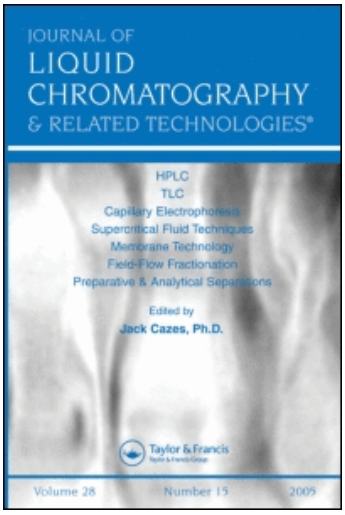

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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Online publication date: 22 April 1999

To cite this Article Uno, Tsukasa , Ohkubo, Tadashi and Sugawara, Kazunobu(1999) 'ENANTIOSELECTIVE HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF MANIDIPINE IN HUMAN PLASMA', Journal of Liquid Chromatography & Related Technologies, 22: 8, 1117 — 1128
To link to this Article: DOI: 10.1081/JLC-100101721
URL: http://dx.doi.org/10.1081/JLC-100101721

J. LIQ. CHROM. & REL. TECHNOL., 22(8), 1117–1128 (1999)

ENANTIOSELECTIVE HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF MANIDIPINE IN HUMAN PLASMA

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ABSTRACT

A sensitive and enantioselective method for the determination of the dihydropyridine calcium antagonist manidipine in human plasma was developed by high performance liquid chromatography (HPLC). The method involves a rapid and simple extraction in human plasma based on C8 bonded-phase extraction, and extraction samples were purified and concentrated on the C1 pre-column by using the column-switching technique. (+)-Manidipine, (-)-manidipine and (+)-barnidipine as an internal standard were detected by ultraviolet detection at 254 nm, and enantiomers of manidipine were quantitatively separated by HPLC on a Sumichiral OA-4500 column, containing chemically modified Pirkle-type stationary phase. Calibration graphs were linear for each concentration of enantiomer from 1.0 to 25 ng/mL and the detection limit in human plasma was 0.5 ng/mL for each enantiomer. The recoveries of (+)-manidipine and (-)-manidipine added to plasma were 93.6%-99.3% and 91.5%-99.1%, respectively, with coefficients of variation of less than 9.5%, respectively.

The method is applicable to drug level monitoring in the plasma of patients treated with manidipine and of healthy volunteers for the experimental analysis of stereoselective pharmacokinetics.

INTRODUCTION

Manidipine hydrochloride, 2-[4-(diphenylmethyl)-1-piperazinyl] ethyl-methyl (±) - 1,4-dihydro - 2,6-dimethyl - 4 -(m-nitrophenyl)-3,5-pyridine-carboxylate (I) (Figure 1), is a newly developed potent 1,4-dihydropyridine calcium antagonist having long-lasting activity.[1] The drug, as a racemic mixture of (+)- and (-)-manidipine, is undergoing clinical evaluation for antihypertensive effects.[2] The pharmacological effects of the enantiomers of calcium antagonists are known to be different, as demonstrated for verapamil,[3,4] nimodipine,[5] nilvadipine[6] and amlodipine.[7]

The pharmacological potency of the (+)- enantiomer of manidipine has been found to be 30-fold more potent than that of the (-)-enantiomer in rats.[8] In a previous paper, we have described a highly sensitive and selective method for the determination of manidipine by high performance liquid chromatography.[9] The method was based on the combination of column-switching technique and selective electrochemical determination of manidipine in human plasma. However, this method could not be applied to the pharmacokinetic determination of manidipine enantiomers. Therefore, it became necessary to develop an enantioselective determination method for determining manidipine enantiomers in human plasma.

Recently, methods for enantioselective chromatographic determination of calcium antagonists in plasma, including manidipine, have been reported using gas chromatography,[10,11] gas-chromatography-mass spectrometry (GC-MS),[12,13] and HPLC.[14,15] Optical resolution of dihydropyridine derivatives in previous methods using a xylan- or cellulose (phenylcarbamate)-type column by HPLC[16] could not obtain highly sensitive enantiospecific determination because of broadening of the peak on the chromatogram. Therefore, these methods have only been used in the highly sensitive HPLC-GC-MS system. However, this method is very tedious and time-consuming.

We established a highly sensitive chromatographic separation of dihydropyridine enantiomers with sharp peaks in our previous study by using Pirkle-type chiral stationary phase.[17] The previous methods for determination of dihydropyridine in plasma using liquid-liquid extraction method,[10-15] however, required tedious procedures. Recently, we described an extraction method using a solid bounded phase for the efficient recovery of several drugs from plasma or serum samples.[18-20] This method is satisfactory in terms of simplicity and

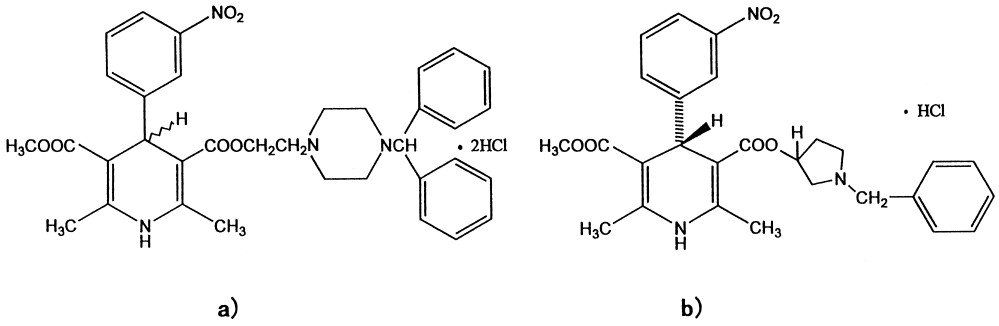

Figure 1. Chemical structures of racemic manidipine a) and (+)-barnidipine b) as an internal standard.

rapidity. In the present paper, we describe a sensitive and stereoselective method for the determination of manidipine enantiomers in human plasma by HPLC using modified Pirkle-type chiral stationary phase. This method was applied to an enantioselective pharmacokinetic study in healthy volunteers.

MATERIALS AND METHODS

Reagents and Materials

Racemic manidipine, (+)-manidipine and (-)-manidipine were kindly donated by Takeda Chemical Industries (Osaka, Japan). (+)-Barnidipine, used as an internal standard, was obtained from Yamanouchi Pharmaceutical Co. Ltd. (Tokyo, Japan). Manidipine tablet (20 mg of manidipine tablet brand of CALSLOT(r), Takeda) was purchased from Takeda Chemical Industries (Osaka, Japan). Bond Elut™ C8 cartridge was purchased from Varian (Harbor City, CA, USA). Other reagents and chemicals of analytical grade were purchased from Wako Pure Chemical Industries (Osaka) or Nakarai Tesque (Kyoto, Japan).

Stock standard solutions of racemic manidipine in methanol (1 mg/mL) and of (+)-barnidipine in methanol (1 mg/mL) were prepared by diluting to appropriate concentrations and stored at 4°C until time of analysis.

Apparatus

The apparatus used for HPLC analyses consisted of a Jusco Model 880-PU chromatography pump, a Jusco Model 875-UV detector (Jusco, Tokyo, Japan),

and a Rheodyne model 7125 injector (Rheodyne Inc., Cotati, CA, USA) with an effective volume of a 100 μL loop. The HPLC precolumn contained a trimethylsilylated silica stationary phase (5 μm, 10x4.6 mm I.D.) which was prepared in our laboratory, and packed by a low-pressure packing technique. The analytical column contained SUMICHIRAL OA-4500 chiral stationary phase (5 μm, 250x4.6 mm I.D.) (Sumika Chemical Analysis Service, Osaka, Japan). The mobile phase consisted of n-hexane-1,2-dichloroethane-methanol-trifluoroacetic acid (250:140:12:1, v/v), and flow rate was 1 mL/min at room temperature.

Preparation of Sample

(+)-Barnidipine (20 ng) in methanol (10μL) was added to the plasma sample (1.0 mL) as an internal standard, and allowed to stand at room temperature (10 min). The plasma sample was then diluted with water (5 mL) and the solution was briefly mixed. The mixture was applied to a Bond Elut™ C8 cartridge that had previously been activated with 5 mL of each of methanol and water. The cartridge was then washed with water (10 mL) and 40% methanol (10 mL), and then the desired fraction was eluted with 80% methanol (6 mL). The elute was evaporated to dryness in vacuum at 60°C. The residue was dissolved in chloroform (0.2 mL) and aliquots of 60 μL were loaded onto the precolumn for the elimination of interfering substances from the plasma sample.

After thorough washing for 25 seconds, (+)-, (-)-manidipine, and (+)-barnidipine were eluted from the precolumn to the chiral stationary analytical column by a column switching technique using the previously described mobile phase.

Calibration Graphs

Known amounts of racemic manidipine in the range of 2-50 ng/mL[1-25] ng/mL of (+)- and 1-25 ng/mL of (-)-manidipine] were added to blank plasma samples. These plasma samples were treated according to the extraction procedure described above. The peak height ratios of each manidipine enantiomer to (+)-barnidipine were measured and plotted against the concentration of analyte.

Preparation of Quality Control and Calibration Samples

Duplicate samples were prepared in 1 mL of plasma, by adding aliquots of the stock solution of racemic manidipine to drug-free plasma at five different concentrations - 2, 5, 10, 20, and 50 ng/mL of racemic manidipine, equal

amounts of (+)- and (-)-manidipine–to determine the accuracy and precision of the method. These samples are designated quality control samples. The quality control samples were stored at -40°C. Calibration samples containing 2, 5, 10, 20, and 50 ng/mL of racemic manidipine were prepared. The calibration samples were treated in the same manner as the quality control samples. For each validation run, a quality control sample was thawed and extracted.

Recovery Experiments

Plasma was spiked by adding a known amount of racemic manidipine to drug-free plasma to obtain a total volume 1 mL. These samples were extracted by the above-described method. Control samples were prepared by adding a known amount of racemic manidipine to 1 mL of methanol. These control samples were not extracted but were directly dried by evaporation at 60°C, then the residues were reconstituted in 0.2 mL of chloroform. An external standard, (+)-barnidipine was added to all of the samples before being dried by evaporation. Recoveries were determined by comparison between solid-phase extraction and no extraction as control.

Method Validation

The accuracy of the method was determined by injection of the racemic manidipine calibration samples and the five different quality control samples after extraction on five separate days. Calibration curves of (+)- and (-)-manidipine were required to have a correlation value of at least 0.993. The accuracy was calculated as a percentage of the nominal concentration. Accuracy = (conc. obs./conc. nominal)·100%. The same data used for the accuracy determinations was used for the calculation of the between-run percentage relative standard deviation [% R.S.D., R.S.D.= (S.D./mean)·100%]. The within-run % R.S.D. was derived from analysis of five quality control samples at each concentration with injection on the same day.

The detection limit of the HPLC assay after extraction was estimated as the drug quantity in plasma giving a signal five times baseline noise. The lower limit of quantitation was defined as the quantity of the sample, after preparation and extraction, which could be quantified with a deviation and precision of less than 20%.

Drug Administration and Sampling

The study protocol was approved by the Ethics Committee of Hirosaki University Hospital, and each subject gave written consent to participate in the

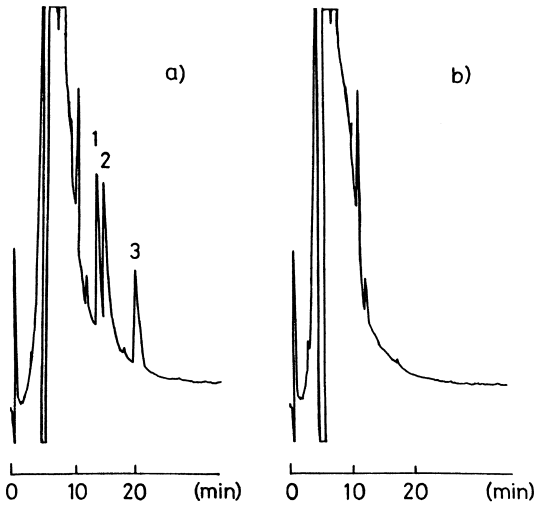

Figure 2. Typical chromatograms of (a) added stds. In plasma, (b) plasma blank. Peaks: 1 = (-)- manidipine, 2 = (+)- manidipine, 3 = (+)-barnidipine (I.S.).

study. Racemic manidipine 40 mg (two 20mg tablets of CALSLOT(r) brand of manidipine, Takeda) were orally administered to six male healthy volunteers. Blood samples (5 mL) were collected by venipuncture at 0.5, 1, 2, 4, 6, and 8 hours after oral administration. Plasma samples were separated by centrifugation at 1900 g for 15minutes and stored at -40°C until analysis.

RESULTS

We established that racemic manidipine was clearly separated into its enantiomers on a Pirkle-type chiral column (SUMICHIRAL OA-4500) with a non-aqueous mobile phase (n-hexane - 1,2-dichloroethane - methanol - trifluoroacetic acid, 250:140:12:1). The resolution factor of (+)- and (-)-manidipine in human plasma extraction was 1.13, and the time of analysis was less than 25 min. Typical chromatograms of manidipine enantiomers and (+)-barnidipine in human plasma are shown in Figure 2. Retention times for each enantiomer were 15.2 min for (-)-manidipine, 16.3 min for (+)-manidipine, and 19.3 min for (+)-barnidipine as an internal standard, and there were no interfering peaks from human plasma (Figure 2).

Calibration graphs for (+)- and (-)-manidipine in human plasma were linear in the range of 1-25 ng/mL with a correlation coefficient of at least r=0.993. The limit of detection for manidipine enantiomers was 0.5 ng/mL (signal-to-

Table 1

Accuracy and Precision of Manidipine Enantiomers in Human Plasma

	(+) Manidipine					(-) Manidipine		
Added ($ng \cdot mL^{-1}$)	Found (Mean±S.D.) ($ng \cdot mL^{-1}$)	Accuracy (%)	Precision (%)	Between-run Precision (%)	Within-run (Mean±S.D.) ($ng \cdot mL^{-1}$)	Found Accuracy (%)	Between-run Precision (%)	Within-run Precision (%)
1	0.99 ± 0.07	99.3	7.0	5.3	0.99 ± 0.08	99.1	7.8	6.4
2.5	2.48 ± 0.24	99.3	4.7	9.5	2.47 ± 0.23	98.8	5.5	9.5
5	4.81 ± 0.29	96.2	3.6	6.1	4.86 ± 0.27	97.2	5.6	5.6
10	9.47 ± 0.41	94.7	2.6	4.5	9.27 ± 0.34	92.7	3.3	3.7
25	23.4 ± 0.74	93.6	1.9	3.2	22.8 ± 0.93	91.5	2.9	3.2

n = 5.

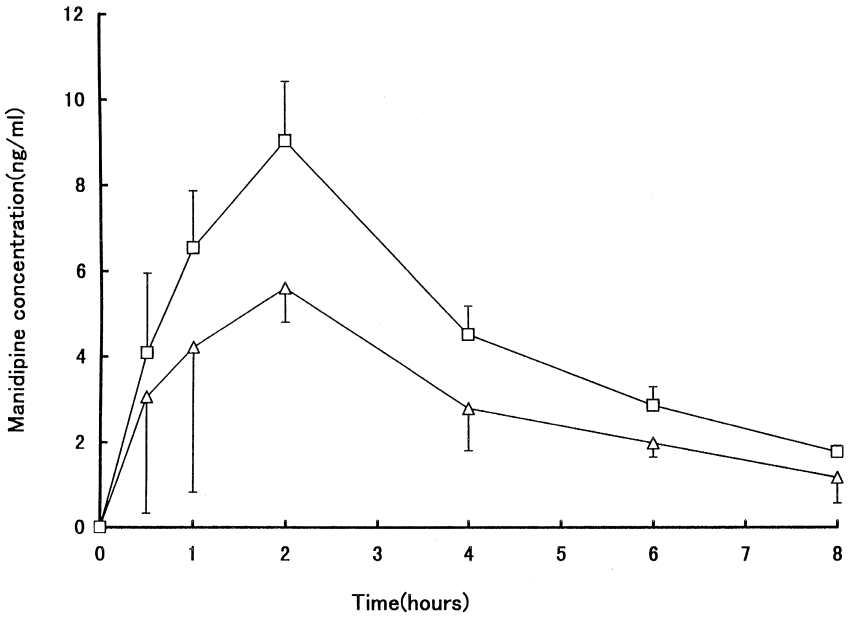

Figure 3. Mean plasma concentration-time profile of (+)-manidipine and (-)- manidipine after a single 40-mg oral dose of racemic manidipine to six healthy volunteers. □: (+)-manidipine, Δ: (-)- manidipine.

noise ratio=5). The results of recovery studies are shown Table 1, and the precision of this method was satisfactory. The recoveries of manidipine enantiomers were determined by adding the five known levels of 1, 2.5, 5, 10, and 25 ng/mL to blank plasma. The recovery values were 93.6-99.3% for (+)-manidipine and 91.5-99.1% for (-)-manidipine, respectively (Table 1). The within-run precision for spiked plasma samples was <7.4% (coefficient of variation) for both enantiomers, and also the between-run precision was <9.5% (coefficient of variation).

Representative plasma concentrations-time profiles for (+)- and (-)-manidipine after an oral administration of racemic manidipine 40mg were determined using the proposed method (Figure 3). Pharmacokinetic parameters of the six healthy volunteers were calculated and are shown in Table 2.

Mean peak concentrations of the (+)-manidipine and (-)-manidipine were reached at 1.8 h with mean values of 9.3 and 5.6 ng/mL, respectively. The area

Table 2

Pharmacokinetic Parameters of Manidipine Enantiomers in Human Plasma

Parameters	Mean ± S.D.* (+)-Manidipine	(-)- Manidipine
C_{max}(ng·mL^{-1})	9.3 ± 2.1	5.6 ±1.2
T_{max}(h)	1.8 ± 0.4	1.8 ± 0.4
$T_{1/2}$(h)	2.6 ± 0.4	2.9 ± 0.4
AUC_{0-8}(ng·h·mL^{-1})	36.4 ± 5.9	24.1 ± 4.1
AUC_{0-oo}(ng·h·mL^{-1})	43.1 ± 7.6	29.0 ± 4.5
Cl_{total}(mL·min^{-1}·kg^{-1})	134.9 ± 28.0	198.9 ± 33.9

* n = 6.

under the curve ($AUC0_{0-\infty}$) value of (+)-manidipine was about 1.5-fold higher than that of (-)-manidipine (43.1 ng·hr·mL^{-1} for (+)-manidipine, 29.1 ng·hr·mL^{-1} for (-)-manidipine, respectively), and the plasma concentration of (+)-manidipine was always higher than (-)-manidipine. On the other hand, elimination constant (Ke) and the half-life ($t_{1/2}$) values were no different between (+)-manidipine and (-)-manidipine.

DISCUSSION

First of all, a method of efficient enantiomer separation of manidipine chromatogram needed to be developed that would produce a sharp peak on a HPLC chromatogram, because previous chiral column HPLC determination systems were insufficiently sensitive in biological samples due to broadening of both enantiomer peaks on chromatogram. Also, these methods needed an off-line sensitive detection system after isolation of both enantiomers, using of GC-MS,[12,13] GC,[10,11] and reversed phase HPLC-UV[21] detection. However, off-line detection method is very tedious and time consuming.

In our previous paper,[17] we established enantiomeric separation of a 1,4-dihydropyridine, nicardipine, using Pirkle-type column (Sumichiral OA series), giving a satisfactory chromatogram with sharp peaks suitable for on-line ultraviolet detection. In the present study, this enantiometric separation method was applied for separation of manidipine enantiomers, and then a highly sensitive chromatographic system having sharp peaks by on-line ultraviolet detection was developed. Our present enantio-separation HPLC system gave a

higher detection limit (0.5 ng/mL in plasma) than previous studies have reported. Under our chromatographic conditions, (+)-manidipine eluted at 16.3 min and (-)-manidipine at 15.2 min. The observed stereochemical selectivity (α) was 1.04 and the stereochemical resolution factor (R) was 1.13. Therefore, this HPLC system could be applicable to the measurement of (+)- and (-)-manidipine in human plasma using an on-line ultraviolet detection system.

Subsequently, we directed our project towards establishing an extraction method for racemic manidipine and (+)-barnidipine, and the elimination of endogenous substances from plasma. Böcker et al.[22] reported a simple extraction method for nitrendipine and its metabolite in an incubation mixture of liver microsomes using a Bond Elut™ C18 extraction column. However, in our present study, the high interference peak of endogenous components in plasma was not removed with Bond Elut™ C8 extraction.

In our previous paper, we described a serum sample direct injection HPLC system coupled with a column-switching method for analysis of several drugs,[23-25] and a solid-phase extraction method coupled with a column-switching technique.[9,17] No interference of the chromatogram occurred with these methods. Therefore, we used a column-switching technique after extraction in the present study and no interference of the chromatograms was observed at the retention time of the (+)-, (-)-manidipine, and (+)-barnidipine (Figure 2). The plasma concentration of (+)- and (-)-manidipine was reported by Yamaguchi et al.[21] The range of plasma concentration of manidipine enantiomers for therapeutic drug monitoring may be 0.5-25 ng/mL for each enantiomer in the therapeutic dose range of 10-40 mg. The sensitivity and calibration ranges of the present method are appropriate for therapeutic drug monitoring of manidipine enantiomers in patients.

The results of recovery studies (Table 1) show that the proposed method is satisfactory with respect to accuracy and precision. Furthermore, the method is rapid, the extraction is efficient, and the system simple. The method described was used to study the pharmacokinetics of (+)- and (-)-manidipine in an oral dose of 40mg of manidipine racemate given to six healthy volunteers (Figure 3). The pharmacokinetic parameters obtained in the present study (Table 2) are similar to those of a previous paper.[21] The $AUC0_{0-\infty}$ value of (+)-manidipine (43.1ng·hr mL^{-1}) and (-)-manidipine (29.1ng·hr mL^{-1}) are nearly the same values as in the previous paper (AUC_{0-24} of (+)-manidipine 57.4 ± 5.1 ng·hr mL^{-1} and (-) manidipine 28.6 ± 2.8 ng•hr•mL^{-1}). The value of C_{max} and T_{max} are similar to the previously found values. The enantiomeric ratio of (+)/(-)-manidipine is about 1.5 in $AUC0_{0-\infty}$ and 1.7 in C_{max} in the present study, and then 2.0 in $AUC0_{0-\infty}$ and 1.8 in C_{max} in previous study.[21]

The validity of the previous enantioselective pharmacokinetic study of manidipine was proved by our stereoselective pharmacokinetic study using analytical methods.

The stereoselective activity of 1,4-dihydripyridine isomers has been described in a previous paper.[26] The pharmacokinetic analysis of 1,4-dihydripyridine enantiomer is useful for understanding the stereoselective contribution of both enantiomers to the pharmacological activity and for understanding the different contribution each enantiomer has on the pharmacokinetics of the racemic drug. From the results, it was shown that the proposed method for the determination of (+)- and (-)-manidipine could be applied to pharmacokinetic study in patients receiving racemic manidipine treatment. Further pharmacokinetic studies of drug interaction will be carried out in these laboratories, and the details will be reported elsewhere.

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Received July 29, 1998
Accepted August 6, 1998
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