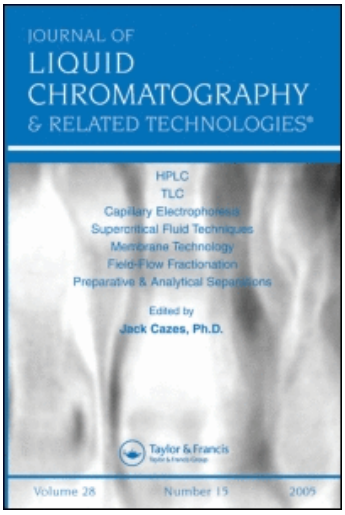

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DETERMINATION OF VERAPAMIL ENANTIOMERS IN SERUM FOLLOWING RACEMATE ADMINISTRATION USING HPLC

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ABSTRACT

A method for quantitation of (+)-(R)-verapamil and (-)-(S)-verapamil concentrations in serum was developed for the purpose of determining verapamil enantiomer pharmacokinetics after racemate administration. Two high-performance liquid chromatographic (HPLC) systems were used. A reversed-phase HPLC system was first used to quantitate summed concentrations of the enantiomers ((R+S)-verapamil) using fluorescence detection. Mobile phase eluate containing the (R+S)-verapamil was collected from the detector outlet, and the individual enantiomers in the residue were separated using a chiral column with a stationary phase of silica-bound α_1-acid glycoprotein. Peak heights were measured and peak height ratios of R/S were determined. The measured (R+S)-verapamil concentration from the achiral system was used with the R/S ratio from the chiral system to determine the serum concentrations of the individual enantiomers. The pharmacokinetics of (+)-(R)-verapamil and (-)-(S)-verapamil were studied in a healthy volunteer who received a single oral dose (120 mg) and a 30 minute intravenous infusion (15 mg) of racemic verapamil hydrochloride. The pharmacokinetic parameters obtained for the individual enantiomers (half-life, steady-state volume of distribution, body clearance,

bioavailability) agreed well with reported values from studies in which enantiomers were administered on separate occasions or in which one enantiomer was dideuterated.

INTRODUCTION

Verapamil is a calcium channel antagonist that is widely used as an antianginal and antihypertensive agent (1-4). The negative dromotropic effects of verapamil make it useful as an antiarrythmic agent to control supraventricular tachyarrythmias. It is currently administered to patients as a racemic mixture of the (+)-(R)- and the (-)-(S)-enantiomers. Previous studies have shown that the two enantiomers possess different pharmacokinetic and pharmacodynamic properties (5-10). In-vitro and in-vivo animal studies have shown that the (-)-(S)-enantiomer has a greater effect on atrioventricular conduction than does the (+)-(R)-enantiomer (6,11,12). Investigations with dideuderated pseudoracemic verapamil in humans have demonstrated that the (-)-(S)-enantiomer is responsible for the negative dromotropic effects of verapamil on AV conduction (5-7,11) and that the (-)-(S)-enantiomer has a greater plasma clearance and a lower bioavailability than the (+)-(R)-enantiomer (5-8,10). Conventional methods of assay for verapamil measure the summed serum concentrations of the enantiomers and are unable to quantitate the active (-)-(S)-enantiomer concentrations (13,14) following racemate intake. Gas chromatography with specific mass fragmentation detection has been used to measure individual enantiomer serum concentrations (6,15). However this method requires the administration of a pseudoracemic mixture containing one dideuterated and one unlabelled enantiomer making it unsuitable for studies in patients who receive true racemic verapamil.

A coupled achiral-chiral high-performance liquid chromatographic (HPLC) system for the determination of verapamil enentiomers was developed by Chu and Wainer (16) for patients on chronic oral therapy who have relatively high serum verapamil concentrations. A less labor-intensive, automated method using several pumps has also been reported (17). In this report we describe a more sensitive HPLC method which separates and quantitates the individual

enantiomers of verapamil in human serum following racemic verapamil administration. The method is specific and sensitive enough for use in pharmacokinetic studies using single doses.

MATERIALS AND METHODS

Chemicals and Materials

Racemic verapamil HCl and imipramine HCl were purchased from Sigma Chemical Co., St. Louis, MO. (-)-(S)-verapamil HCl and (+)-(R)-verapamil HCl and eight metabolites: D 617-HCl, D 620-HCl, PR 21-HCl, PR 22-HCl PR 23-HCl, PR 24-oxalate, PR 25-HCl, and norverapamil HCl were donated by Knoll Pharmaceuticals, Ludwigshafen, G.F.R. Methanol, tetrahydrofuran and acetonitrile were HPLC grade and purchased from Fisher Scientific, Fair Lawn, NJ. HPLC-grade methyl t-butyl ether was obtained from Baxter Healthcare Corp., Burdick and Jackson Division, Muskegon, MI. Concentrated sulfuric acid, glacial acetic acid, ethanol, triethylamine, and sodium hydroxide were A.C.S. reagent grade. Water was double-distilled and demineralized and stored in polypropylene containers. Polypropylene test tubes, 8-ml and 13-ml, were purchased from Kew Scientific Inc., Columbus, OH.

Phase I, Achiral HPLC: Isolation and Quantitation of Summed Verapamil Enantiomers

Chromatography. A model 222B isocratic HPLC pump equipped with a model XL sample injector valve with a 50 μl loop (Scientific Systems Inc., State College, PA) and a Spectroflow 980 fluorescence detector (Applied Biosystems, Ramsey, NJ) were used. Detector excitation wavelength was 209 nm and no emission cut-off filter was used. Detector output was recorded using a model 4290 integrator (Varian Instrument Group, Walnut Creek, CA).

A Dynamax Microsorb CN column (5-μm particle diameter, 250 mm x 4.6 mm) was purchased from Rainin Instrument Co., Woburn, MA. The mobile phase was composed of 0.3 M sulfuric acid:tetrahydrofuran:acetonitrile (0.4:15:84.6, v/v/v), and was degassed by sonication under vacuum for 5 min. The mobile phase flow rate was 1 ml/min.

Drug Standards. A stock solution of racemic verapamil base (200 μg/ml) was prepared in 50% ethanol in water. The stock solution was serially diluted with 50% ethanol in water to produce spiking solutions ranging in concentration from 0.04 to 5 μg/ml of racemic verapamil. Fifty microliters of each solution added to 1.0 ml of drug-free serum produced calibration standard concentrations ranging from 2 to 250 ng/ml. A stock solution of imipramine base (200 μg/ml) was prepared in methanol. A working internal standard solution was prepared by diluting the stock with water to a final concentration of 200 ng/50 μl. A reference mixture containing eight verapamil metabolites (0.93 μg/ml of each) was prepared in water. All solutions were stored at -20°C in polypropylene tubes.

Extraction. The method of Bremseth et al. (14) was used with some modifications. One milliliter of serum was pipetted into 8-ml polypropylene tubes which contained 50 μl (200 ng) of imipramine and 400 μl of 5 N NaOH. After vortexing for 10 sec, 5 ml of methyl t-butyl ether were added and the tubes were agitated for 10 min on a reciprocating shaker. After centrifugation for 10 min at 1200 g, the aqueous layer was frozen in a bath of dry ice and acetone. The ether layer was decanted into an 8-ml polypropylene tube and evaporated to dryness in a dry-block sample concentrator set at 45° C under a stream of filtered air. The residue was reconstituted with 50 μl of methanol, vortexed for 1 min, centrifuged briefly, and the entire volume was injected onto the column.

Quantitation and Verapamil Isolation. Standard curves were constructed using peak heights from the integrator output. Peak height ratios of racemic verapamil to internal standard were plotted against known concentrations of racemic verapamil. Non-weighted linear regression analysis was used for determination of summed verapamil enantiomer concentrations ((R+S)-verapamil) in patient samples. For patient samples, mobile phase effluent corresponding to the (R+S)-verapamil peak on the integrator was collected from the detector outlet (approximately 800 μl) into a 13-ml polypropylene tube, and processed as described below for injection onto the chiral Phase II system.

Analytical Variables. Extraction recoveries of racemic verapamil and imipramine were determined by comparing the peak heights of extracted samples (adjusted for transfer volumes) to those obtained by direct injection of unextracted standards. Extraction recovery of racemic verapamil was determined at serum concentrations of 15 and 100 ng/ml; recovery of the internal standard was determined at a serum concentration of 200 ng/ml. Within-day precision for racemic verapamil serum concentrations of 15 and 100 ng/ml was determined by analyzing 10 samples of each on a given day. Day-to-day precision for racemic verapamil serum concentrations of 15 and 100 ng/ml was determined by analyzing 30 samples on 30 different days over a 6-month period. Coefficients of variation (CV) were determined for the corresponding mean concentrations. Specificity of the method with respect to verapamil metabolites was evaluated by injection of all isolated metabolites of verapamil: norverapamil, D617, D620, PR21, PR22, PR23, PR24, PR25. The minimum detectable amount and the maximum linear amount of verapamil were determined by injecting verapamil quantities ranging from 1 ng to 1.5 μg. The minimum detectable amount was defined as the amount of verapamil coresponding to a peak height that is twice the baseline noise. The maximum linear amount was determined as described by Poole and Schuette (18).

Phase II, Chiral HPLC: Individual Verapamil Enantiomer Concentrations

Chromatography. A model 2010 HPLC pump from Varian Instruments, Walnut Creek, CA, equipped with a model 7125 sample injection valve with a 100 μl loop (Rheodyne, Cotati, CA) and a model 980 programmable fluorescence detector (Applied Biosystems, Ramsey, NJ) were used. Excitation wavelength was set at 230 nm, and an emission cut-off filter of 295 nm was used. A model 4290 integrator (Varian Instrument Group, Walnut Creek, CA) was used to record detector output.

A Chiral AGP® column (5-μm particle diameter, 100 mm x 4.0 mm) was purchased from Advanced Separation Technologies Inc., Whippany, NJ. The mobile phase consisted of

acetonitrile:0.01 M triethylamine (11.5:88.5, v/v). The 0.01 M triethylamine was adjusted to a pH of 6.50 ± 0.02 with glacial acetic acid. The mobile phase was set at a flow rate of 0.9 ml/min and was continuously purged with helium during use. The signal to noise ratio for detection was optimized by setting the high voltage to 10% above the manufacturer's recommended setting, and using a rise time of 10 sec.

Quantitation of Enantiomer Ratios and Concentrations. Collected eluate corresponding to verapamil from the Phase I system was placed in a rotary mixer with a water bath temperature of 37° C, and concentrated for 10 min under a stream of filtered air. The sides of the tube were rinsed down with 200 μl of acetonitrile, and the sample was concentrated again for 5 min. The residue was immediately diluted with 50 μl of double distilled water, and the entire volume, if necessary, was injected onto the Phase II HPLC system.

Stock solutions of (+)-(R)-verapamil and (-)-(S)-verapamil (200 μg/l each) were prepared in 50% ethanol in water. They were further diluted with 50% ethanol in water and combined in different proportions to produce solutions of R/S verapamil ratios ranging from 1:1 to 7:1. Each stock solution had a final (R+S)-verapamil concentration of 2 μg/ml. All solutions were stored at -20°C in polypropylene tubes. Standard curves were created by plotting measured peak height ratios of (+)-(R)-verapamil to (-)-(S)-verapamil (from the integrator output) against the known R/S concentration ratios. Non-weighted linear regression was used for determination of R/S enantiomer concentration ratios from R/S enantiomer peak height ratios in patient samples. Once the R/S concentration ratio was determined, the individual verapamil enantiomer concentrations were calculated from the total (R+S)-verapamil concentrations that had been determined using the Phase I system according to:

$$(S) = \frac{(R+S)}{R/S + 1}$$

$$(R) = (R+S) - (S)$$

Analytical Variables. Coefficients of variation of enantiomer peak height ratios were determined for replicate injections (n=5) of stock solutions that were prepared to contain the following R/S verapamil enantiomer concentration ratios: 1:1, 3:1, 5:1 and 7:1. The effect of injected amount on the ratios was evaluated by performing the precision experiments for (R+S)-verapamil amounts of 10 ng and 50 ng.

The possibility that an enantiomer ratio could change during sample processing and chromatography was also evaluated. Sera spiked with solutions of known enantiomer concentration ratios were extracted and taken through both HPLC systems as described. The resulting enantiomer peak height ratios were compared to those obtained by direct injection of the corresponding stock solutions. Processed and nonprocessed samples were compared by non-weighted linear regression analysis.

Application to a Pharmacokinetic Study

The pharmacokinetics of (+)-(R)-verapamil and (-)-(S)-verapamil were studied in a healthy volunteer who had received a single oral dose of 120 mg racemic verapamil hydrochloride (Calan®, Searle Pharmaceuticals, Chicago, IL) and a 30 min intravenous infusion of 15 mg of racemic verapamil hydrochloride (Isoptin®, Knoll Pharmaceuticals, Whippany, NJ) on two separate occasions separated by one week. Blood samples were drawn for up to 12 hours through an indwelling catheter (kept patent with heparin in normal saline, 10 units/ml) and allowed to clot in polypropylene tubes. Separated sera were stored in polypropylene tubes at -20°C until analyzed according to the previously described procedure. Concentrations of (R+S)-verapamil were determined by the Phase I HPLC system and the ratios of the enantiomers were determined by the Phase II system as previously described.

The elimination half-life ($t_{1/2}$) of verapamil was calculated from the terminal values of the serum concentration-time curves using non-weighted linear regression. The areas under the serum concentration-time curves (AUC) were calculated using the linear trapezoidal rule

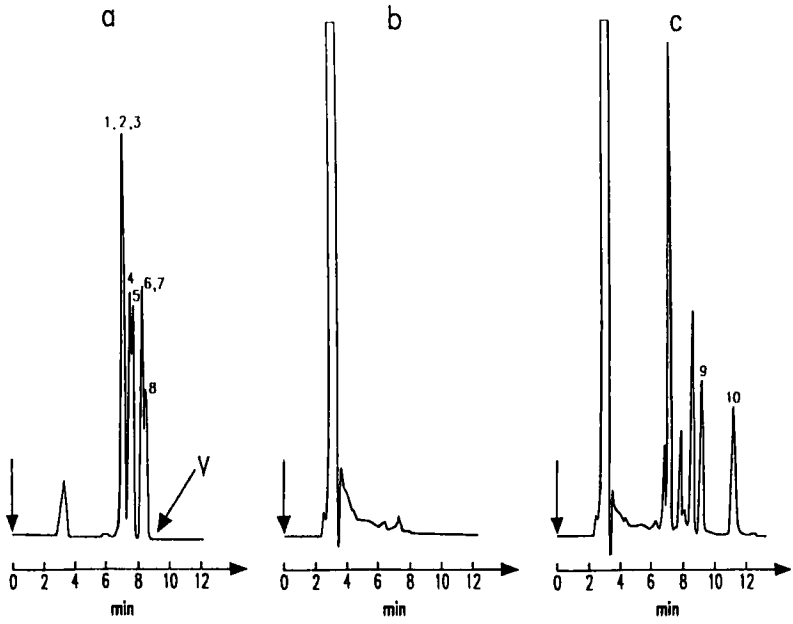

Figure 1a. Chromatogram of an unextracted reference mixture of eight metabolites of verapamil. The peaks are: norverapamil (1), P-620 (2), PR-22 (3), PR-21 (4), PR-25 (5), PR-23 (6), PR-24 (7), D-617 (8). The arrow refers to the elution time for verapamil.

Figure 1b. Chromatogram of extracted drug-free pre-dose serum.

Figure 1c. Chromatogram of extracted serum sample taken 4 hrs after a dose of 120 mg oral racemic verapamil HCl. The peaks are: verapamil (9), imipramine (internal standard) (10). The mobile phase consisted of 0.3 M sulfuric acid:tetrahydrofuran: acetonitrile (0.4:15:84.6, v/v/v). The flow rate was 1 ml/min.

with extrapolation to infinity using the elimination rate constant (19). Body clearance, steady-state volume of distribution (Vss) and bioavailable fraction were determined using non-compartmental methods described by Gibaldi and Perrier (19).

TABLE 1

Extraction Recovery, Phase I HPLC

Compound	Concentration (μg/l)	Recovery MEAN%	SD	CV%
Verapamil	15	100	5.5	5.0
	100	104	1.1	1.0
Imipramine	200	101	3.9	3.8

TABLE 2

Precision of Assay for (R+S)-Verapamil, Phase I HPLC

Within-day (n=10)			Day-to-day (n=30)		
MEAN (μg/l)	SD	CV%	MEAN (μg/l)	SD	CV%
18.8	0.7	3.7	18.1	1.4	7.5
124.3	2.6	2.1	112.7	5.0	4.4

RESULTS

Phase I HPLC: Quantitation and Isolation of R+S-Verapamil

Sample chromatograms are shown in Fig. 1. As shown in Fig. 1a, verapamil did not co-elute with any of the tested metabolites. No plasma or other interferences with verapamil or the internal standard were evident in chromatograms of extracted pre-dose serum, as shown in Fig. 1b. Fig. 1c shows a chromatogram of an extracted serum sample obtained 4 hrs after administration of the single 120-mg oral racemic verapamil hydrochloride dose to the subject. Peaks 9 and 10 correspond to verapamil and imipramine (internal standard). The measured serum concentration of (R+S)-verapamil was 42.1 ng/ml. Total chromatography time was 12 min.

Extraction recoveries of verapamil and internal standard are summarized in Table 1. Verapamil and internal standard were completely recovered from plasma. Recovery of verapamil ranged from 100% to 104% and did not significantly change with

concentration. The recovery of the verapamil was better than that achieved by earlier investigators (16,17).

The precision data for (R+S)-verapamil concentration measurements is presented in Table 2. Within-day CV values ranged from 2.1% to 3.7%, while day-to-day CV values ranged from 4.4% to 7.5%. These data compare favorably with precision data for the HPLC assay method of Bremseth et al. (14). Since there were no changes in concentrations of the quality control samples with time, it appears that verapamil in serum is stable at -20° C for at least 6 months. The maximum linear concentration was 1 μg/ml and the limit of sensitivity was 0.2 ng/ml.

Phase II HPLC: Individual Verapamil Enantiomer Concentrations

Separations of the enantiomers of verapamil on the chiral HPLC (Phase II) are shown in the chromatograms in Fig. 2. Peaks (1) and (2) correspond to (+)-(R)-verapamil and (-)-(S)-verapamil respectively. The resolution (R) and separation factors (α) for verapamil enantiomers are 1.53 and 1.23, respectively. As shown in Fig. 2a, injection of a stock solution of racemic verapamil yielded a peak height ratio of R/S of 1.08. An R/S ratio of 6.4 was determined for an extracted serum sample taken from a normal subject who received a single oral dose of 120 mg racemic verapamil (Fig. 2b). The R/S ratio following intravenous administration of racemic verapamil to the same subject was 1.25, as shown in Fig. 2c.

Precision data for R/S enantiomer peak height ratios are presented in Table 3. Coefficients of variation for all R/S ratios were less than 5%. At an R/S enantiomer concentration ratio of 7:1, an injected amount of 10 ng total (R+S)-verapamil (1.25 ng of (-)-(S)-verapamil and 8.75 ng of (+)-(R)-verapamil) had a coefficient of variation of 3.3%.

The individual concentration ratios were not affected by the amount of (R+S)-verapamil, as seen by the superimposability of the standard curves in Fig. 3. Enantiomer ratios in samples taken through the complete analytical procedure were identical to those obtained by direct injection of the enantiomer ratio stock solutions.

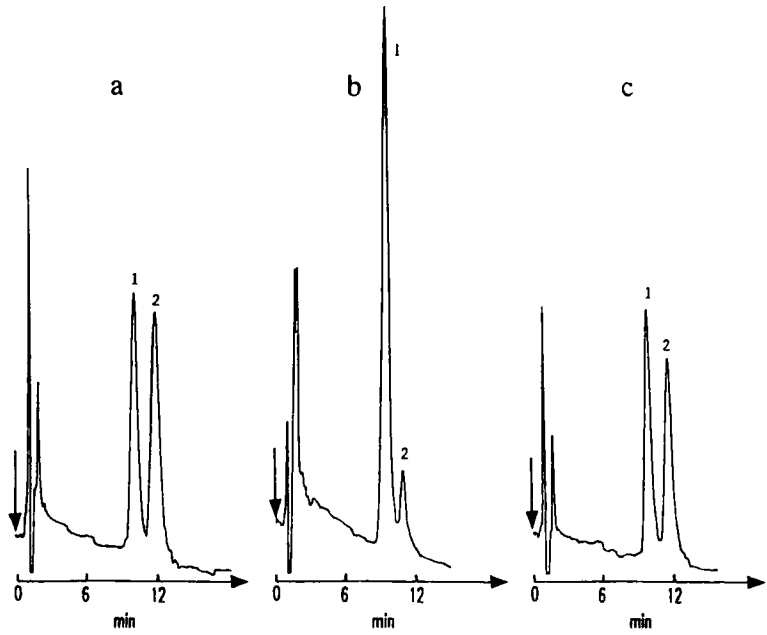

Figure 2. Chromatogram showing the resolution of verapamil enantiomers from the collected eluate of the Phase I system. The peaks are: (+)-(R)-verapamil (1), and (-)-(S)-verapamil (2).

Figure 2a. Chromatogram of reference solution containing 50 ng of racemic verapamil. The peak height ratio is 1.08.

Figure 2b. Chromatogram of a serum extract from a sample taken 4 hours after an oral 120 mg racemic verapamil dose. The (R+S)-verapamil concentration is 42.1 ng/ml and the ratio of concentrations (R/S) is 6.7; (+)-(R)-verapamil concentration is 36.6 ng/ml and the (-)-(S)-verapamil concentration is 5.5 ng/ml.

Figure 2c. Chromatogram of a serum extract of a sample taken 15 min after the end of a 30 min intravenous infusion of 15 mg racemic verapamil HCl. The (R+S)-verapamil concentration is 60.9 ng/ml and the ratio of the concentrations R/S is 1.25; the (+)-(R)-verapamil concentration is 33.8 ng/ml and the (-)-(S)-verapamil concentration is 27.1 ng/ml.

TABLE 3

Within-day Precision for R/S Peak Height Ratios, Phase II HPLC

Enantiomer Concentration Ratio (R/S)	Peak Height Ratio (R/S) 10ng R+S (n=5) MEAN	SD	CV%	50ng R+S (n=5) MEAN	SD	CV%
1:1	1.1	0.0	1.1	1.1	0.0	0.7
3:1	2.8	0.1	2.7	2.7	0.1	1.9
5:1	4.4	0.2	4.9	4.7	0.1	2.3
7:1	5.9	0.2	3.3	5.8	0.3	4.4

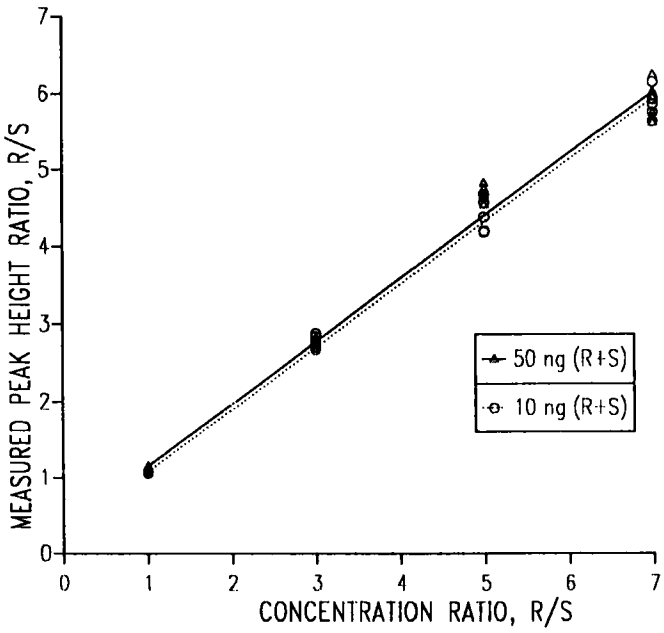

Figure 3. Comparison between the measured peak height R/S ratio to the known R/S concentration ratio at R+S-verapamil amounts of 10 ng and 50 ng. Non-weighted linear regression lines are shown.

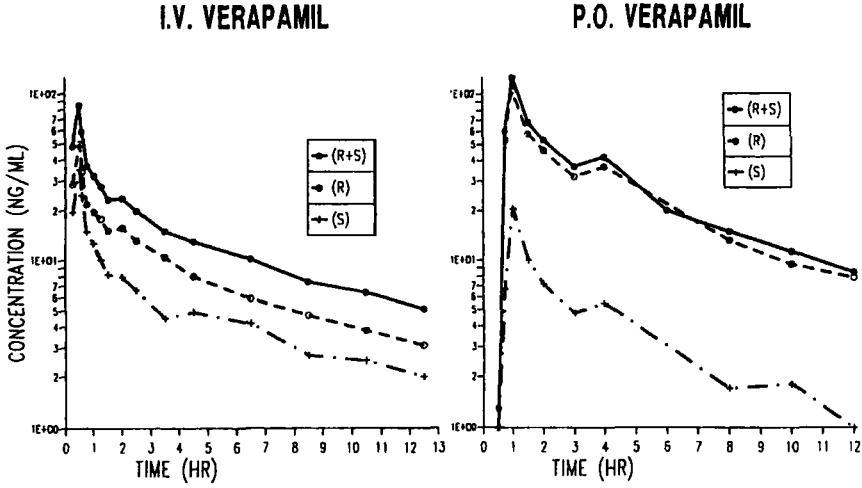

Figure 4. (R+S)-, (+)-(R)- and (-)-(S)-verapamil serum concentrations in a healthy individual following intravenous infusion of 15 mg racemic verapamil HCl over 30 min and following oral administration of 120 mg racemic verapamil HCl.

Application To Pharmacokinetic Study

The plasma concentration-time curves of (R+S)-, (+)-(R)- and (-)-(S)-verapamil after intravenous and oral racemic verapamil administration are shown in Fig. 4. After a single intravenous dose, the peak (R+S)-verapamil concentration at the end of the infusion was 84.4 ng/ml. The R/S ratio at that time was 1.5. Two hours after the infusion, the R/S ratio reached a maximum of 2.0 and the concentration had dropped to 20 ng/ml. The concentration 10 hours later was 5 ng/ml and the R/S ratio was 1.5.

After a single oral dose, the peak (R+S)-verapamil concentration of 124 ng/ml occurred 1 hour after ingestion of the dose. The R/S ratio at that time was 5.5. Concentrations fell to 8.4 ng/ml at 12 hours with a R/S ratio of 5.2.

The calculated pharmacokinetic parameters are listed in Table 4. The half-lives were similar for (+)-(R)-, (-)-(S)-, and (R+S)-verapamil. The body clearances were different: the (-)-(S)-verapamil clearance was 1.6 times

TABLE 4

Pharmacokinetic Parameters for (R+S)-, (R)-, and (S)-Verapamil After Oral and Intravenous Administration

	$T_{1/2}$,iv (hr)	$T_{1/2}$,po (hr)	CL (ml/min/kg)	Vss (L/kg)	F
R+S	6.0	4.8	12.4	5.3	0.22
R	5.9	4.7	10.0	4.2	0.33
S	6.1	4.8	16.2	7.2	0.07

greater than that of the (+)-(R)-enantiomer. The steady-state volumes of distribution differed as well: the volume of distribution of the (-)-(S)-enantiomer was 1.7 times greater than that of the (+)-(R)-enantiomer. The (+)-(R)-enantiomer had a bioavailable fraction that was almost five times that of the (-)-(S)-enantiomer.

DISCUSSION

Our method for determination of verapamil enantiomers in human serum differs from other methods reported previously. Chu and Wainer (16) reported an HPLC system which permitted the use of a column switching valve to selectively divert eluate corresponding to verapamil and norverapamil from an achiral HPLC column to a Chiral-AGP^R column. This method was successfully applied to the determination of verapamil and norverapamil enantiomer concentrations in sera taken from patients who were taking 480 mg per day of oral racemic verapamil. The sensitivity limit for each enantiomer was reported to be 25 ng/ml (16). Oda et al. (17) offered an alternative achiral-chiral coupled HPLC system which used automated column switching for determination of verapamil and five metabolites. An ovomucoid column was used for the chiral separation (17). While this method was less labor intensive, it required four pumps, three columns, and gradient capabilities. Sensitivity limits and applications to patient studies were not reported by Oda et al. (17).

Our method is technically more simpler than the other methods since the achiral and chiral HPLC systems are not coupled by a column switch valve. Although an additional step

of eluate collection and concentration is required, the method is not any more labor intensive than the method described by Chu and Wainer (16) since both methods require two separate injections of sample. Chu and Wainer (16) require one injection to quantitate (R+S)-verapamil concentrations, and a separate injection to determine the R/S ratio. Our method requires one injection to determine the (R+S)-verapamil concentration and to isolate the (R+S)-verapamil eluate, and a second injection on the chiral system to determine the R/S ratio.

The resolution (R) and separation factors (α) for verapamil enantiomers using our method are comparable to those reported by Chu and Wainer (16) and Oda et al. (17). Chu and Wainer reported R and α values for verapamil of 1.35 and 1.29, respectively, while Oda et al. reported an α value of 1.25. We report R and α values of 1.53 and 1.23, respectively. The slightly better resolution using our method is likely caused by less band broadening due to the use of smaller injection volumes than is possible when column switching procedures are used.

We have shown precise measurements of R/S ratios as high as 7:1 following injection of as little as 10 ng of R+S-verapamil. This corresponds to S-verapamil amounts as low as 1.25 ng. Thus, our method is suitable for measurement of (R+S)-verapamil serum concentrations as low as 2 ng/ml with R/S ratios of 1.5 (as might be seen following intravenous administration) or 4.5 to 10 ng/ml with R/S ratios of 3.5 to 7 (as might be seen following oral adminsitration).

We have also shown our method to be specific with respect to norverapamil. While we have not applied our method to determination of enantiomers of norverapamil, the same eluate isolation procedure could be used.

The results of the pharmacokinetic study agree well with the results obtained by other investigators who have administered each enantiomer separately (6) or who have concomitantly administered labelled and unlabelled enantiomers (7,8). The lower bioavailability of (S)-verapamil relative to the (R)-enantiomer seen in our subject was consistent with the results of other investigators who have determined that the

(S)-verapamil undergoes a stereoselective first-pass metabolism, and is therefore preferentially metabolised (9,10). The higher body clearance and volume of distribution of the (S)-verapamil compared to the (R)-verapamil is consistent with the report of Eichelbaum, Echizen and others (6-8). Because of the demonstrated sensitivity and reliability of our method, we conclude that it can be used for single dose pharmacokinetic studies after administration of intravenous or oral doses of racemic verapamil or in studies where lower concentrations of verapamil may be observed.

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