Journal of Chromatography, 564 (1991) 338–345
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5625

Short Communication

Determination of diltiazem hydrochloride enantiomers in dog plasma using chiral stationary-phase liquid chromatography

KAZUHIRO ISHII*, KIYOSHI BANNO, TAKAAKI MIYAMOTO and TOSHIO KAKIMOTO

Analytical Chemistry Research Laboratory, Tanabe Seiyaku Co., Ltd., 16–89, Kashima 3-chome, Yodogawa-ku, Osaka (Japan)

(First received June 21st, 1990; revised manuscript received August 29th, 1990)

ABSTRACT

The separation and determination of *d*- and *l*-diltiazem hydrochloride in dog plasma by a two-column high-performance liquid chromatographic technique are described. Diltiazem hydrochloride and its metabolites were extracted from dog plasma and analyzed on a conventional column (Nucleosil $5C_{18}$) with a volatile buffer system. The column effluent of diltiazem hydrochloride was collected and evaporated. The enantiomeric ratio of the collected diltiazem was determined using a chiral column (Chiralcel OC). The method was accurate and sensitive.

INTRODUCTION

Diltiazem hydrochloride, *d*-3-acetoxy-*cis*-2,3-dihydro-5-[2-(dimethylamino)-ethyl]-2-(*p*-methoxyphenyl)-1,5-benzothiazepin-4(5H)-one hydrochloride, is a calcium channel blocker, which is mainly used in the treatment of angina pectoris [1–4]. Diltiazem hydrochloride is also effective in the treatment of hypertension and supraventricular arrhythmias [5].

Diltiazem hydrochloride has 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 *d*- and *l*-forms (Fig. 1). Diltiazem hydrochloride is *d-cis* form.

The efficacy of the *d-cis* and *l-cis* forms was investigated. It was found that only the *d-cis* form had the coronary vasodilating effect. That is, the coronary vasodilating effect of *dl-cis*-diltiazem is further dependent on the absolute configurations of position 2 and position 3, and only the *d-cis* form is active. On the other hand, it was found that the acute toxicity of the *d-cis* form was almost equal to that of the racemate. The efficacy and toxicity of the *d-cis* and *dl-cis* forms are shown in Table I [6,7].

The stereoselective disposition of the enantiomers result in different pharma-

0378-4347/91/$03.50

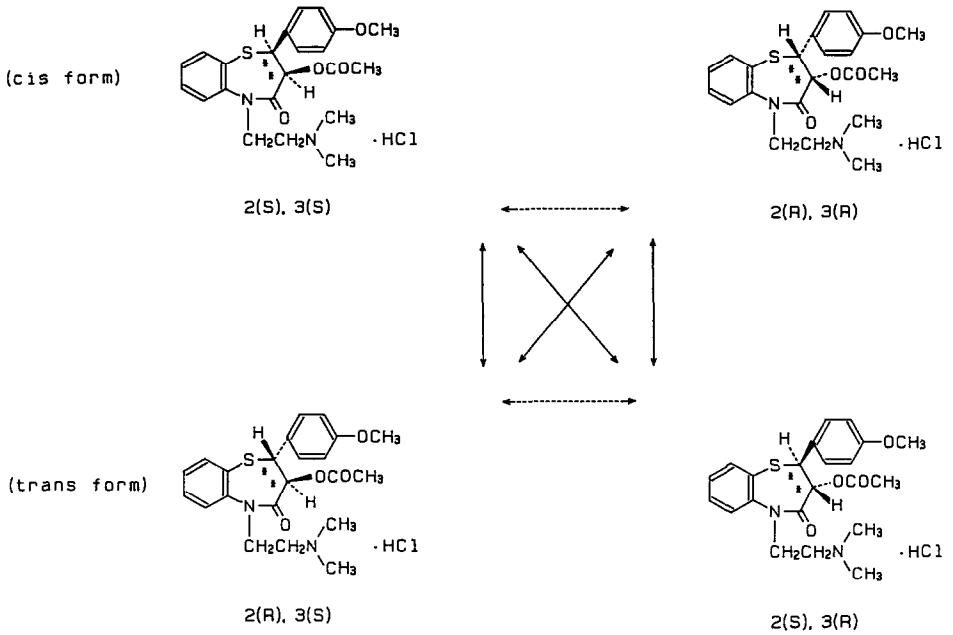


Fig. 1. Stereochemical structures of diltiazem hydrochloride.

cological profiles owing to the different rates of absorption, stereoselective presystemic metabolism, distribution or clearance. Several methods have been published in order to determine the presence of diltiazem in plasma by gas chromatography (GC) [8,9] and high-performance liquid chromatography [10–12]. Up to date, there is no information on the disposition kinetics of individual diltiazem enantiomers, in man or in animals, after the administration of the racemic drug.

We have already reported the method used for determining optical purity by

TABLE I

EFFICACY AND TOXICITY OF DILTIAZEM HYDROCHLORIDE AND ITS OPTICAL ISOMER

d = Diltiazem hydrochloride, *l* = *l*-diltiazem hydrochloride, *dl* = *dl*-diltiazem hydrochloride.

Effects	Activities
Vasodilating effects	$d > dl > l$
Smooth muscle relaxing effects	$d > dl > l$
Local anesthetic effects	$d = dl = l$
LD_{50}	$d = dl \geq l$

derivatization [13,14]. The direct enantiomeric separation and quantitation of *d-cis*- and *l-cis*-diltiazem hydrochloride was accomplished in this study using a chiral column. The specific and sensitive determination of enantiomers in dog plasma was developed using a two-column HPLC technique.

EXPERIMENTAL

Materials

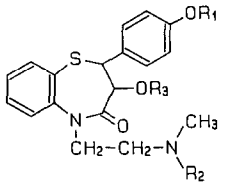
The synthesis of diltiazem hydrochloride and its optical isomers have been reported previously [15–17]. The N- and O-desmethyl metabolites (M_A, M_2, M_4 and M_6), desacetyldiltiazem hydrochloride, and 8-chlorodiltiazem hydrochloride (internal standard) were obtained from the Organic Chemistry Research Laboratory (Tanabe Seiyaku, Saitama, Japan). Chemical structures of diltiazem and its metabolites are shown in Fig. 2. All solvents and reagents were either of HPLC grade or analytical grade.

Apparatus

An LC-6A HPLC system (Shimadzu, Kyoto, Japan) equipped with a UV detector set at 240 nm and a C-R5A integrator were employed. A Rheodyne injection valve with a 500-μl sample loop (Rheodyne, Cotati, CA, U.S.A.) was used.

HPLC conditions

Method A. The column was a Nucleosil $5C_{18}$ (150 mm × 4.6 mm I.D.) (Macherey-Nagel, Düren, Germany) and the mobile phase was 0.1% trifluoroacetic acid (TFA)–acetonitrile (7:3, v/v).



	R_1	R_2	R_3
Diltiazem	CH_3	CH_3	$COCH_3$
M_A	CH_3	H	$COCH_3$
M_1	CH_3	CH_3	H
M_2	CH_3	H	H
M_4	H	CH_3	H
M_6	H	H	H

Fig. 2. Chemical structures of diltiazem metabolites.

Method B. The column was a Chiralcel OC (250 mm × 4.6 mm I.D., 5 μm) (Daicel Chemical Industries, Osaka, Japan) and the mobile phase was ethanol–diethylamine (100:1, v/v).

Standard solution

Diltiazem hydrochloride A 10-mg amount of *dl-cis*-diltiazem hydrochloride was dissolved in 1000 ml of water. A 5-ml aliquot of this solution was then diluted to 100 ml to give a final solution of 500 ng/ml. A 50–100 μl aliquot (25–100 ng/each isomer) was added to each 1-ml aliquot of plasma.

Internal standard. A 1-mg amount of 8-chlorodiltiazem hydrochloride was dissolved in 100 ml of water. A 5-ml aliquot of this solution was then diluted to 250 ml to give a final solution of 200 ng/ml. A 500-μl aliquot (100 ng) was added to each 1-ml aliquot of plasma standard or specimen.

Extraction procedure

Method A. A 1-ml aliquot of plasma, 0.5 ml of water, 0.5 ml of the internal standard solution and 0.5 ml of 2 *M* dipotassium hydrogenphosphate solution were placed in a 10-ml centrifuge tube, and the mixture was extracted with 6 ml of hexane–diethyl ether (5:1, v/v) by mixing for 10 min on a mechanical shaker. After centrifugation for 5 min at 250 *g*, the organic layer was transferred to another centrifuge tube and back-extracted with 0.5 ml of 0.01 *M* hydrochloric acid. The upper organic layer was discarded. A 400-μl aliquot of the lower aqueous layer was then injected into the liquid chromatograph (see *HPLC conditions, Method A*). The column effluent fraction corresponding to the diltiazem was collected for chiral separation. The column effluent was evaporated to dryness under vacuum at 30°C. The absolute recovery of the two enantiomers from the plasma was greater than 97% in method A.

Method B. The residue obtained in method A was then dissolved in 100 μl of the mobile phase of HPLC method B and all of the resultant solution were injected into the liquid chromatograph (see *HPLC conditions, Method B*).

Stability and reproducibility of the Chiralcel OC column

More than 300 samples of plasma extracts and standard solutions have been injected without any significant changes in the retention or stereoselectivity. The column-to-column stereoselectivity was unchanged, but the retention times for the enantiomers varied. It is possible to adjust the capacity factors by changing the concentrations of diethylamine.

Linearity, reproducibility and sensitivity

Method A. The calibration curves were linear in the range tested, 10–100 ng for diltiazem hydrochloride. A regression line, $y = 0.011x + 0.001$ was found with a correlation coefficient of 0.995.

Method B. dl-Diltiazem hydrochloride was added to diltiazem hydrochloride

TABLE II

ACCURACY AND PRECISION OF THE METHOD

In each case $n = 5$.

Concentration (ng/ml per enantiomer)	Measured concentration (ng/ml)		Intra-day C.V. (%)	
	d	*l*	*d*	*l*
25	26.3	24.3	12.6	8.0
50	49.8	50.6	3.4	4.6
100	101.8	96.7	5.4	2.0

to give concentrations of *l*-form in the range 10–50%. The resulting mixtures (25 and 50 ng) were injected into the chiral column. The relationship between the known content and the found value of the *l*-form was investigated. A regression line with slope 1 shows that the peak-area ratio is equal to the ratio of the enantiomers of diltiazem hydrochloride.

Reproducibility studies were performed at three different concentrations (25, 50 and 100 ng of enantiomer per ml of plasma) with five samples using the time limit described in methods A and B. Coefficients of variation (C.V.) were calculated and have been summarized in Table II.

The minimum detectable quantities that have to be injected in order to give a response three times the noise were 2 ng of *l*-diltiazem and 3 ng of *d*-diltiazem.

RESULTS AND DISCUSSION

The choice of mobile phase compositions is rather limited for a chiral column and the plasma constituents and metabolites interfere with the determination of the enantiomeric ratio of diltiazem hydrochloride. Therefore, a two-column HPLC technique was developed for the determination of the enantiomeric ratio of diltiazem hydrochloride in dog plasma.

The first chromatographic conditions were chosen to achieve a rapid separation of diltiazem and the various metabolites under isocratic conditions, and to minimize the interference from plasma constituents. Diltiazem and its metabolites are weakly basic amines. The retention behavior was strongly influenced by the pH of the mobile phase, the ionic strength and the concentration of the organic solvent in the eluent. The use of the volatile buffer system is favorable for the isolation of the substance and can be easily monitored by the second HPLC method. Since Bennet *et al.* [18] introduced the use of TFA into the reversed-phase HPLC of proteins and peptides, a great number of authors have reported its successful application in various separation problems [19–21]. TFA undoubtedly has some major advantages over other organic and inorganic acids used as mobile phase additives. It has (1) a low UV cut-off, (2) is an excellent solvent for

polypeptides, (3) is a strong ion-pairing agent and (4) easily removed by lyophilization. Furthermore, it does not block the amino terminus and it is miscible with most common organic modifiers [22]. Fig. 3 shows a typical chromatogram of diltiazem hydrochloride and its metabolites. The first HPLC technique (method A) was used for the determination of the racemic concentration of diltiazem hydrochloride.

The eluate containing diltiazem was collected and handled as described under Experimental. The resolution of the enantiomers of *dl-cis*-diltiazem was accomplished on a Chiralcel OC. The resolution factor R_s for diltiazem was 2.1. Fig. 4 shows the chiral separation of the enantiomers of diltiazem, isolated from a dog plasma samples.

The concentration of the enantiomers was calculated by multiplying the fraction of the total area ($d + l$) of each enantiomer by the plasma concentration of the racemic diltiazem, determined by reversed-phase HPLC. The connected use of the reversed-phase system and the chiral system avoids the disturbances resulting from metabolites and endogenous compounds in the plasma. The degree of

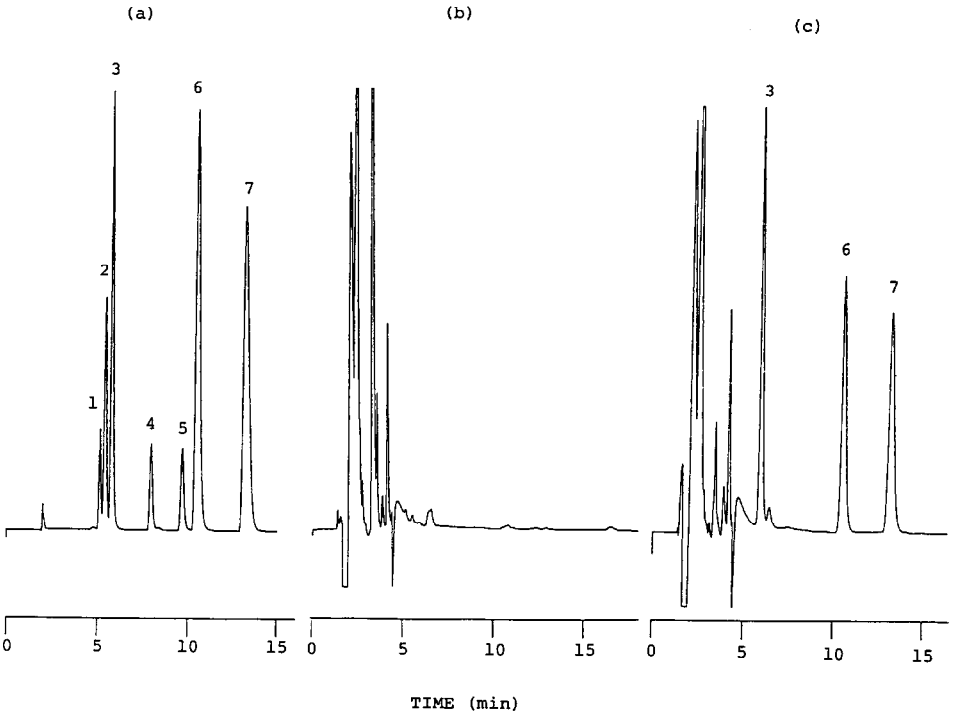


Fig. 3. Typical chromatograms of diltiazem hydrochloride and its metabolites. Column: Nucleosil $5C_{18}$ (150 mm × 4.6 mm I.D.). Mobile phase: 0.1% TFA–acetonitrile (7:3, v/v). Column temperature: 40°C. Detection: 240 nm. Peaks: 1 = M_6; 2 = M_2 and M_4; 3 = M_1; 4 = M_5; 5 = M_A; 6 = diltiazem; 7 = I.S. (internal standard). (a) Chromatogram of standard (0.32 a.u.f.s.). (b) Chromatogram of blank plasma (0.02 a.u.f.s.). (c) Chromatogram of plasma extract, 50 ng (0.02 a.u.f.s.).

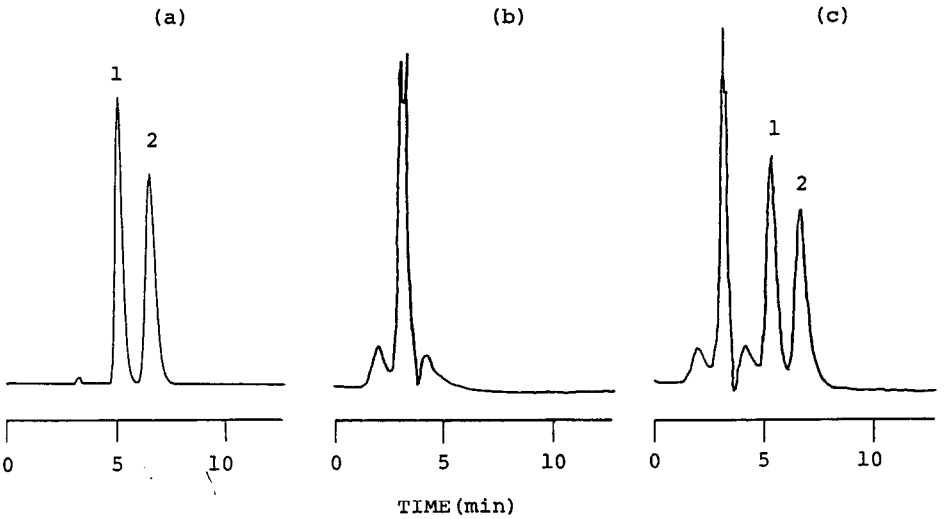


Fig. 4. Separation of enantiomers of diltiazem isolated from dog plasma. Column: Chiralcel OC (250 mm × 4.6 mm I.D.). Mobile phase: ethanol–diethylamine (100:1, v/v). Detection: 240 nm. Column temperature: ambient. Flow-rate: 1.0 ml/min. Peaks: 1 = *l*-form; 2 = *d*-form. (a) Chromatogram of standard (0.32 a.u.f.s.). (b) Chromatogram of blank plasma (0.02 a.u.f.s.). (c) Chromatogram of plasma extract, 50 ng (0.02 a.u.f.s.).

extraction of the enantiomers of diltiazem from plasma was more than 97% (50 ng/ml). The reproducibility was determined by assaying blank plasma spiked with different concentrations of racemic diltiazem. The samples were analysed in replicates of five as described earlier. The relative standard deviations obtained are summarized in Table II.

The assay method used for determining the enantiomeric component in dog plasma was accurate and sensitive. The method is applicable to stereoselective pharmacokinetic studies of diltiazem hydrochloride.

REFERENCES

1 P. Theroux, D. D. Waters, G. S. Affaki, J. Crittin, R. Bonan and H. F. Mizgala, *Circulation*, 60 (1980) 504.
2 K. Kohno, Y. Takeuchi, A. Etoh and K. Noda, *Arzneim.-Forsch*, 27 (1977) 1424.
3 S. J. Rosenthal, R. Ginsburg, I. H. Lamb, D. S. Baim and J. S. Schroeder, *Am. J. Cardiol.*, 46 (1980) 1027.
4 D. D. Waters, J. Szlachic, P. Theroux, F. Dauwe and H. F. Mizgala, *Am. J. Cardiol.*, 47 (1981) 179.
5 M. Chaffman and R. N. Brogden, *Drugs*, 29 (1985) 387.
6 T. Nagao, M. Sato, H. Nakajima and A. Kiyomoto, *Jpn. J. Pharmacol.*, 22 (1972) 1.
7 T. Nagao, M. Sato, Y. Iwasawa, T. Takada, R. Ishida, H. Nakajima and A. Kiyomoto, *Jpn. J. Pharmacol.*, 22 (1972) 467.
8 J. P. Clozel, G. Caille, Y. Taeymans, P. Theroux, P. Biron and J. G. Besner, *J. Pharm. Sci.*, 73 (1984) 207.

9 R. Calaf, P. Marie, Cl. Ghiglione, M. Bory and J. Reynand, *J. Chromatogr.*, 272 (1983) 385.
10 S. C. Montamat, D. R. Abernethy and J. R. Mitchell, *J. Chromatogr.*, 415 (1987) 203.
11 S. M. Johson and S. K. Whaba Khalil, *J. Liq. Chromatogr.*, 10 (1987) 673.
12 A. M. Rustum, *J. Chromatogr.*, 490 (1989) 365.
13 R. Shimizu, K. Ishii, N. Tumagari, M. Tanigawa and M. Matsumoto, *J. Chromatogr.*, 253 (1982) 101.
14 R. Shimizu, T. Kakimoto, K. Ishii, Y. Fujimoto, H. Nishi and N. Tsumagari, *J. Chromatogr.*, 357 (1986) 119.
15 H. Kugita, H. Inoue, M. Ikezaki, M. Konda and S. Takeo, *Chem. Pharm. Bull.*, 18 (1970) 2028.
16 H. Kugita, H. Inoue, M. Ikezaki, M. Konda and S. Takeo, *Chem. Pharm. Bull.*, 18 (1970) 2284.
17 H. Kugita, H. Inoue, M. Ikezaki, M. Konda and S. Takeo, *Chem. Pharm. Bull.*, 19 (1971) 595.
18 H. P. J. Bennett, A. M. Hudson, C. McMartin and G. E. Purdon, *Biochem. J.*, 168 (1977) 9.
19 N. Takahashi, Y. Takahashi and F. W. Putnam, *J. Chromatogr.*, 266 (1983) 511.
20 M. A. Phelan and K. A. Cohen, *J. Chromatogr.*, 266 (1983) 55.
21 G. Winkler, F. X. Heinz and C. Kunz, *J. Chromatogr.*, 297 (1984) 63.
22 G. Winkler, P. Wolschann, P. Beriza, F. X. Heinz and C. Kunz, *J. Chromatogr.*, 347 (1985) 83.