatography with ovomucoid column

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ABSTRACT

A method is proposed for the direct enantiomeric separation of *cis*-(±)diltiazem in plasma. The method is based on a commercial Ultron ES-OVM column with a mobile phase of different mixtures of ethanol–phosphate buffer. The degree of extraction of the enantiomers was more than 86%. The detection limit of the method used was 5 ng of racemic mixture on column (coefficient of variation = 13%), corresponding to 3.2 ng for *cis*-(+)diltiazem and 1.8 ng for *cis*-(−)diltiazem.

INTRODUCTION

Interest in the high-performance liquid chromatographic (HPLC) separation of chiral isomers has been growing rapidly in recent years. These separations are particularly important to the pharmaceutical industry because the biological activity of two chiral isomers is often significantly different and it is necessary to quantitate 0000the amount of each isomer in a formulation.

Diltiazem hydrochloride [(+)-3-acetoxy-*cis*-2,3-dihydro-5-(2-dimethylaminoethyl)-2-(*p*-methoxyphenyl)-(5*H*)-1,5-benzothiazepin-4-one hydrochloride], an inhibitor of membrane transport of calcium, is used in the therapy of a number of cardiovascular disorders, including angina in its several forms [1], hypertension [2] and peripheral cardiovascular disorders and some cardiac arrhythmias [3].

The great difference in pharmacological effect and pharmacokinetics between the two enantiomeric forms of many drugs has in recent years emphasized the need for methods of enantioselective separation and determination [4,5]. Diltiazem has two asymmetric carbon atoms at positions 2 and 3; two geometric isomers of the form *cis* and *trans* exist, depending on the relative positions of the substituents at these positions. Each isomer also has optical isomers, the (+) and (−) forms, but only the *cis* (+) form causes the pharmacological effect. Hence the interest is in the enantiomeric separation of *cis*-(±)-diltiazem (Fig. 1).

Several methods have been published for the determination of diltiazem in plasma by gas chromatography (GC) [6] and HPLC [7–9].

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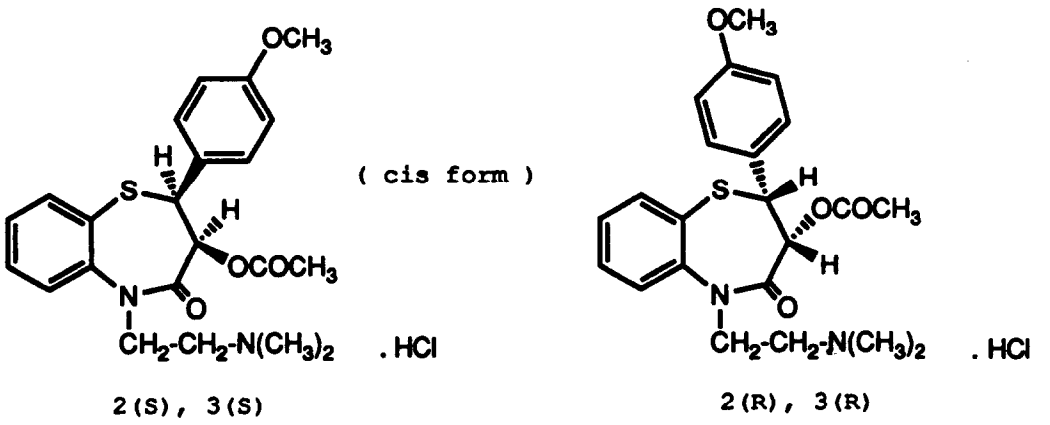

Fig. 1. Schematic representation of the enantiomeric forms of *cis* (±)-diltiazem hydrochloride.

The direct enantiomeric separation of *cis*-(±)-diltiazem was accomplished in this study using a chiral column, the Ultron ES-OVM. This bonded phase has numerous chiral recognition sites, including those for hydrogen-bonding, polar ionic, and hydrophobic groups, as well as the three-dimensional structure of the sample molecule.

EXPERIMENTAL

Reagents

cis-(±)-Diltiazem hydrochloride was supplied by Synthelabo (Le Plessis Robinson, France) and *cis*-(+)-diltiazem hydrochloride by Wassermann (Barcelona, Spain). Absolute ethanol (HPLC grade) was obtained from Scharlau (Barcelona, Spain) and potassium dihydrogenphosphate, phosphoric acid and potassium hydroxide of analytical-reagent grade were obtained from E. Merck (Darmstadt, Germany). Bidistilled water was purified with a Milli-Q system (Millipore, Milford, MA, USA).

Apparatus

The HPLC system consisted of a high-pressure pump (Model M-45, Waters), a variable-wavelength UV detector (Model 480, Waters), a six-port switching valve (Rheodyne, Cotati, CA, USA) with a 20-μl sample loop and a Data Module integrator (Waters). HPLC column was a 150 mm × 4.6 mm I.D. Ultron ES-OVM in which ovomucoid is chemically bonded to aminopropylsilica gel of particle size 5 μm, with an Ultron ES-OVM guard column (18 mm × 0.4 mm I.D.) of the same quality (Shinwa Kako, Kyoto, Japan).

Samples

cis-(+)-Diltiazem and *cis*-(±)-diltiazem hydrochloride solutions were prepared by dissolution in water of known amounts, and the solutions were diluted with the eluent to the desired concentration. A 20-μl aliquot of the sample solution was injected into the column.

Plasma samples

Aliquots of human plasma (0.5 ml) were pipetted into glass tubes, and 5 μl of standard solutions of *cis*-(±)-diltiazem hydrochloride were added. The solution was mixed with 6 ml of hexane–2-propanol (98:2, v/v). After stirring (15 min) and centrifugation (1500 *g* for 10 min), the organic layers were transferred to new glass tubes and evaporated to dryness under a stream of nitrogen at 50°C. The residue was reconstituted in 0.2 ml of the mobile phase (see HPLC conditions). The reconstitution was achieved by vortex-mixing the tube contents for 1 min. An aliquot (20 μl) was injected into the column.

HPLC conditions

The samples were detected at 237 nm. Mobile phases were prepared by mixing aqueous 20 m*M* potassium dihydrogenphosphate with various

TABLE I

EFFECT OF ETHANOL CONCENTRATION IN THE MOBILE PHASE ON RETENTION AND ENANTIOSELECTIVITY OF DILTIAZEM

20 m*M* phosphate buffer–ethanol (v/v, %) (pH 4.5) was used as the eluent. Each result is the mean of three determinations.

Ethanol (%)	t_0	k' (2*S*,3*S*)	k' (2*R*,3*R*)	α
3	2.60	0.33	1.80	5.64
7	2.72	0.47	1.30	2.80
10	2.40	0.37	0.92	2.48

TABLE II

EFFECT OF pH OF MOBILE PHASE ON RETENTION AND ENANTIOSELECTIVITY OF DILTIAZEM

20 m*M* phosphate buffer–ethanol (93:7, v/v) was used as the eluent. Each result is the mean of three determinations.

pH	t_0	k' (2*S*,3*S*)	k' (2*R*,3*R*)	α
4.5	2.72	0.47	1.30	2.80
5.0	2.47	0.41	1.25	3.04
5.5	2.40	1.71	5.30	3.08
6.0	2.64	1.44		1.00

amounts of ethanol, and adjusting the pH by the addition of phosphoric acid or potassium hydroxide. They were filtered through a Millipore 0.45-μm filter under reduced pressure before use (see Tables I and II). The flow-rate was 1.0 ml/min. All operations were carried out at ambient temperature.

Capacity factors were calculated from the equation of $k' = (t_R - t_0)/t_0$, where t_R and t_0 are the elution times of the retained and unretained solutes, respectively; k'_1 and k'_2 correspond to the capacity factors of the first- and second-eluted peaks, respectively. The retention time of an unretarded solute, t_0, was measured by injecting a solution of which the organic modifier content was slightly different from the eluent used. The enantioseparation factor is calculated from the equation $\alpha = k'_2/k'_1$.

Stability, reproducibility and sensitivity of the ES-OVM column

Several samples of product were injected without any significant changes in the retention or enantioselectivity in the mobile phase studied.

Recoveries were determined using four different concentrations of racemic solute. Intra-day variabilities were determined in the same way with four different plasma samples (Table III). Inter-day variabilities were determined using one concentration during four days. The detection limit and linearity of the method were also investigated.

RESULTS AND DISCUSSION

Diltiazem is a weakly basic amine ($pK_a = 7.7$) so its retention behaviour was highly influenced

TABLE III

RECOVERY AND VARIABILITY OF THE METHOD

Concentration (μg/0.5 ml of plasma)	Recovery (mean ± S.D.) (%)		C.V. (%)		*n*
	(2*S*,3*S*)	(2*R*,3*R*)	(2*S*,3*S*)	(2*R*,3*R*)	
0.7	96.0 ± 8.5	96.8 ± 12.2	8.9	13.2	6
1.0	89.0 ± 3.0	86.4 ± 2.7	3.4	3.1	6
1.5	95.3 ± 4.5	93.5 ± 5.6	4.7	6.0	11
3.0	113.4 ± 3.4	109.0 ± 12.6	2.9	14.9	4

by the pH of the mobile phase, and the ionic strength and concentration of the organic solvent in the eluent. But there are limitations on the choice of the mobile phase composition owing to the chiral column and the possible interference of the plasma constituents with the determination of enantiomeric ratio.

Table I shows the effect of organic modifiers on the retention and enantioselectivity of diltiazem. The eluents used were 20 m*M* phosphate buffer containing 3, 7 and 10% ethanol (pH 4.5).

The results show that the use of ethanol as the organic modifier gives a very good enantioselectivity. Moreover, the enantioselectivity was dependent on the concentration of the organic modifier (Fig. 2). The effect of decreasing the amount of ethanol in the eluent is to increase the value of α, owing to a significant retardation in the peak of the (−) form compared with the peak of the (+) form.

In Table II, the effect of the pH on retention and enantioselectivity is indicated. We tested pH values between the isoelectric point of ovomucoid, pI = 3.4–4.3 [10], and the pK_a value of diltiazem.

Diltiazem is extensively ionized (99.94%) at pH 4.5, and the column pH is close to that of the isoelectric point of the ovomucoid protein. This pH gives a good resolution between the two enantiomeric forms, with a relatively small retention time (Fig. 3A). As the pH increased (5.5), the ratio of ionization of diltiazem decreases, and the greater difference from the isoelectric point of the ovomucoid protein causes the retention times to

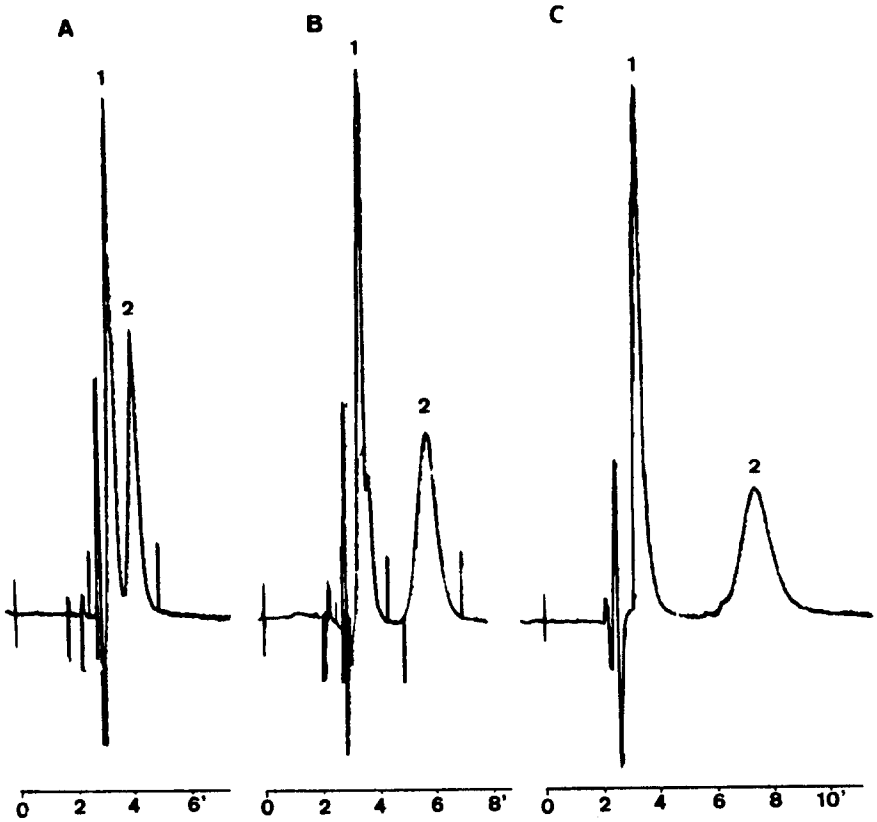

Fig. 2. Effect of concentration of ethanol in the mobile phase ethanol–potassium phosphate buffer (v/v) (pH 4.5): (A) 10:90; (B) 7:93; (C) 3:97. Detection, 237 nm; column temperature, ambient; flow-rate, 1 ml/min. Peaks: 1 = (+)-form: 2 = (−)-form. Chromatograms of *cis*-(±)-diltiazem in methanol (10 μg/ml) (0.02 a.u.f.s.).

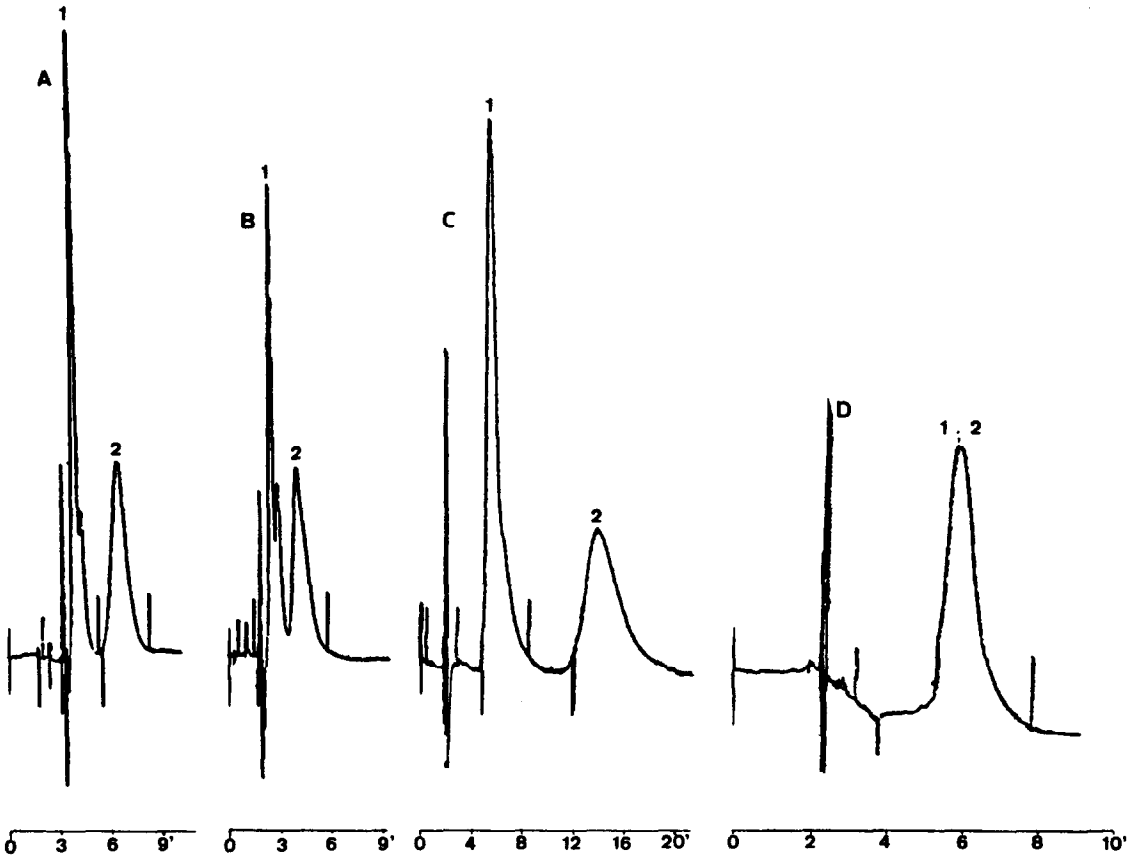

Fig. 3. Effect of pH of mobile phase ethanol–potassium phosphate buffer (7:93, v/v): (A) pH 4.5; (B) pH 5; (C) pH 5.5; (D) pH 6.0. Detection, 237 nm; column temperature, ambient; flow-rate, 1 ml/min. Peaks: 1 = (+)-form; 2 = (−)-form. Chromatograms of *cis*-(±)-diltiazem in methanol: (A) 10 μg/ml (0.02 a.u.f.s.); (B) 10 μg/ml (0.02 a.u.f.s.); (C) 7.5 μg/ml (0.01 a.u.f.s.); (D) 7.5 μg/ml (0.02 a.u.f.s.).

be longer, especially for the (−) enantiomer. At pH 6 the two enantiomers coelute with a longer retention time (Fig. 3D).

Calibration curves prepared from standard solutions (*cis*-(±)-diltiazem in methanol) were linear: $r = 0.997$ and $r = 0.964$ for enantiomers (2*S*,3*S*) and (2*R*,3*R*), respectively, in the concentration ranges studied (0.3–15 μg/ml). (+)-Diltiazem hydrochloride (2*S*,3*S*) was added in the range 5–25% to (±)-diltiazem hydrochloride. The resulting mixtures were injected into the chiral column. The first-eluted peak corresponds to the peak of the (2*S*,3*S*) enantiomer form, and the second peak is that of the (2*R*,3*R*) form, *i.e.* the (+) and (−) forms respectively.

Chromatograms obtained from a blank plasma sample and a plasma sample spiked with diltiazem (1 μg per 0.5 ml of plasma) are shown in Fig. 4A and B. These chromatograms indicate that the drug peaks are well resolved and endogenous interference was absent.

The recoveries of these compounds and the intra-day variabilities are shown in Table III. The inter-day variability was determined with one concentration of *cis*-(±)-diltiazem (1 μg per 0.5 ml of plasma) during four days. The recovery values found are 90.6 ± 3.1 and 84.0 ± 6.1% with C.V. of 3.5 and 7.2% for the enantiomers (2*S*,3*S*) and (2*R*,3*R*), respectively.

Using our extraction procedure, the degree of

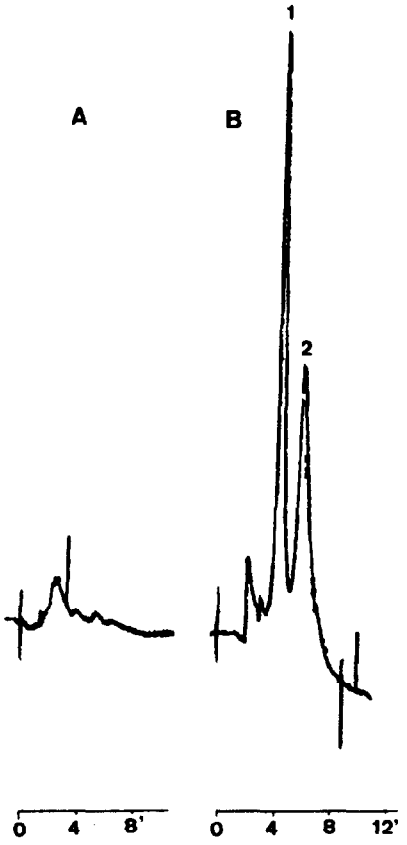

Fig. 4. Separation of enantiomers of diltiazem. Column, Ultron ES-OVM (150 mm × 4.6 mm I.D.); mobile phase, ethanol–potassium phosphate buffer (7:93, v/v); detection, 237 nm; column temperature, ambient; flow-rate, 1.0 ml/min. Peaks: 1 = (+)-form; 2 = (−)-form. (A) Chromatogram of black plasma. (B) Chromatogram of plasma spiked with 1 μg of *cis*-(±)-diltiazem (0.01 a.u.f.s.).

extraction of the enantiomers of diltiazem from plasma was higher than 86% . A similar finding was reported by Ishii *et al.* [8] but their method required a two-step extraction sequence and a two-column HPLC technique for the determination of the enantiomeric ratio of diltiazem in plasma. In our assay, only one extraction and one column are required.

The absolute detection limit was established at 50 ng per 0.5 ml of plasma (5 ng of diltiazem on column, C.V. = 13%), of the racemic mixture at a signal-to-noise ratio of 3. The detection limit of the first enantiomer, the (2*S*,3*S*) form, was 3.2 ng, and that of the second enantiomer, the (2*R*,3*R*) form, was 1.8 ng.

This method demonstrates adequate performance with respect to precision, accuracy and stereospecificity. It is linear and it has a good reproducibility. The method is fast, sensitive and applicable to the determination of the pharmacokinetics of diltiazem.

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