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Chiral analysis of drug substance in clinical plasma extracts using achiral HPLC with circular dichroism detection

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Abstract

A circular dichroism (CD) detector in series with a UV detector was used with reversed-phase gradient HPLC to do the chiral analysis of unresolved enantiomers of a single stereoisomer drug substance in extracts of clinical plasma samples. The CD/UV peak area ratio of the unresolved enantiomers in the plasma extracts was calculated and compared with pure drug substance standards to show that there was no racemization of the chiral center during pharmacokinetic studies. The CD detector can be used only with chiral compounds with a UV chromophore and the limit of detection was approximately 5 ng with the drug substance and chromatographic conditions used in this study.

Keywords: Circular dichroism; HPLC detector; Chiral analysis; Pharmaceutical; Metabolism; Pharmacokinetics

1. Introduction

Chiral compounds can exist in two enantiomeric forms, both of which have the same physical and chemical properties in an achiral environment, but frequently have different biological properties [1]. Most chiral compounds are now being developed as single stereoisomer compounds because of difficulties in development of a racemate in drug metabolism, pharmacokinetic and safety assessment studies [2,3]. Since the unwanted enantiomer is considered an impurity, chiral analysis of metabolized samples is needed if significant racemization of the chiral center occurs during metabolism. For this reason, regulatory guidelines suggest that a chiral analysis of a single stereoisomer drug substance be done early in the phase I clinical patient studies in order to establish the stability of the chiral center during metabolism and pharmacokinetic studies[4–6].

Chiral analysis of pharmaceutical compounds is usually done with chiral separation techniques where enantiomers are separated and individually measured. Chiral HPLC is the most widely used

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method for chiral analysis in metabolism studies. However, method development of chiral separations with sufficient sensitivity and selectivity for analyzing the low levels of drug and enantiomer in plasma or microsomal incubation extracts can be challenging and time consuming. A simple achiral reversed phase separation coupled to a sensitive chiral detector is a technique, which potentially would require less method development time in early clinical studies. Chiral detection is based upon the interaction of polarized electromagnetic radiation with a chiral center in the analyte [7]. Chiroptical methods include polarimetry, optical rotary dispersion (ORD) and circular dichroism (CD). While polarimetry and ORD are based upon refractive index differences [8] between right circularly polarized light (RCP) and left circularly polarized light (LCP), CD detection depends on the UV absorbance difference between RCP and LCP [9]. CD is more stable and sensitive than either ORD or polarimetry although it is necessary to have a UV absorbing chromophore in the chiral molecule [9].

Polarimetric and ORD detectors for HPLC have been available but lack the sensitivity [10] necessary for most metabolism studies. CD spectropolarimeters with flow-through cells for use as HPLC detectors have been investigated and described by Yeung and Synovec [11], Westwood et al., [12], Takahashi et al., [13], Horvath et al., [14] and Szokan et al., [15]. Recently, a new CD detector specifically designed for HPLC and with improved sensitivity has become commercially available [16]. This detector can be used with gradient elution of aqueous and organic mobile phases for chiral analysis and should require less method development than chiral HPLC. The purpose of this study was to evaluate this CD detector with reversed-phase gradient HPLC as a convenient technique for the chiral analysis of plasma sample extracts in phase I clinical studies.

2. Experimental

2.1. Equipment

A model CD-995 detector for HPLC from Jasco Inc. (Easton, MD) was used for chiral analysis of plasma extract samples. The detector has a Hg–Xe lamp and can be used for CD detection in the 220–420 nm UV wavelength range. This detector has autoscanning capabilities for determining the best wavelength for optimal signal to noise CD response. The detector was used in series with a Hewlett Packard (Wilmington, DE) model 1100 HPLC system equipped with an autosampler, gradient elution and a variable wavelength UV detector. Limited volume (300 μl) glass inserts from Hewlett Packard were used in the autosampler vials. Fisons Multichrom software was used with a VAX6000 computer for the data reduction and generation of chromatograms. Waters (Milford, MA) Symmetry C18 columns (150×3.9 mm and 150×2.1 mm with 5 μ size particles) were used for the separation of standards and plasma extracts. A Chirobiotic V column, 250×4.6 mm ID, from ASTEC (Whippany, NJ) was used for the chiral HPLC.

2.2. Chromatographic conditions

A 10 min reversed-phase HPLC gradient on a Waters Symmetry C18 column, 150 by 3.9 mm ID, was used for separation and analysis of the DPC 961 in the clinical plasma extracts. The chromatographic conditions are listed in Fig. 2. Similar conditions were used for the high sensitivity analysis except that a Waters Symmetry C18 column, 150 by 2.1 mm, was used with conditions listed in Fig. 5.

A chiral HPLC separation on a Chirobiotic V column, 250 by 4.6 mm ID, with a UV detector was also developed for chiral analysis of the plasma extracts. The chromatographic conditions are listed in Fig. 4.

2.3. Chemicals

HPLC grade methanol (MeOH), chlorobutane, and acetonitrile (ACN) were obtained from VWR corporation (South Plainfield, NJ). Trifluoroacetic acid (TFA), HPLC grade was obtained from Sigma Chemical (St. Louis, MO).

DMP 961 (structure in Fig. 1) is a development drug of the DuPont Pharmaceutical Company (Wilmington, DE). It is a nonnucleoside reversed transcriptase inhibitor used in the treatment of HIV infection. It has a molecular weight of 314.7, a water solubility of 10 μg/ml, and a UV molar absorptivity of 1.5×10^4 l/M cm at 250 nm. DMP 961 has one chiral center and is in the S configuration; the R enantiomer is maintained at less than 0.05% in the synthesis of the drug substance. A standard of the R enantiomer was obtained from the Chemical Process Department of the DuPont Pharmaceutical company.

2.4. Plasma extraction procedure

A 500 μl aliquot of clinical patient plasma sample (subjects were administered multiple oral doses of DPC 961 and plasma samples were withdrawn after 3–9 days of dosing) was mixed with 0.1 ml of 0.01 N NaOH and 5 ml of chlorobutane, vortexed for 20 min, and then centrifuged at 2000 rpm for 10 min. The plasma was frozen with dry ice and the organic layer transferred to dry tubes and evaporated to dryness with N_2 at 40°C. The extracts were reconstituted by vortexing the glass tubes with 300 μl of 50:50 water/MeOH. The reconstituted samples were transferred to limited volume inserts in the autosampler vials. Aqueous standards of DMP 961 in the same concentration range as in the plasma samples were also extracted and handled with the same procedure as the plasma extracts.

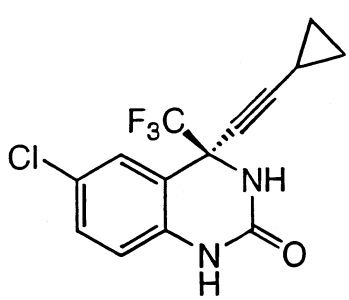

Fig. 1. Structure of DPC 961.

3. Results and discussion

3.1. CD detection and chiral analysis

Enantiomers of a compound with a single chiral center, such as DPC 961, will have CD peaks that are equal in area but opposite in direction [9] as shown in the reversed-phase gradient chromatograms in Fig. 2. The CD response of 50:50 racemate of the S enantiomer (DPC 961) and the R enantiomer would cancel and be zero. At the same time, the peak area response of a UV absorbance detector in series with the CD detector would be the same for drug, enantiomer and the racemate since the UV spectra are identical for the three. A ratio of the CD peak area/UV peak area can then be used for chiral analysis of drug substance samples [7]. A plot of the CD/UV ratio versus DPC 961 and R enantiomer composition is shown in Fig. 3. The plot is linear and can be used as calibration curve to determine the enantiomeric composition of the drug substance peak. A single point calibration curve can then be made by measuring the CD/UV ratio for the drug substance standard (100% S enantiomer) and designating the zero intercept to be the 50:50 mixture of R and S enantiomer. This calibration curve can then be used for the chiral analysis of drug substance peak to determine if racemization of the chiral center has occurred in metabolism or pharmacokinetic studies.

Samples and standards must be within the linear range of both detectors to obtain an accurate CD/UV calibration curve. A Beer–Lambert's law plot of DPC 961 concentration versus CD peak area response was found to be linear only to 1 μg of DPC 961 injected on column while the UV detector was linear to over 6 μg under the same conditions. The CD/UV calibration curve is made using three aqueous DPC 961 standard samples that are within the 1 μg linear concentration range of the CD detector; the CD/UV ratios of these three standards are averaged to give the single point CD/UV calibration curve. If the amount of DPC 961 injected on column is expected to exceed the 1 μg linear range of the CD detector, then it may be necessary to inject smaller volumes of sample or to reduce the volume of plasma used in the extraction.

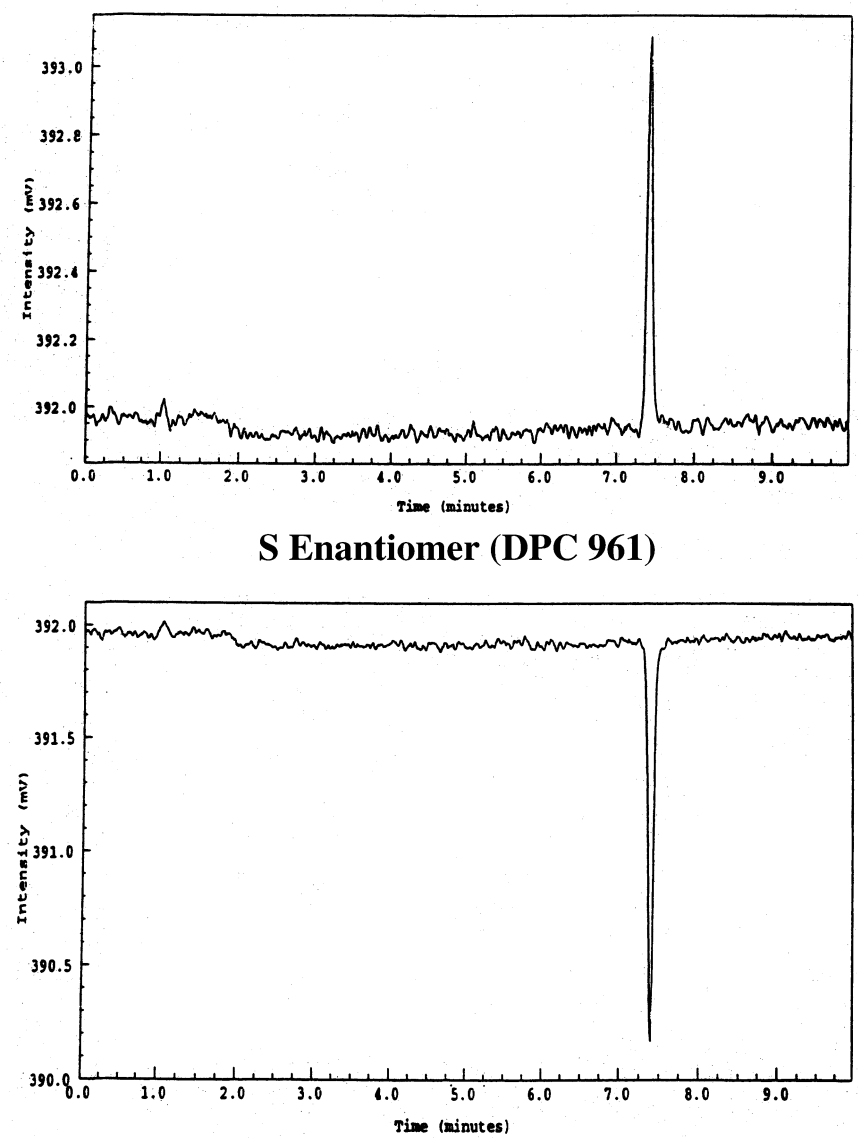

Fig. 2. Reverse-phase gradient HPLC separation of S enantiomer (DPC 961) and R enantiomer with CD detection. Chromatographic conditions: Symmetry C18 column 150 × 3.9 mm; mobile phase A = 0.05% aqueous TFA; B = ACN linear gradient 10–80% B in 10 min; flow rate of 1.5 ml/min; temperature of 35°C; CD detection at 240 nm; 50 ng of each enantiomer.

The optimum CD wavelength for maximum signal to noise response is not necessarily the same wavelength of maximum UV absorbance [7]. The optimum CD wavelength can be determined by stopping the mobile phase flow with the drug substance peak in the detector flow cell. The detector can then scan the CD spectra to find the wavelength maxima in the 220–420

nm wavelength limit of the detector. For DPC 961, the CD maxima was determined to be 240 nm while the UV maxima is 250 nm.

3.2. Chiral analysis of plasma extracts

The CD/UV calibration curve was established with three aqueous DPC 961 standards of 1000, 2500 and 5000 ng/ml concentration. A 0.50 ml aliquot of each standard was extracted and reconstituted with 300 μl of sample solvent as outlined in the experimental section. A 100 μl volume of each reconstituted standard and extract was injected and analyzed. Ten clinical plasma samples, two blank plasma samples and two blank plasma spiked with DPC 961 were also extracted using the same procedure. The analysis of all standards and samples was accomplished in one overnight run with the chromatographic conditions outlined in the experimental section. The blank plasma extracts showed that there were no plasma interferences for the DPC 961 analysis. The CD/UV peak area ratio was then calculated for each of three standards of the single stereoisomer drug substance (S enantiomer) and then averaged and used to construct the single point CD/UV calibration curve. The CD/UV ratio for the drug substance peak in each plasma extract sample was calculated and the chiral composition of each drug substance peak was then determined with the calibration curve. Five replicate injections of a 900 ng aqueous standard were also analyzed to determine that the system precision of the method was 0.5% R.S.D. at these conditions. The clinical plasma samples were taken from patients at 3 to 9 days after administration of drug and ranged from 1920 to 2942 ng/ml in drug substance concentration. After extraction and reconstitution this corresponded to 319–490 ng injected on column and was within the linear range of the CD detector.

The chiral compositions of the drug substance peak in the ten clinical plasma samples are listed in Table 1 and were averaged to give a composition of 100.5% S enantiomer (DPC 961) with a 2.7% RSD. The chiral compositions of the drug substance peak in the two spiked plasma samples were also averaged to give a value of 101.0% S enantiomer (DPC 961). The

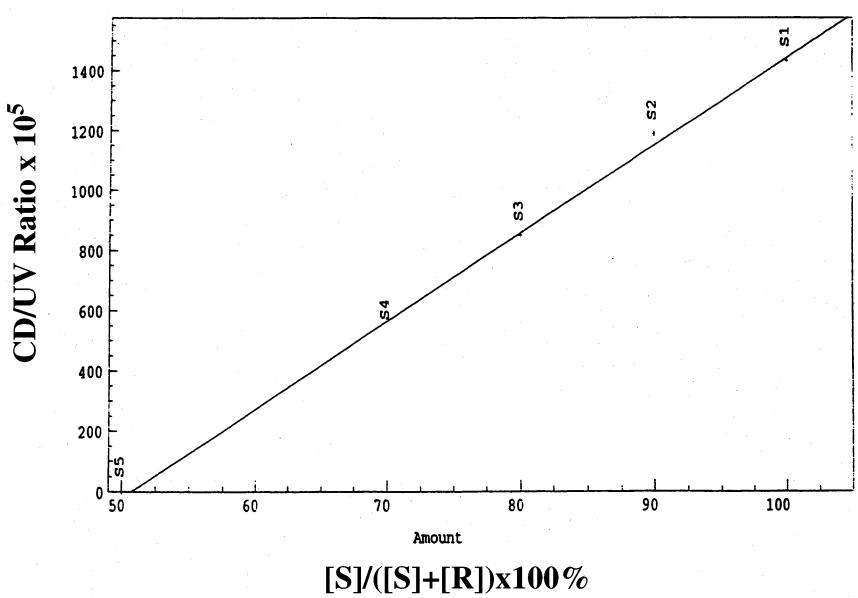

Fig. 3. Plot of CD/UV peak area ratio versus S enantiomer (DPC 961) and R enantiomer composition. Same chromatographic conditions as Fig. 2.

Table 1
Chiral analysis of drug substance peak in clinical plasma extracts

Sample			% S enantiomer (DPC 961) by CD/UV analysis
Patients	Days	Time (h)	
A	5	12	99.3
A	6	0	101.4
B	8	10	96.0
B	9	0	100.3
C	4	5	99.6
C	5	12	100.0
D	5	12	100.0
D	8	10	98.9
E	3	10	103.6
E	4	0	106.1
Mean			100.5
R.S.D.			2.7

results show that there was no racemization of the chiral center in DPC 961 drug substance during metabolism. This was confirmed by doing a chiral HPLC separation and analysis of the same clinical plasma extracts with the chiral chromatographic conditions out-lined in the experimental section. Chiral HPLC chromatograms of standards and a plasma extract are shown in Fig. 4. This chiral HPLC analysis confirmed that there was no measurable amount of the R enantiomer in any of the clinical plasma samples.

The clinical plasma samples contained from 319 to 490 ng of DPC 961 injected on column, which is well above the limit of quantitation (LOQ) of the UV and CD detectors. In order to challenge the sensitivity of the CD detector, 5 μl volumes of these same standards and clinical extract samples were injected and analyzed with the high sensitivity chromatographic conditions listed in the experimental section. These samples contained from 16 to 24 ng of the drug substance. The CD and UV chromatograms of an extract containing 24 ng drug substance are shown in Fig. 5 and the signal to noise response is close to the LOQ of the CD detector for DPC 961. The CD/UV peak area ratios were calculated and the calibration curve used to determine the chiral composition of the drug substance peak. The results of the ten samples were averaged to show the peak contained at least 97.5% of the S enantiomer (DPC 961). The RSD of these measurements was 4.9%. The system precision for five replicate injections of a 25 ng aqueous standard was determined to be 5.2% RSD. The results again show that there was no measurable racemization of the chiral center within the precision of the method. The CD/UV detection can be used for chiral analysis even with samples that approach the LOQ of the CD detector. The LOQ and LOD of the CD detector will depend on the CD response of the analyte as well as the chromatography. The LOD of the CD detector for DPC 961 at 240 nm was approximately 5 ng injected on column with the chromatographic conditions in this study. The LOD for the UV detector at 250 nm was approximately 0.2 ng under the same conditions.

4. Conclusions

Availability of a CD detector specifically designed for HPLC with improved sensitivity has led to development of reversed phase HPLC methods with tandem detection (CD/UV) for chiral analysis of UV absorbing drug substance in clinical samples. The use of the tandem detection scheme eliminated the need for methods with complete chromatographic resolution of the two enantiomers to conduct pharmacokinetic

studies to determine, if racemization occurred during metabolism. The CD detector is selective for chiral compounds and the baseline is not affected by gradient elution of the mobile phase. The sensitivity of the detector is dependent upon the analyte and the chromatographic conditions. The limit of detection for DPC 961 in this study is 5 ng. Since reversed-phase gradient HPLC is used for the separation of the drug substance from metabolites and matrix, method development is

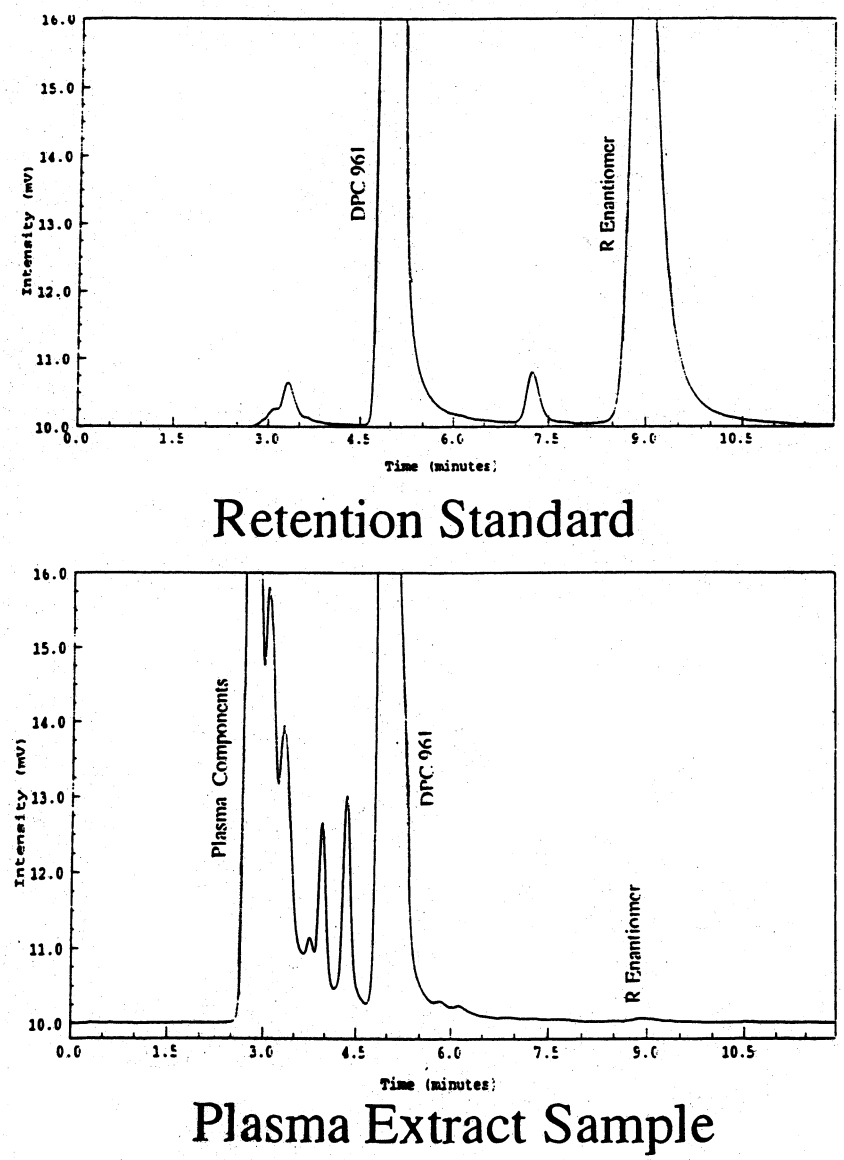

Fig. 4. Chiral HPLC separation of DPC 961 and R enantiomer standards and clinical plasma extract. Chromatographic conditions: Chirobiotic V column 250 × 4.6 mm; mobile phase of 10:90 MeOH/ACN; flow rate of 1 ml/min; temperature of 30°C; UV detection at 250 nm; 1000 ng of DPC 961 and 100 ng of R enantiomer.

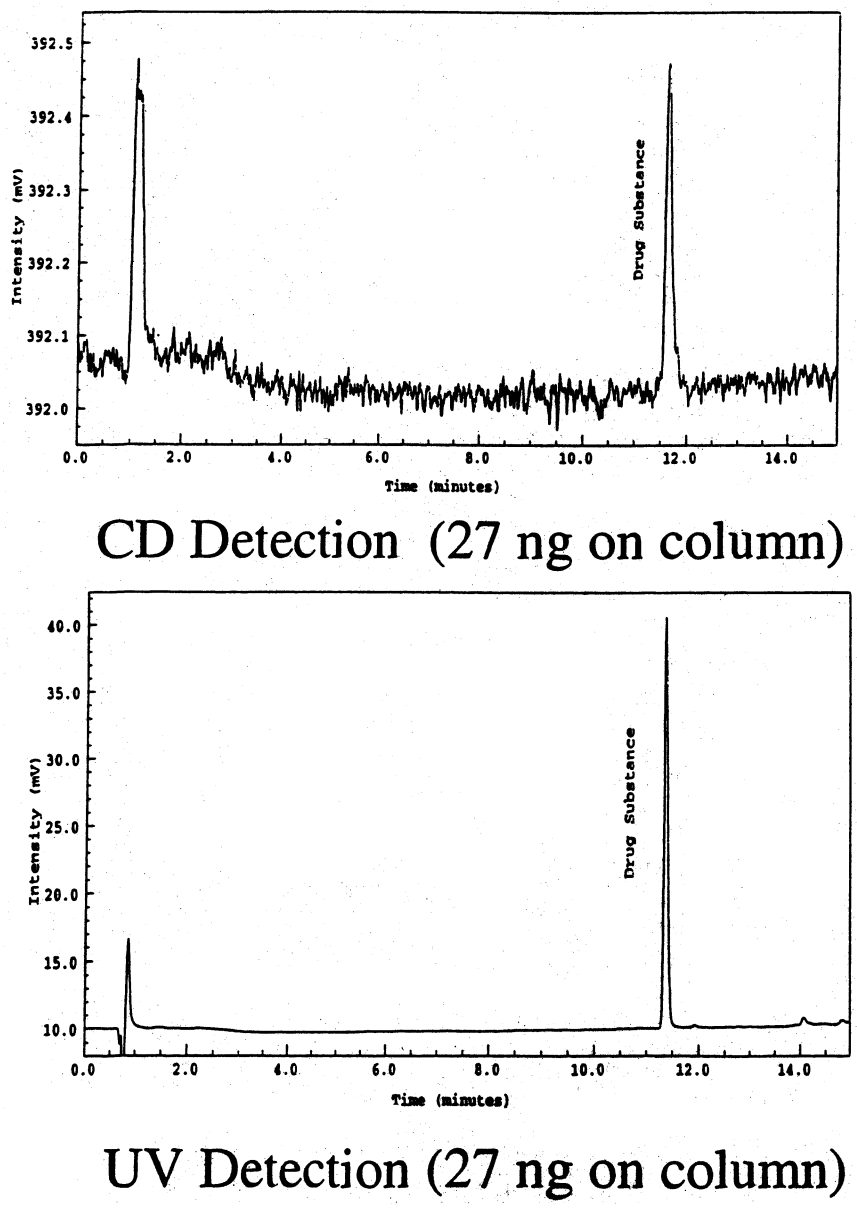

Fig. 5. Reversed-phase HPLC separation of clinical plasma extract with high sensitivity CD and UV detection. Chromatographic conditions: Symmetry C18 150 × 2.1 mm; mobile phase A = 0.05% aqueous TFA; B = ACN linear gradient from 10 to 80% B in 15 min, low rate of 0.6 ml/min; temperature of 35°C; CD detection at 240 nm and UV detection at 250 nm; 5 μl injection volume of 300 μl extract sample.

relatively simple and fast. The utility of this detection method demonstrated in phase I pharmacokinetic studies with DPC 961. The tandem detection method (CD/UV) confirmed that there was no racemization of the chiral center of DPC 961 during metabolism.

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