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STEREOSELECTIVE PHARMACOKINETICS AND METABOLISM OF THE ENANTIOMERS OF CYCLOPHOSPHAMIDE

PRELIMINARY RESULTS IN HUMANS AND RABBITS

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Abstract—[*R*(+),*S*(−)]-Cyclophosphamide [(*R*,*S*)-CP] is an anticancer drug, containing a chiral phosphorous atom, which is prepared and used clinically as the racemic mixture. A new high-performance liquid chromatographic assay suitable for pharmacokinetic studies of CP enantiomers in plasma has been reported recently by this laboratory (Reid *et al.*, *Anal Chem* **61**: 441–446, 1989). Briefly, the assay involves ethyl acetate extraction of CP enantiomers from plasma followed by derivatization to diastereomers in a two-step process utilizing chloral and (+)-naproxen acid chloride. Chromatographic analysis was performed on a reversed phase (ODS) column with detection at 232 nm. In the present study, preliminary results on the applicability of this assay to pharmacokinetic studies are presented. Several rabbits were used to compare the influence of i.p., i.v., and oral routes of administration on the stereoselective disposition of (*R*,*S*)-CP. Following i.p. administration, *S*-CP was cleared faster than *R*-CP. Following oral administration, only *R*-CP was detectable in plasma, while i.v. administration resulted in minor or no stereoselective disposition. These results indicated that there was a marked stereoselective metabolism of the *S*-CP enantiomer, with the i.p. and oral routes producing the greatest differences due to first-pass metabolism. Incubation of rabbit-liver microsomes with (*R*,S)-CP demonstrated that the monooxygenase system can exhibit marked stereoselectivity in its metabolism of CP. The ratio of *R*-CP to *S*-CP in the incubation medium increased during the incubation period from 1:1 initially to 4.5:1 after 60 min. The results from the experiments with rabbits indicate that the first-pass metabolism of this drug is highly stereoselective; in contrast, cancer patients who had received (*R*,S)-CP as an i.v. infusion showed no stereoselectivity in the elimination of the enantiomers. Pharmacokinetic studies with cancer patients, receiving (*R*,S)-CP as an oral dose, are in progress in order to determine if stereoselective first-pass metabolism of this drug also occurs in humans.

[*R*(+),*S*(−)]-Cyclophosphamide [(*R*,*S*)-CP] is an alkylating agent commonly used as an antineoplastic agent in cancer chemotherapy. It is composed of an oxazaphosphorine ring with a nitrogen mustard side chain (Fig. 1). The phosphorous atom is chiral and, because of the method of synthesis, a racemic mixture is obtained during preparation of the commercial product [2, 3]. The mechanism of action of this drug has been the subject of numerous investigations which have been reviewed recently [4, 5]. CP itself is inactive and requires metabolic activation to exhibit alkylating activity. The proposed mechanism of CP activation is by an initial hydroxylation at C-4, catalyzed principally by the hepatic microsomal cytochrome P450 monooxygenase system. The resulting 4-hydroxy-CP exists in equilibrium with its ring-opened tautomer, aldophosphamide, which may subsequently undergo hydrolysis to produce acrolein and phosphoramide mustard (PM), which is believed to be responsible for the alkylating activity observed *in vivo*. Additionally, the two tautomer intermediates may be enzymatically oxidized to the inactive metabolites, 4-keto-CP and carboxyphosphamide, found in urine.

One aspect of CP metabolism that has not been investigated extensively, however, is the question of stereoselectivity in the metabolism and pharmacokinetics of the CP enantiomers. Previous studies [6–8] have shown that enantiomers of compounds containing a chiral phosphorous atom exhibit differences in biological activity. For example, the *S*-(−)-enantiomers of some alkyl-*S*-alkyl methylphosphonothioates have been found to have greater inhibitory activity towards certain acetylcholine esterases [6]. Comparative metabolic studies on the enantiomers and racemates of one such compound, cyanofenphos (*O*-*p*-cyanophenyl *O*-ethyl phenylphosphonothioate), have shown that its metabolism can also be stereoselective [7]. Additionally, several reports discussing stereoselectivity in the metabolism of various drugs by the cytochrome P450 enzymes have been presented recently [9–12].

At present, only three studies have investigated the question of stereoselectivity in the metabolism of CP enantiomers [8, 13, 14]. Utilizing optical rotation and ^{31}P-NMR analysis, two of these studies found little or no stereochemical differences in the enantiomer levels in the urine of cancer patients after an i.v. dose of CP [8, 13]. On the other hand, Cox *et al.* [14] found that a stereochemical difference is

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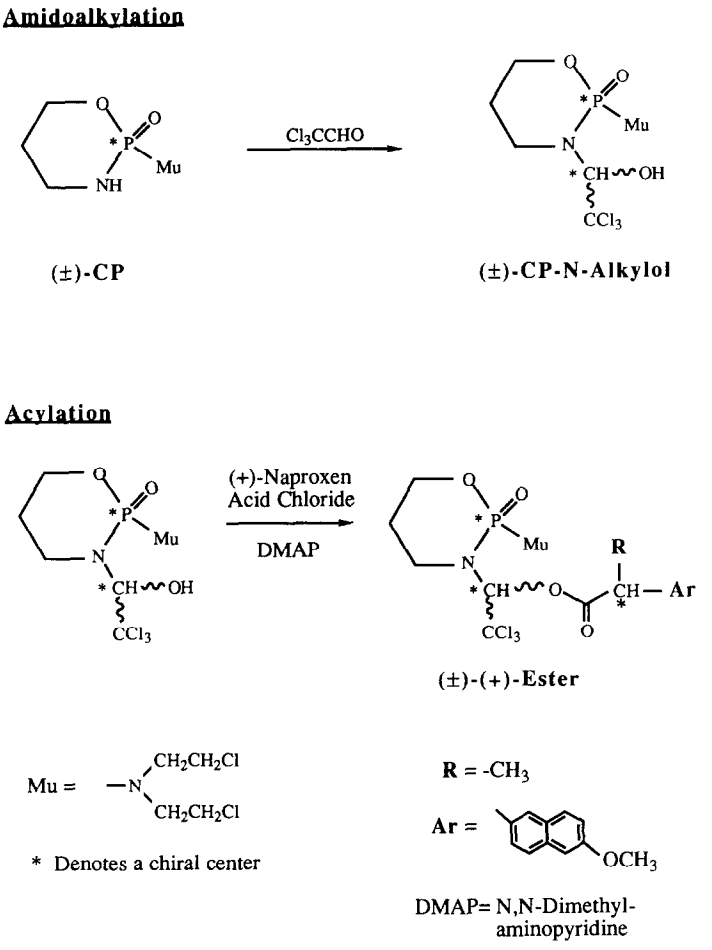

Fig. 1. Structures of cyclophosphamide and derivatives after reaction with chloral and (+)-naproxen acid chloride. The reaction with chloral has been shown to be stereospecific, and the new chiral center introduced by chloral produces a new pair of enantiomers which are unresolvable by HPLC [1]. Therefore, the diastereomeric designations refer only to the chiral centers of the cyclophosphamide and naproxen moieties.

exhibited by mice and rabbits during the metabolism of the CP enantiomers. This study [14] utilized a pseudoracemate* of CP and examined extracts from incubations with liver microsomes from mice, rats, and rabbits as well as urine samples obtained after i.p. treatment of these animals. Enantiomer quantitation in the latter study [14] was by mass spectrometry. Because the clinical studies involved only a few patients, the results with humans are inconclusive. To examine stereoselective pharmacokinetics in a larger group of patients, an alternative method that is less affected by interferences than optical rotation and is less time consuming and more economical than the NMR and mass spectroscopic methods used previously would be helpful.

Research in this laboratory has focused on the development of a sensitive and stereospecific HPLC-based assay utilizing plasma samples and suitable for pharmacokinetic studies involving large numbers of subjects. An assay which can monitor plasma levels of the CP enantiomers is preferable to methods for the analysis of urine samples as it will be able to detect stereoselectivity in the pharmacokinetics of the parent racemic drug. An HPLC-based method has been developed recently by Reid *et al.* [1] and, in this report, the preliminary results of several studies applying this assay to the metabolism and plasma pharmacokinetics of the CP enantiomers in rabbits and cancer patients are reported.

* A racemate in which one enantiomer is labeled with deutrium.

MATERIALS AND METHODS

Chemicals

(*R,S*)-CP monohydrate, containing sodium chloride (90 mg/200 mg CP), was obtained as the USP injection (NEOSAR) from Adria Laboratories (Columbus, OH) and used for all the animal studies. (*R,S*)-Cyclophosphamide monohydrate (98+%, Aldrich Chemical Co., Milwaukee, WI), dissolved in Invenex bacteriostatic water for injection USP (LyphoMed, Inc., Rosemont, IL), was used for the

microsomal experiments. Both preparations were shown to be racemic mixtures by HPLC [1]. Chloral hydrate was obtained from Mallinckrodt, Inc. (Paris, KY). (+)-Naproxen [(+)-6-methoxy-α-methyl-2-naphthaleneacetic acid], 4-dimethylaminopyridine (99%), neutral activated Brockman type I aluminum oxide, oxalyl chloride (99+%, gold label), *N,N*-dimethylformamide (DMF, anhydrous, 99+%, gold label), and dichloromethane (anhydrous, 99+%, gold label) were obtained from the Aldrich Chemical Co. Benzene (thiophene free, 99 mol% pure), and HPLC grade solvents (ethyl acetate, dichloromethane, methanol, acetone, and acetonitrile) were obtained from the Fisher Scientific Co. (Fair Lawn, NJ), Silica gel spe cartridges (3 mL) were obtained from J.T. Baker (Phillipsburg, NJ). Other chemicals and buffer components were of reagent grade and obtained from various sources.

Preparation of derivatizing reagents

Anhydrous chloral. Chloral hydrate was dried and distilled as previously described [1]. Briefly, chloral hydrate crystals (25.0 g) were added to concentrated H_2SO_4 (15 mL) and mixed until dissolved. The resulting liquid chloral was separated after centrifugation for 10 min at 1000 *g* and washed with H_2SO_4 two additional times. The chloral was further purified by distilling the sodium twice (fractions boiling above 90° were collected). Anhydrous DMF was added to the freshly distilled chloral, such that the DMF concentration was 1% (v/v). This chloral–DMF solution was stored at −70° until used (this frozen reagent is stable for 3 months [1]).

Synthesis of (+)-naproxen acid chloride. (+)-Naproxen was converted from the free acid to the acid chloride form according to the procedure described by Reid *et al.* [1]. The chiral acid (1.0 g, 4.34 mmol) was added to a dry round-bottom flask protected from moisture with a calcium chloride drying tube. Dry benzene (2 mL) was added followed by oxalyl chloride (0.95 mL, 5.1 mmol) and the contents were stirred for 2 hr at ambient temperature. The round-bottom flask was then attached to a rotary evaporator (Buchi, Fort Lee, NJ) and solvents were removed under reduced pressure. The product, a pale yellow powder (m.p. 94–96%), was used without recystallization.

Pharmacokinetic experiments

Rabbits. Female New Zealand White rabbits (4.0 to 4.5 kg, Becker's Rabbitry, Centralia, KS) were housed in standard rabbit cages and given a restricted diet (6 oz./day) of high fiber Purina rabbit chow and water *ad lib.* For all experiments CP was dissolved immediately before use and administered as follows: for i.v. administration, 200 mg CP was dissolved in 6 mL sterile water and injected into an ear vein; for i.p. administration, 200 mg CP was dissolved in 10 mL sterile water and injected into the peritoneal cavity; oral administration was performed on fasted rabbits using 200 mg CP dissolved in 10 mL sterile water and delivered into the stomach with a rubber feeding tube (French size 8, Sherwood Medical Industries, Inc., St Louis, MO). Immediately after dosing, the animals were placed in plastic restrainers, and blood samples (1.8 mL) were taken from the medial artery of the ear, with syringes containing 0.2 mL of 0.1 M potassium oxalate to prevent clotting. The blood samples were centrifuged for 10 min at 1000 *g* to obtain plasma which was stored at −20° prior to analysis.

Two rabbits were used for the i.p. and i.v. dosing experiments. Rabbit No. 1 was treated initially by i.p. injection, and 8 weeks later it was treated by i.v. injection. Rabbit No. 2 was treated initially by i.v. administration, and 8 weeks later the i.p. administration was made. Two additional rabbits were used for the oral dosing experiments.

The sampling times following i.p. administration were 0, 4, 8, 12, 16, 20, 30, 40, 60, 90, 120, 150, 180, and 240 min. The sampling times following i.v. infusion were identical except for an additional sample taken at 1 min after dosing. The sampling times following oral administration were 0, 4, 8, 12, 16, 20, 25, 35, 45, 60, 90, 120, and 150 min.

The extent of stereoselectivity in the disposition of the CP enantiomers following each route of administration was assessed by calculating the total area under the plasma concentration–time curve (AUC) and the elimination half-life ($T_{1/2,\beta}$) for each enantiomer. The data expressing the decline in the plasma concentration (C_p) of the CP enantiomers with time (t), following i.v. administration, were fitted to a two-compartment open model (equation 1) by least-squares linear regression, using the method of residuals for the determination of A and α:

$$C_p = Ae^{-\alpha t} + Be^{-\beta t} \quad (1)$$

and the AUC was determined using the equation

$$\text{AUC} = A/\alpha + B/\beta \quad (2)$$

where the concentrations (A,B) and the rate constants (α,β) are the coefficients of the regression analysis. The elimination half-life was calculated from the expression

$$T_{1/2} = 0.0693/\beta. \quad (3)$$

The AUC for the data obtained following the i.p. and oral administrations were calculated using the trapezoidal rule. The AUC from the last time point to infinity was approximated by C_t^*/k where C_t^* is the last measured plasma concentrations. The elimination rate constant, k, was determined by linear regression analysis of the terminal slope. The elimination half-life was determined from the equation

$$T_{1/2} = 0.693/k. \quad (4)$$

The absolute bioavailability of each enantiomer, following i.p. administration, was calculated by dividing the $AUC_{i.p.}$ value by the $AUC_{i.v.}$ value and expressing the result as a percentage.

Human samples. Plasma samples were obtained from nine patients treated for various neoplastic diseases at the Veterans Administration Hospital (Kansas City, MO). The treatment regimens and blood collection times are summarized in Table 1. The plasma samples were stored at −20° until analyzed.

Microsomal studies

Microsomes were prepared from rabbit liver as

Table 1. Dose and blood collection times of cancer patients receiving (*R*,*S*)-CP by i.v. injection*

Patient No.	Sex	Dose (mg)	Other chemotherapy	Blood collection times (min)
1	M	614	Adriamycin®	Pre-dose; 0, 7, 20
2	F	700	Adriamycin®	Pre-dose; 5, 15, 30, 120
3		†	Adriamycin®	Pre-dose; 15, 30, 60, 120
4	F	800	Dactinamycin	Pre-dose; 5, 15, 30
5	M	1270	Adriamycin®	Pre-dose; 5, 15, 30, 60, 120, 180, 240
6	F	550	Adriamycin®	Pre-dose; 5, 15, 30, 120
7	M	700	Adriamycin®	Pre-dose; 10, 20, 30, 60, 120, 180, 240
8	M	1040	VP-16	Pre-dose; 0, 5, 15, 30, 60
9	M	1200	Adriamycin®	Pre-dose; 5, 15, 30

* Each patient received a dose equivalent to 500 mg/m^2.
† Dose and sex are unknown.

described by Burstein and Kupfer [15]. The liver was removed, washed in ice-cold distilled water, and kept on ice prior to homogenization in ice-cold 0.25 M sucrose (5 mL/g tissue). Subsequent steps and centrifugations were performed on ice or at 4°. The homogenate was transferred to screw-cap tubes and centrifuged for 20 min at 10,000 *g* in a Beckman model J2-21 centrifuge (Beckman Instruments, Palo Alto, CA). The supernatant fraction was then centrifuged for 60 min in a Beckman L5-65 ultracentrifuge at 105,000 *g*. The supernatant fraction was discarded, and the pellet (microsomal fraction) was washed by re-suspension in 1.15% aqueous KCl followed by an additional centrifugation for 60 min at 105,000 *g*. The supernatant fraction was discarded, and the microsomal pellet was covered with fresh 1.15% KCl and stored at −70°. Prior to use the microsomes were thawed and resuspended in fresh KCl solution. Protein determinations were made by modification [16] of the method of Lowry *et al.* [17], using bovine serum albumin as a standard. Cytochrome P450 determinations were carried out as described by Omura and Sato [18].

Incubations were carried out in 20-mL glass scintillation vials containing the following components: 200 μg (*R*,*S*)-CP added in 0.05 mL of potassium phosphate buffer (0.1 M, pH 7.4); 1.5 mg microsomal protein added in 0.2 mL from a suspension in 1.15% aqueous KCl; 0.55 mL of a solution containing 0.45 mL of potassium phosphate buffer (0.1 M, pH 7.4) and 0.1 mL of an aqueous solution of $MgCl_2$ (0.1 M); and 0.1 mL of H_2O for a final volume of 0.9 mL. The reaction vials were incubated for 2 min in a shaking water bath (37°) prior to addition of 0.1 mL of an NADPH-regenerating system (glucose-6-phosphate, 38.3 μmol; NADPH, 1.2 μmol; and glucose-6-phosphate dehydrogenase, 3 I.U.) in water. Subsequently, duplicate vials were removed from the water bath at 0, 5, 10, 15, 30, 45, and 60 min, and the reaction was terminated by the addition of 4 mL ethyl acetate.

Control incubations contained either (a) the complete system containing boiled microsomes (10 min at 100°) analyzed at time zero, (b) the complete system containing boiled microsomes analyzed after the 60-min incubation, or (c) intact microsomes with a complete system but lacking the NADPH-regenerating system and analyzed after the 60-min incubation. A sample containing only plasma and (*R*,*S*)-CP and incubated for 60 min was also included in order to compare the CP enantiomer extraction efficiency from microsomes with that from plasma.

Sample preparation

Solvent extraction and derivatization. Cyclophosphamide was extracted from 1 mL of plasma or the microsomal incubation medium with 4 mL of ethyl acetate. The derivatization (Fig. 1) of the enantiomers was performed as described previously by Reid *et al.* [1] with the following modifications: (a) the amidoalkylation of the CP enantiomers in the plasma extract with anhydrous chloral was conducted at room temperature rather than 20°, and (b) the elution of the CP-chloral derivative from the silica cartridge was preceded by a wash with 1 mL dichloromethane:methanol (49:1). The derivatized extracts were dissolved in 1 mL of the chromatographic mobile phase [acetonitrile:potassium phosphate buffer (10 mM, pH 6.65):methanol (50:35:15, by vol.)], and 20 μL was analyzed by HPLC.

Standard curves. Three milligrams of (*R*,*S*)-CP (Neosar formulation) was dissolved in buffered saline (0.1 M KCl + 0.05 M potassium phosphate, pH 7.4) at a final concentration of 1.5 mg/mL (a substock solution of 0.1 mg/mL was also prepared). A standard curve was prepared at concentrations (with respect to total CP) of 150, 100, and 50 μg/mL from the 1.5 mg/mL stock, and 10 and 5 μg/mL from the 0.1 mg/mL stock. Solutions were adjusted to 100 μL with buffered saline followed by the addition of 900 μL plasma (rabbit or human) to give a final volume of 1 mL. The standards were prepared and processed along with each set of animal and human samples.

Chromatography

The derivatized CP enantiomers were determined by high-performance liquid chromatography using a Waters model M-6000A pump, a model 712 WISP auto-sampler (Waters Associates, Milford, MA) equipped with a Kratos Spectroflow 783 variable

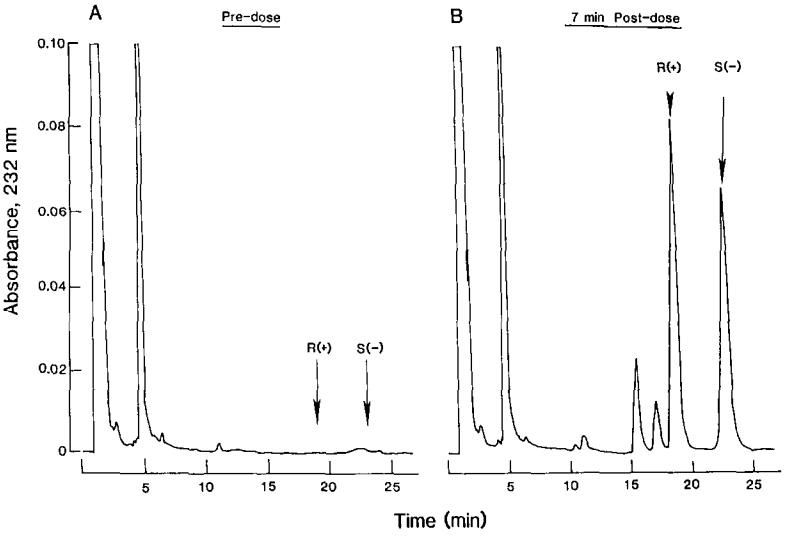

Fig. 2. HPLC chromatograms of derivatized CP enantiomers from human plasma obtained from a cancer patient treated with 614 mg of CP by i.v. infusion. (A) Plasma sample obtained prior to dosing. (B) Plasma sample obtained at 7-min post-dose. Retention times: *R*-CP = 18.8 min; *S*-CP = 22.9 min. The concentrations of both *R*- and *S*-CP were 31 μg/mL.

wavelength UV/VIS absorbance detector (Kratos Analytical Instruments, Ramsey, NJ) and a Shimadzu C-R3A chromatopac computing integrator (Shimadzu, Kyoto, Japan). The analytical column (150 × 4.6 mm) contained 5 μm octadecyl-bonded silica (Shandon Hypersil ODS, Keystone Scientific, Inc., State College, PA) packed by an upward slurry technique [19]. Samples were eluted with a mobile phase of potassium phosphate buffer (10 mM, pH 6.65) : acetonitrile : methanol (35 : 50 : 15, by vol.) at a flow rate of 1.3 mL/min, detected by UV absorbance at 232 nm, and quantified by peak height measurements.

RESULTS AND DISCUSSION

In the present study, the HPLC assay developed in this laboratory for the measurement of CP enantiomers in plasma was applied to pharmacokinetic studies in rabbits and humans as well as studies on the differential metabolism of CP enantiomers by rabbit liver microsomes. As shown in Fig. 2, derivatized plasma extracts from the blood samples of a cancer patient, taken before treatment and at 7 min after i.v. infusion of (*R*,*S*)-CP, produced an HPLC chromatogram that showed excellent separation of the derivatized enantiomers and was relatively interference-free. Some interfering material, which co-eluted with the *S*-CP diastereomer, was observed in the extract from plasma obtained prior to CP administration. This interfering material was also present in extracts from buffered aqueous solutions and appears to be a by-product of the derivatization process. The interference was always present at a constant level (equivalent to 0.5 μg/mL of the *S*-CP enantiomer) and resulted in a slight intercept in the calibration curve for *S*-CP.

Calibration curves (Fig. 3), based on peak heights and enantiomer concentrations, were constructed over the concentration range of 2.5 to 75 μg/mL. Duplicate samples were prepared at each of six concentrations, and the peak height values for each enantiomer were averaged. The peak height responses were linear over this concentration range with coefficients of determination (r^2) of 0.998 observed for both the *R*- and *S*-CP derivatives. The overall efficiency of the extraction and derivatization was found to be approximately 38%. To minimize errors due to any variation in the overall efficiency of the process, standard curves were always constructed with each of samples.

Figure 4 shows the plasma CP enantiomer concentration–time curves for two rabbits following the i.v. and i.p. administrations. The AUC and $T_{1/2,\beta}$ values obtained from these curves are shown in Table 2. Figure 4 shows that for rabbit No. 1, following i.p. administration, the observed plasma concentration of *R*-CP was consistently greater than the *S*-CP concentration. The *R*-CP : *S*-CP ratio increased from 1.8 : 1 to 2.5 : 1 during the course of this experiment (Fig. 5). The higher concentrations of *R*-CP are also reflected in the $AUC_R : AUC_S$ ratio (2.5 : 1) (Table 2). Following i.v. administration to this rabbit, the first plasma sample analyzed showed that the enantiomer concentrations were identical but after 1 hr the *R*-CP : *S*-CP ratio had increased to 2.7 : 1. This increase in the *R*/*S* ratio was apparently due to an elimination half-life of the *S* enantiomer that was twice that for the *R* enantiomer, 16 min versus 30 min, respectively (Table 2). The studies with rabbit No. 2 yielded differences in the plasma concentrations and elimination half-lives of *R*- and *S*-CP that were not as pronounced as with rabbit No. 1 for both routes of administration (Figs. 4 and 5 and Table 2). However, for the i.p. route of administration, the *R*/*S*-CP ratio throughout the analysis

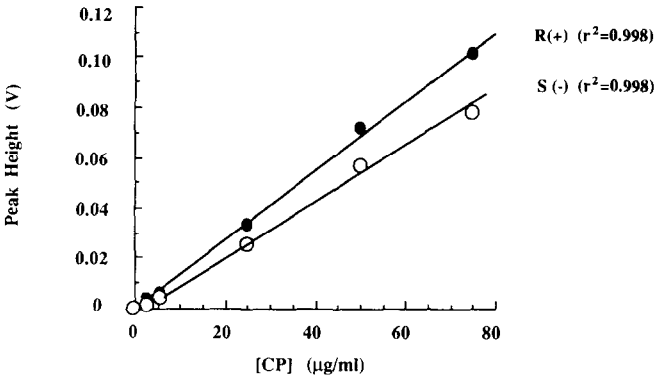

Fig. 3. Calibration curves for (*R*,*S*)-CP extracted from human plasma. Total CP concentrations ranged from 5 to 150 μg/mL in spiked plasma samples. Extraction and derivatization of CP enantiomers were as described in Materials and Methods.

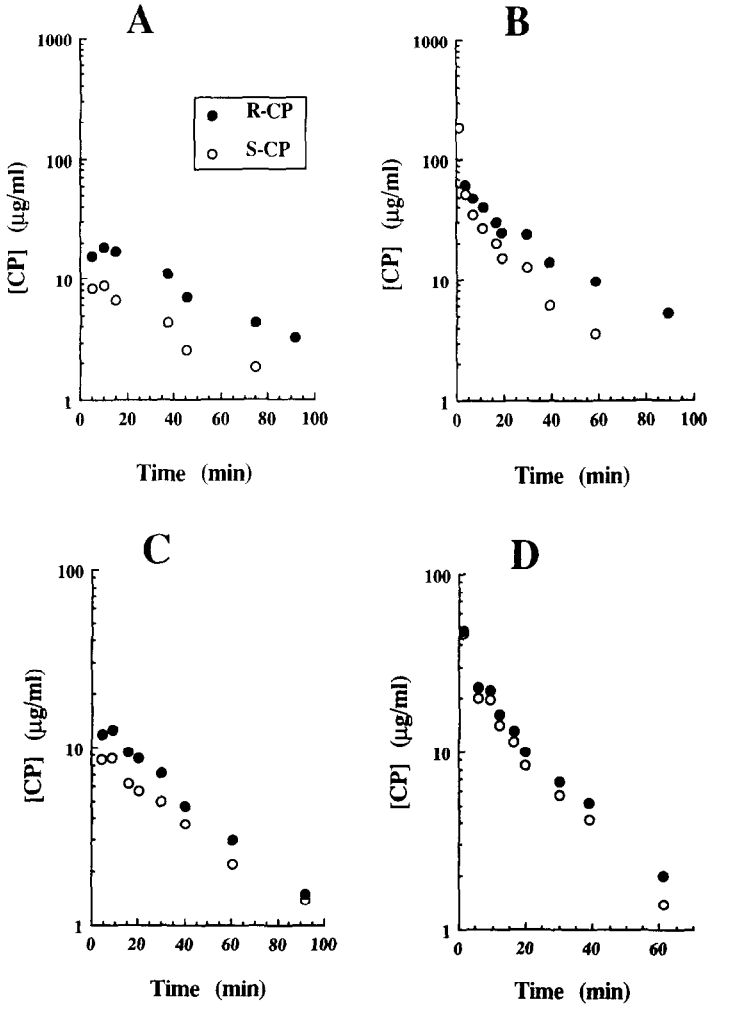

Fig. 4. Plasma concentration–time profile of *R*- and *S*-CP for two rabbits (No. 1 and No. 2) dosed either i.v. or i.p. with (*R*,*S*)-CP at 45 mg/kg. Panels A and B are the profiles observed with rabbit No. 1 following i.p. and i.v. administrations respectively (i.v. administration was given 8 weeks after i.p. treatment). Panels C and D are the profiles observed with rabbit No. 2 following i.p. and i.v. administrations respectively (i.v. administration was given 8 weeks before i.p. treatment). Dosing and blood collection procedures are given in Materials and Methods.

Table 2. Pharmcokinetic parameters obtained following i.p. and i.v. administration of (*R*,*S*)-CP to two rabbits*

	i.v. Administration			i.p. Administration			
	AUC ($\mu g \cdot mL^{-1}) \cdot min$	$T_{1/2,\beta}$ (min)	$AUC_R : AUC_S$	AUC ($\mu g \cdot mL^{-1}) \cdot min$	$T_{1/2,\beta}$ (min)	$AUC_R : AUC_S$	Bioavailability† (%)
Rabbit No. 1							
R	2112	30	2.0	964	29	2.5	46
S	1077	16		392	29		36
Rabbit No. 2							
R	670	17	1.1	541	26	1.4	81
S	589	15		374	26		63

* Rabbit No. 1 was treated with (*R*,*S*)-CP by i.p. administration initially and by i.v. administration 8 weeks later. Rabbit No. 2 was treated with (*R*,*S*)-CP by i.v. administration initially and by i.p. administration 8 weeks later.

† Bioavailability = $(AUC_{i.p.}/AUC_{i.v.}) \cdot 100$.

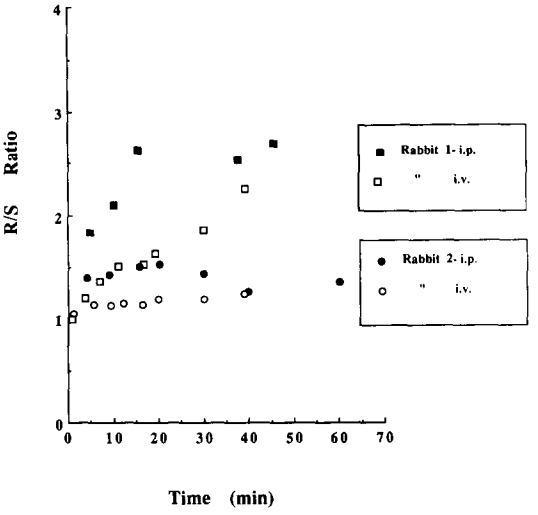

Fig. 5. Time profile of the changes in the enantiomeric ratio (*R*-CP/*S*-CP) in rabbits No. 1 and No. 2 following the i.p. and i.v. treatments shown in Fig. 4.

with both rabbits was greater than the ratio obtained after i.v. administrations of (*R*,*S*)-CP, with the largest difference observed between 5 and 30 min (Fig. 5). The significance of the stereoselective differences observed in these studies will require a larger study involving more rabbits and the use of a cross-over administration technique.

The i.p. bioavailabilities of *R*-CP and *S*-CP were 46 and 36%, respectively, in rabbit No. 1 (Table 2). In rabbit No. 2 the i.p. bioavailabilities of both enantiomers were substantially higher; on the other hand, the bioavailability of *R*-CP was again greater than that observed for *S*-CP (81% for *R*-CP and 63% for *S*-CP; Table 2). Of greater interest, however, are the values of the ratio of the areas under the curve ($AUC_R : AUC_S$) following i.p. administration which were greater than the value observed following i.v. administration, in both animals.

Because the disposition of the CP enantiomers, following i.p. administration, proceeded with some gree of stereoselectivity, oral-dosing experiments were conducted. Two rabbits were used in this experiment, and the results are shown in Fig. 6. This figure shows the presence of only the *R* enantiomer which reached a maximum concentration of 3–4 μg/mL after approximately 30 min and thereafter decreased to levels below the limit of detection (0.5 μg/mL) after 2.5 hr. The *S* antipode was detectable only in the region of the highest concentrations of the *R* enantiomer, which were 3–4 μg/mL at 25–50 min after administration. The concentrations of the *S* enantiomer in plasma following oral administration were not reported because they were below the limit of quantitation (0.5 μg/mL). These values for the maximum concentrations of *R*-CP and *S*-CP correspond to a minimum ratio of the concentration of the two enantiomers of 6:1. These results suggest a large stereoselective effect in the first-pass metabolism of (*R*,*S*)-CP which was similar but more dramatic than the stereoselectivity observed following

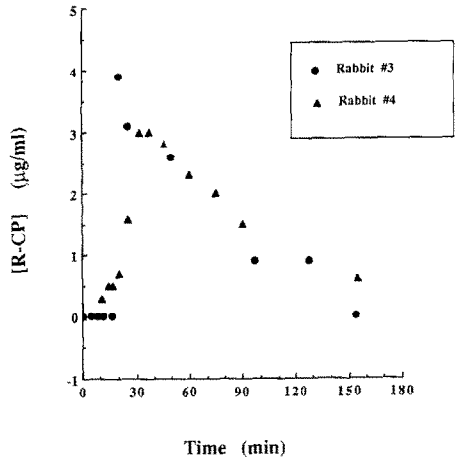

Fig. 6. Plasma concentration–time profile of *R*-CP in two rabbits (No. 3 and No. 4) following oral administration of 45 mg/kg of (*R*,*S*)-CP. *S*-CP was detectable in samples obtained between 25 and 50 min in each rabbit, but the concentration was not quantified because the level was below the limit of quantitation.

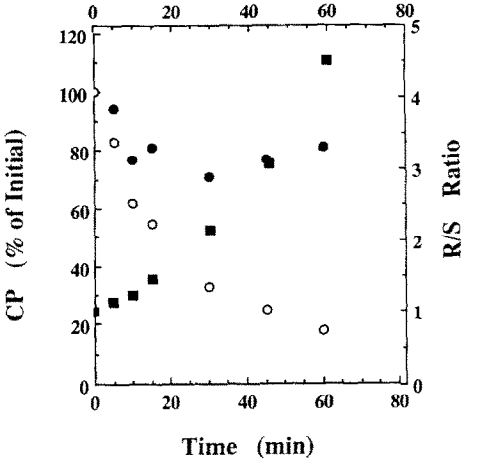

Fig. 7. Stereoselectivity in the metabolism of (*R*,*S*)-CP by rabbit liver microsomes. Microsomal protein (1.5 mg) was incubated with 200 μg of (*R*,*S*)-CP at 37° as described in Materials and Methods. Each value is the mean of duplicate determinations. Key: (●) *R*, (○) *S*, and (■) *R*/*S* ratio.

the i.v. and i.p. dosing experiments. The AUC values for *R*-CP for each rabbit 271 and 265 ($\mu g \cdot mL^{-1}$)·min, while the $T_{1/2,\beta}$ values were 48 and 49 min. The variability in the disposition of *R*-CP between these two rabbits was less than in the animals used for the i.p. and i.v. dosing experiments.

It was not possible to assess the absolute oral bioavailability of the *R*-CP isomer in these two rabbits since they did not receive i.v. doses. A full oral biovavailability study is being initiated and the results will be presented at a later date. Nevertheless, the presence of dramatic stereoselectivity in the handling of (*R*,*S*)-CP following i.p. and oral administration and the demonstration that CP is initially metabolized by the hepatic cytochrome P450 monooxygenases [4], which have been shown to be stereoselective in the metabolism of some chiral substrates [10], suggest that these hepatic enzymes may be involved in the stereoselective metabolism of CP. This possibility was examined in a study by Cox *et al.* [14] who reported that rabbit liver microsomes exhibit a marked stereoselctive preference for metabolism of the *S*-enantiomer over the *R*-enantiomer of CP after incubation of liver microsomes with a pseudoracemic mixture of the CP enantiomers. To determine if our chiral derivatization assay would yield results similar to those of Cox *et al.* [14], who had used mass spectrometry to quantitate enantiomeric differences, a similar study was performed with sample analysis by our methodology.

It was observed that the *S*-CP enantiomer was preferentially metabolized at each time point of the experiment (Fig. 7). While the percentage of *R*-CP in the reaction mixture has decreased 29% after 30 min, after which no further metabolic conversion was observed*, the percentage of *S*-CP in the reaction mixture had decreased by 67% after 30 min and continued to decrease such that only 20% remained after 60 min, at which point the experiment was terminated. At the end of the incubation (1 hr), the *R*-CP:*S*-CP ratio was 4.5:1. The various control incubations included in this experiment showed no changes in the concentrations of derivatized CP enantiomers indicating that (a) no degradation of the enantiomers occurred during the 60-min incubation, (b) NADPH was required for expression of enzymatic activity, and (c) *R*- and *S*-CP were equally extractable from both the microsomal suspension and plasma samples.

Following the rabbit experiments, plasma samples obtained from nine cancer patients treated i.v. with (*R*,*S*)-CP were examined. Table 3 shows the CP enantiomer concentrations found in these patient samples at various time points between the end of drug administration (zero time) and up to 4 hr after drug administration. The concentration of each enantiomer decreased from 60 to 2 μg/mL during the 4-hr period. Between patients there was substantial variation in the enantiomer concentrations determined at each time point, with the greatest variation occurring at 30 min where both the *R*- and *S*-CP concentrations ranged from 3 to 17 μg/mL. However, Table 3 shows that the *R*-CP:*S*-CP ratio in each patient sample was close to 1:1 at all times, illustrating the lack of any stereoselectivity in the disposition of the CP enantiomers following i.v. administration of the racemate to humans. This finding is similar to the results of a previous study in which cancer patients were treated with the individual enantiomers as well as the racemic mixture by i.v. injection [13].

It should be noted that there have been no studies to date which have explored the stereoselectivity in the disposition of the CP enantiomers following oral administration to humans. In light of the data presented here using rabbits and because CP is also

* It has been shown by Tsui *et al.* [20] that no chiral inversion of the CP enantiomers occurs during *in vitro* incubations of liver microsomes with the individual enantiomers.

Table 3. *R*/*S* Ratios and concentrations of CP isomers in plasma samples obtained from nine cancer patients treated with cyclophosphamide

Patient		Concentrations of *R*- and *S*-cyclophosphamide (μg/mL) and the *R*/*S* ratio											
		Sampling times (min)											
		Pre-dose	0	5	7	10	15	20	30	60	120	180	240
1	*R*	*	32.9	†	30.8	†	†	9.8	†	†	†	†	†
	S	*	33.1	†	30.7	†	†	9.9	†	†	†	†	†
	R/*S*	*	0.99	†	1.00	†	†	0.99		†	†	†	†
2	*R*	*	†	*	†	†	†	†	†	130	5.5	†	†
	S	*	†	*	†	†	†	†	†	148	5.7	†	†
	R/*S*	*	†	*	†	†	†	†	†	0.88	0.96	†	†
3	*R*	*	†	*	†	†	11.0	†	8.6	7.2	6.5	†	†
	S	*	†	*	†	†	10.9	†	8.5	6.9	6.5	†	†
	R/*S*	*	†	*	†	†	1.01	†	1.01	1.04	1.00	†	†
4	*R*	*	†	*	†	†	17.0	†	13.4	†	†	†	†
	S	*	†	*	†	†	17.2	†	14.2	†	†	†	†
	R/*S*	*	†	*	†	†	0.99	†	0.94	†	†	†	†
5	*R*	*	†	*	†	†	18.1	†	17.4	13.3	9.5	8.0	2.3
	S	*	†	*	†	†	17.8	†	17.1	13.8	10.3	9.2	3.1
	R/*S*	*	†	*	†	†	1.02	†	1.02	0.96	0.92	0.87	0.74
6	*R*	*	†	*	†	†	*	†	*	†	6.2	†	†
	S	*	†	*	†	†	*	†	*	†	6.9	†	†
	R/*S*	*	†	*	†	†	*	†	*	†	0.90	†	†
7	*R*	*	†	†	†	5.6	†	5.3	4.1	3.6	3.3	2.7	2.0
	S	*	†	†	†	5.6	†	5.5	4.3	4.0	3.6	2.9	2.2
	R/*S*	*	†	†	†	1.00	†	0.96	0.95	0.90	0.92	0.93	0.91
8	*R*	*	*	*	†	†	13.2	†	11.5	6.4	†	†	†
	S	*	*	*	†	†	13.1	†	12.4	6.3	†	†	†
	R/*S*	*	*	*	†	†	1.01	†	0.93	1.02	†	†	†
9	*R*	*	†	60.0	†	†	8.9	†	3.1	†	†	†	†
	S	*	†	60.3	†	†	8.9	†	2.9	†	†	†	†
	R/*S*	*	†	1.00	†	†	1.00	†	1.07	†	†	†	†
Mean values													
	R	0.00	32.9	60.0	30.8	5.6	13.6	7.6	9.7	7.6‡	6.2	5.4	2.2
	(SEM)						(1.7)		(2.2)	(2.0)	(1.0)		
	S	0.00	33.1	60.3	30.7	5.6	13.6	7.7	9.9	7.8†	6.6	6.1	2.7
	(SEM)						(1.7)		(2.3)	(2.1)	(1.1)		
	R/*S*		0.99	1.00	1.00	1.00	1.00	0.99	0.98	0.97	0.94	0.89	0.81

* Samples with no detectable drug.
† Time points at which no sample was collected.
‡ Outlier values for patient 2 at 60 min were omitted from the analysis.

administered orally to cancer patients, it would be of great interest to investigate the disposition of *R*- and *S*-CP following this route of administration in humans. Studies addressing this question have been initiated in our laboratories.

In conclusion, the HPLC-based procedure described can easily quantify CP enantiomer levels in the plasma of humans and rabbits treated with (*R*,*S*)-CP. This method does not require use of deuterated analogs or expensive and time-consuming GC–MS and NMR methodologies. Additionally, this method has been shown to be sensitive and suitable for application to pharmacokinetic studies involving numerous subjects and samples.

Preliminary results from the experiments with rabbits indicate that (*R*,*S*)-CP undergoes significant stereoselective first-pass metabolism following i.p. and oral administration. Experiments with liver microsomes indicate that hepatic cytochrome P450 monooxygenases may be partially responsible for this stereoselectivity. However, the negigible levels of *S*-CP in plasma, compared with *R*-CP levels, following oral administration to rabbits suggest that the intestinal monooxygenases may also be involved. Because of the significant role that cytochrome P450 monooxygenases appear to play in the stereoselective disposition of CP enantiomers through their participation in the first-pass metabolism of this drug, further studies with cancer patients receiving oral doses of (*R*,*S*)-CP are needed to determine if the human hepatic monooxygenases exhibit similar stereoselectivity.

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REFERENCES

1. Reid JM, Stobaugh JF and Sternson LA, Liquid chromatographic determination of cyclophosphamide enantiomers in plasma by precolumn chiral derivatization. *Anal Chem* **61**: 441–446, 1989.
2. Friedman OM and Seligman AM, Preparation of N-phosphorylated derivatives of bis-β-chloroethylamine. *J Am Chem Soc* **76**: 655–658, 1954.
3. Arnold H and Bourseaux F, Sythese und Abbau cytostatisch wirksamer cydischer *N*-phosphamidester des bis-(β-chloroäthyl)-amins. *Angew Chem (Engl)* **70**: 539–544, 1958.
4. Sladek NE, Oxazaphosphorines. In: *Metabolism and Action of Anti-Cancer Drugs* (Eds. Powis G and Prough R), pp. 48–90. Taylor & Francis, Philadelphia, 1987.
5. Farmer PB, Enantiomers of cyclophosphamide and iphosphamide. *Biochem Pharmacol* **37**: 145–148, 1988.
6. Hall CR, Inch TD, Inns RH, Muir AN, Sellers DJ and Smith AP, Differences between some biological properties of enantiomers of alkyl *S*-alkyl methylphosphonothioates. *J Pharm Pharmacol* **29**: 574–576, 1977.
7. Ohkawa H, Mikami N and Miyamoto J, Stereoselectivity in metabolism of the optical isomers of cyanophenphos (*O*-*p*-cyanophenyl *O*-ethyl phenylphosphonothioate) in rats and liver microsomes. *Agric Biol Chem (Tokyo)* **41**: 369–376, 1977.
8. Cox PJ, Farmer PB, Jarman M, Jones M, Stec WJ and Kinas RW, Observations on the differential metabolism and biological activity of the optical isomers of cyclophosphamide. *Biochem Pharmacol* **25**: 993–996, 1976.
9. Yang SK, Stereoselectivity of cytochrome P-450 isozymes and epoxide hydrolase in the metabolism of polycyclic aromatic hydrocarbons. *Biochem Pharmacol* **37**: 61–70, 1988.
10. Waxman DJ, interactions of hepatic cytochromes P-450 with steroid hormones. Regioselectivity and stereospecificity of steroid metabolism and hormonal regulation of rat P-450 enzyme expression. *Biochem Pharmacol* **37**: 71–84, 1988.
11. Testa B, Substrate and product stereoselectivity in monooxygenase-mediated drug activation and inactivation. *Biochem Pharmacol* **37**: 85–92, 1988.
12. Eichelbaum M, Pharmacokinetic and pharmacodynamic consequences of stereoselective drug metabolism in man. *Biochem Pharmacol* **37**: 93–96, 1988.
13. Jarman M, Milstead RAV, Smyth JF, Kinas RW, Pankiewicz K and Stec WJ, Comparative metabolism of 2-[bis(2-chloroethyl)-amino]tetrahydro-2*H*-1,3,2-oxazaphosphorine-2-oxide (cyclophosphamide) and its enantiomers in humans. *Cancer Res* **39**: 2762–2767, 1979.
14. Cox PJ, Farmer PB, Jarman M, Kinas RW and Stec WJ, Stereoselectivity in the metabolism of th enantiomers of cyclophosphamide in mice, rats, and rabbits. *Drug Metab Dispos* **6**: 617–622, 1978.
15. Burstein S and Kupfer D, Hydroxylation of *trans*-Δ^1-tetrahydrocannabinol by hepatic microsomal oxygenase. *Ann NY Acad Sci* **191**: 61–67, 1971.
16. Stauffer CE, A linear standard curve for the Folin Lowry determination of protein. *Anal Biochem* **69**: 646–648, 1975.
17. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin Phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
18. Omura T and Sato R, The carbon monoxide-binding pigment of liver microsomes. II. Solubilization, purification, and properties. *J Biol Chem* **239**: 2379–2385, 1964.
19. Bristow PA, Brittain PN, Riley CM and Williamson BF, Upward slurry packing of liquid chromatography columns. *J Chromatogr* **131**: 57–64, 1977.
20. Tsui F-P, Brandt JA and Zon G, Effects of enantiomeric homogeneity on the *in vitro* metabolism and *in vivo* anticancer activity of (+)- and (−)-cyclophosphamide. *Biochem Pharmacol* **28**: 367–374, 1979.