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Stereoselective determination of cisapride, a prokinetic agent, in human plasma by chiral high-performance liquid chromatography with ultraviolet detection: application to pharmacokinetic study

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Abstract

We have developed a simple, sensitive, specific and reproducible stereoselective high-performance liquid chromatography technique for analytical separation of cisapride enantiomers and measurement of cisapride enantiomers in human plasma. A chiral analytical column (ChiralCel OJ) was used with a mobile phase consisting of ethanol–hexane–diethylamine (35:64.5:0.5, v/v/v). This assay method was linear over a range of concentrations (5–125 ng/ml) of each enantiomer. The limit of quantification was 5 ng/ml in human plasma for both cisapride enantiomers, while the limit of detection was 1 ng/ml. Intra- and inter-day C.V.s did not exceed 15% for all concentrations except at 12.5 ng/ml for EII (+)-cisapride, which was ~20 and 19%, respectively. The clinical utility of the method was demonstrated in a pharmacokinetic study of normal volunteers who received a 20 mg single oral dose of racemic cisapride. The preliminary pharmacokinetic data obtained using the method we describe here provide evidence for the first time that cisapride exhibits stereoselective disposition.

Keywords: Cisapride

1. Introduction

Cisapride {(±)*cis*-4-amino-5-chloro-*N*-[1[3-(4-fluorophenoxypropyl]-3-methoxy-4-piperidinyl]-2 methoxybenzamide} (Fig. 1) is a gastrointestinal (GI) prokinetic drug that is widely used in adults and children for the treatment of dyspepsia, gastrointestinal reflux disease and other upper GI motility disorders [1,2]. In contrast to other structurally similar but older prokinetic drugs (e.g. metoclopramide), cisapride does not affect psychomotor function or induce central depressant adverse effect [1]. However, there are reports that link the use of cisapride with rare but life-threatening cardiac adverse effects including QT prolongation, ventricular arrhythmias of the torsade de pointes type and sudden death [3], particularly in patients who are taking drugs that inhibit cisapride metabolism (e.g. erythromycin, clarithromycin, ketoconazole and diltiazem) [4–6]. It follows that clear understanding of

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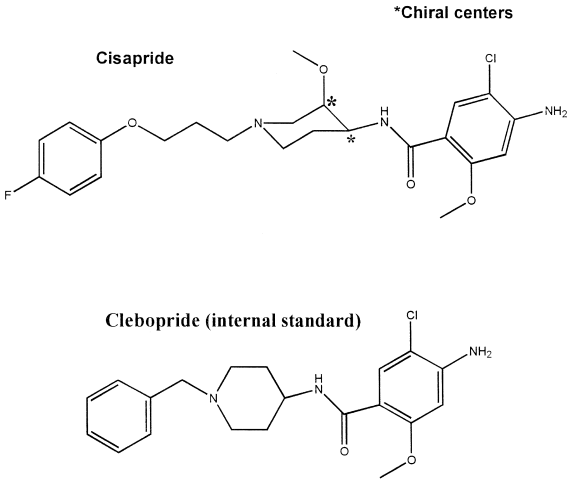

Fig. 1. Chemical structures of cisapride and the internal standard, clebopride.

its disposition and the factors involved is important in order to protect patients from hazardous adverse effect of cisapride.

Cisapride has two chiral centers (Fig. 1) that are administered as a racemate. The enantiomers of drugs can differ in metabolic and pharmacokinetic profiles as well as in potency and the spectrum of effects elicited [7], and it is important to establish the contribution of the individual enantiomers of racemic drugs to the desired and undesired pharmacological properties of the racemate. Although the drug has been marketed for more than a decade and it is possible that three-dimensional structural differences in its enantiomers translate into enantiospecific disposition or effects, the clinical studies conducted have only involved racemic mixture [1,2,4–6]. At present, information about its stereoselective pharmacology is lacking and this is partly due to the lack of a practical quantitation method that will allow researchers to separate and measure the enantiomers of cisapride. A number of methods to measure racemic cisapride in plasma by high-performance liquid chromatography (HPLC) have been published [8–10], but no effort has been made to separate the enantiomers of cisapride. We here present a HPLC method with UV detection suitable for the determination of cisapride in human plasma, which effectively separates the two enantiomers of cisapride. We have tested the utility of this assay by analyzing plasma concentration in two human volunteers administered a 20 mg tablet cisapride by mouth.

2. Experimental

2.1. Chemicals and reagents

Racemic cisapride, (+)*cis*-4-amino-5-chloro-*N*-[1[3-(4-fluorophenoxypropyl]-3-methoxy-4-piperidinyl]-2 methoxybenzamide, was purchased from Research Diagnostic (Flanders, NJ, USA). The internal standard, clebopride was purchased from Sigma (St. Louis, MO, USA). All other chemicals were of analytical grade.

2.2. Chiral separation of cisapride enantiomers

Because there was no published method for chiral separation of cisapride, we used a developmental approach to developing the method. Preliminary experiments aimed at defining optimal conditions with regard to solvents, chiral columns and mobile phases for separation of cisapride enantiomers were performed. Chiral HPLC assay methods published in the literature for other drugs were used as a starting point [11,12]. Two peaks, which were successfully separated, were identified after a solution of racemic cisapride dissolved in 100% ethanol was injected in to an HPLC separation system that consists of a long or short OJ column and a mobile phase containing ethanol–hexane–diethylamine. The spectral characteristics of racemic cisapride (215 and 274 nm) and the two separated peaks (‘‘enantiomers’’) were scanned using a photodiode array detector (Waters Model 996, Waters, Milford, MA, USA) and revealed similar characteristics. A long OJ column (4.6×250 mm, Daicel, Tokyo, Japan) provided better separation than using ChiralCel OJ column (4.6×50 mm). These columns contain the same stationary phase, but they only differ in length. Because the peak resolution was better with the long column, this column was used for subsequent separations. The peak that eluted first was designated as enantiomer I (EI (−)-cisapride) and the peak that appeared there-

after was designated enantiomer II (EII). EI and EII had the same area under the curve (ratio: 1:1.0014), suggesting equal amounts of the enantiomers in the preparation we used.

2.3. Stock solutions

Standard solutions of cisapride were prepared by dissolving 10 mg of racemic cisapride in 10 ml 100% ethanol in a polypropylene tube followed by ultra-sonication for 3 min. Sequential dilutions to 100, 10 and 1 μg/ml and 500, 250, 150, 100, 50, 25, 10 and 5 ng/ml were made in ethanol. Stock solution of the internal standard (1 mg/ml) was prepared by dissolving clebopride (10 mg) in ethanol (10 ml) and the working solution (2 μg/ml) was prepared by diluting the stock solution with ethanol. Solutions were stored at 4°C.

2.4. Instrumentation and chromatographic conditions

The HPLC system consisted of a Waters Model 600 dual-piston multisolvent delivery system, a Waters WISP 710B autosampler and a Waters Model 490 ultraviolet detector (Waters, Milford, MA, USA). The data were collected on the Waters Millennium chromatography software manager. The separation system consisted of a (250×4.6 mm) OJ stainless steel column supplied by Chiral Technologies (Exton, PA, USA) and a Waters CN guard column at room temperature. The mobile phase was prepared by mixing 350 ml of ethanol in 650 ml of hexane. After stirring, this mobile phase was filtered through a 0.45-μm membrane filter. The flow-rate was 1.2 ml/min. The detector was set to measure at a wavelength of 275 nm.

2.5. Extraction procedure

After testing several published extraction methods for racemic cisapride, a slight modification of the method developed by Campanero et al. [8] was used because it produced satisfactory extraction recoveries. In short, 1-ml aliquots of human plasma were pipetted into clean 15-ml glass tubes, and known concentrations of racemic cisapride (0–250 ng) and 50 μl of the internal standard solution (containing 2 μg/ml clebopride in ethanol) were added. The mixture was made alkaline by adding 0.25 ml of 0.1 *M* disodium tetraborate buffer (pH, 13.3) and vortex-mixed. The samples were extracted using *tert.*-butylmethyl ether (3 ml) by use of a vortex mixer for 1 min. The organic phase was transferred in to new tubes following centrifugation for 5 min at 2000 rpm, back-extracted with 0.75 ml of 0.05 *M* sulfuric acid, vortex-mixed and centrifuged at 2000 rpm for 5 min. The organic layer was removed and the remaining acidic phase was then made alkaline with disodium tetraborate buffer (0.1 *M*; 0.75 ml) and re-extracted with 3 ml *tert.*-butylmethyl ether. After vortex mixing and centrifugation for 5 min at 2000 rpm, the organic phase was transferred into new tubes. The samples were dried on speed vacuum and the resulting residue was reconstituted in 200 μl of mobile phase. A 150-μl aliquot was then injected onto the HPLC column.

2.6. Pharmacokinetic study

Two healthy volunteers (one male and one female) were admitted to the General Clinical Research Center of the Georgetown University Medical Center for the study after giving written informed consent. After an 18-gauge sterile indwelling catheter was inserted in one arm and a pre-dose blood samples (~10 ml each) were collected in into tubes that contain ethylenediaminetetraacetic acid (EDTA), subjects were administered a single 20 mg oral dose of racemic cisapride. Blood samples (~10 ml) following drug administration were then collected at 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8 and 24 h. The plasma was separated by centrifugation at 1500 rpm for 20 min at 4°C and subsequent transfer of the plasma to clean 50-ml polypropylene tubes. The tubes were stored at −20°C pending analysis. Since we had no information on the proportion of each enantiomer in the tablets used for the clinical study, we dissolved a 10 mg tablet in 10 ml of 100% ethanol and injected 150 μl into HPLC. On the basis of the area under the curve (ratio of EI/EII, 1/1), the enantiomers ratio appear present in equal amounts.

3. Results and discussion

3.1. Chromatography

We tested the suitability of three chiral columns (ChiralPack AS, ChiralCel OD and ChiralCel OJ) to separate cisapride enantiomers using different mobile phases (ethanol in hexane and isopropanol in hexane and methanol in hexane). Cisapride was soluble in ethanol and methanol, but the stock solution was dissolved in ethanol, as our mobile phase was ethanol in hexane. Injection of racemic cisapride in to ChiralPack AS and ChiralCel OD columns resulted in single peak at retention times of 26.3 and 15.6 min, respectively, with no evidence of chiral separation using any of the mobile phases described. However, effective separation of cisapride enantiomers was achieved using ChiralCel OJ column (long or short) and a mobile phase containing ethanol–hexane–diethylamine (35:64.5:0.5, v/v/v). Qualitatively, both long and short ChiralCel OJ columns provided adequate separation, but we chose the long OJ column because peak resolution was better. Addition of diethylamine improved the peak shape. In Fig. 2, representative chromatograms of extracts of drug free human plasma and of a plasma standard containing 50 ng/ml racemic cisapride and/or the internal standard, clebopride are illustrated. EI and EII peaks represent the enantiomers of cisapride that eluted first and second in the chromatogram respectively. These chromatograms clearly demonstrate the successful separation and well-resolved peaks of cisapride enantiomers in human plasma (Fig. 2C). The retention times were highly dependent on the concentration of ethanol in the mobile phase. Higher concentrations of ethanol shortened and lower concentrations prolonged the retention times of the test compounds. A concentration of 35% ethanol in the mobile phase with 0.5% diethylamine provided sharp symmetrical peaks and was used for all subsequent experiments. Under these conditions, the retention times of clebopride (internal standard, I.S.), EI and EII were 5.3 ± 0.019, 7.2 ± 0.041 and 10.6 ± 0.057 min respectively. Comparison of blank plasma (drug-free) from different subjects (Fig. 2A) with plasma spiked with I.S. (Fig. 2B) or cisapride and the I.S. (Fig. 2C) showed that there is no endogenous source of interference at or near the retention times of cisapride enantiomers or the I.S. Similarly, no interfering peaks were observed in the chromatograms of predose plasma samples (Fig. 3A) and plasma samples obtained 1 h (Fig. 3B) and 8 h (Fig. 3C) after a 20-mg oral dose of cisapride to two human volunteers participated in the pharmacokinetic study. The lack of coeluting peaks from components of drug-free or predose plasma at or near the peaks of the enantiomers or I.S. is an important attribute of specificity of the method for the enantiomers.

3.2. Linearity

The ratio of EI to EII in the racemic cisapride we used, as defined by the area under the peak–height, was 1:1.0014, essentially confirming that the two enantiomers in the cisapride samples are present in equal proportions. The standard curves for each enantiomer were constructed by injecting standard concentrations of racemic cisapride into the HPLC. Linear regression calibration curves based on seven data points were constructed for each enantiomer by plotting the peak height ratio of the enantiomers to the I.S. versus the concentrations of plasma standards of racemic cisapride. The curves were weighted $1/y$ where y = peak height ratio. A typical linear regression equation for EI included a slope of 0.010367 ± 0.000451 and a y-intercept of 0.0149 ± 0.001153. For EII, typical values for slope and y-intercept were 0.010233 ± 0.000651 and 0.0154 ± 0.001769, respectively. Correlation coefficients of >0.99 were obtained for both enantiomers over a concentration range of 10–250 ng/ml racemic cisapride added to human plasma which is equivalent to 5–125 ng/ml of each enantiomer, demonstrating linearity over the entire standard range tested.

3.3. Extraction recovery

Recoveries of cisapride enantiomers from plasma were obtained by comparing the unextracted standard curves with extracted standard curves (duplicate, $n=4$ at each of the seven concentrations). The mean extraction recoveries of EI and EII in plasma were 84 ± 5.2 and 86.6 ± 6.9% respectively (Table 1). The mean extraction ratio of the I.S., clebopride (50 ng/ml), was 85 ± 9.7% ($n=7$ determinations).

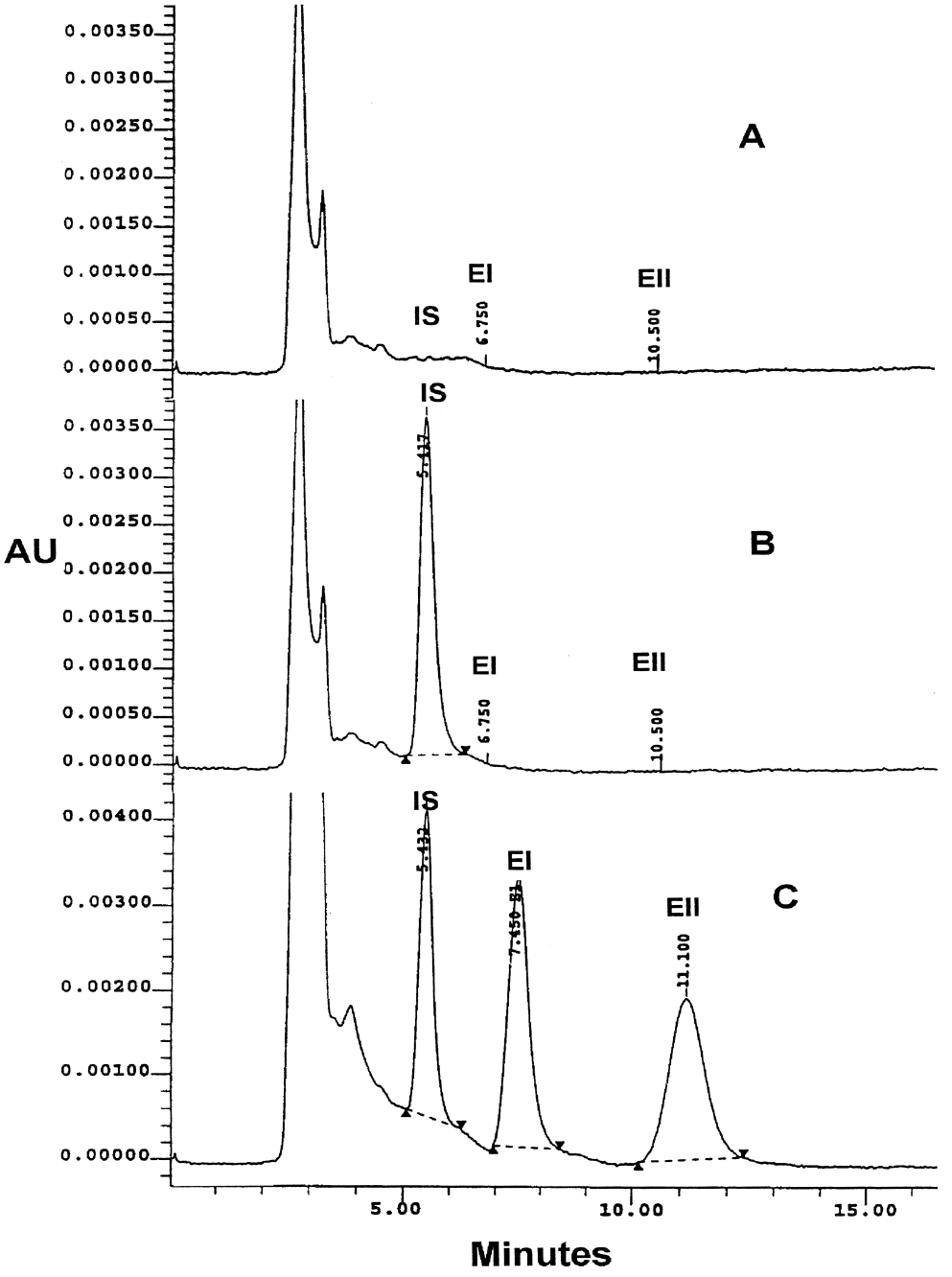

Fig. 2. Representative chromatograms of extracted (A) drug free human plasma, (B) plasma standard containing 50 ng/ml internal standard (IS), clebopride, and (C) plasma standards containing 250 ng/ml racemic cisapride and 50 ng/ml internal standard. EI is the enantiomer of cisapride that appear first in the chromatogram and EII is the enantiomer that follows it.

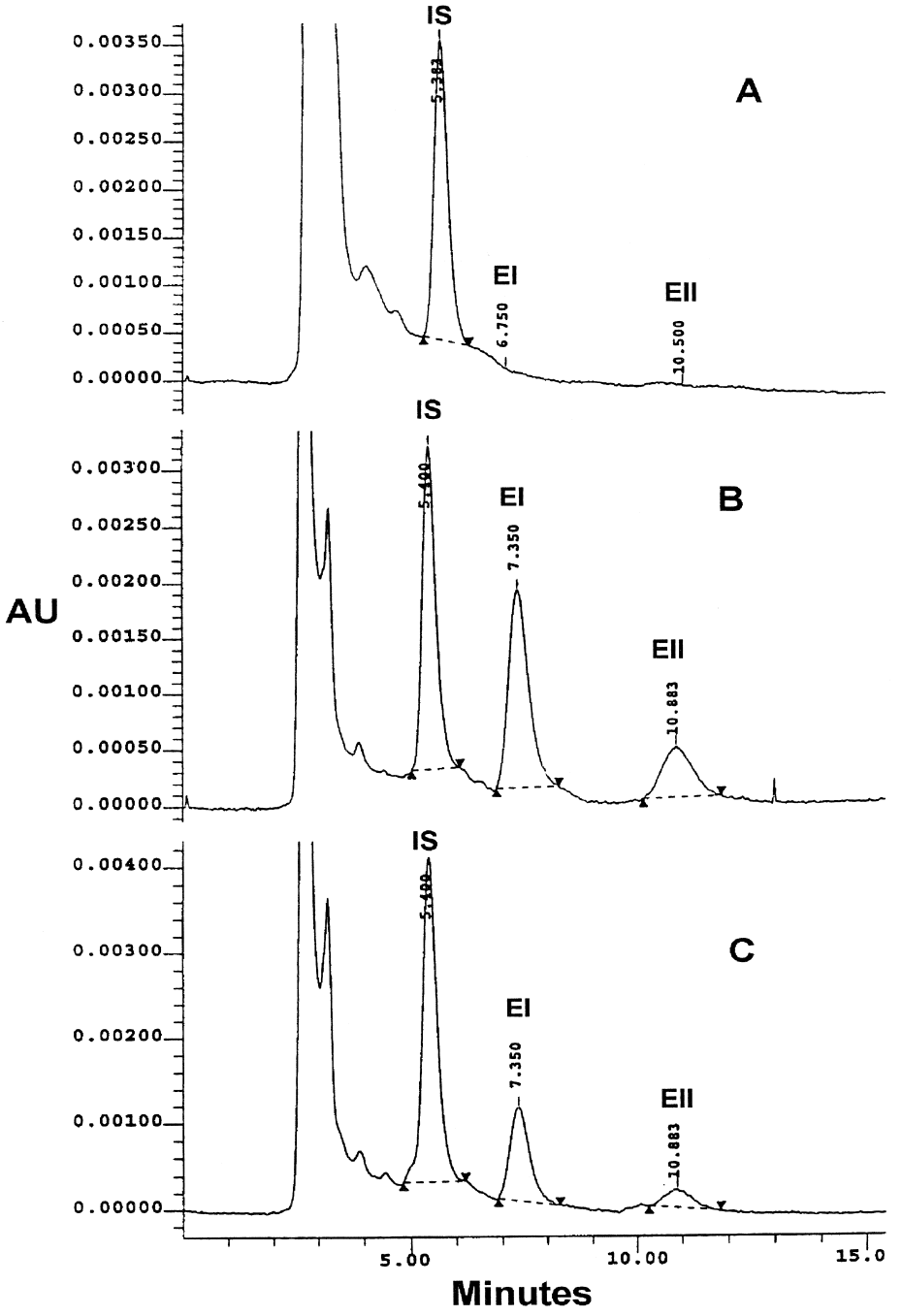

Fig. 3. Representative chromatograms of extracted (A) a predose plasma sample from a subject spiked with the I.S. and a plasma sample obtained from a subject 1 h (B) and 8 h (C) after oral administration of a 20-mg single oral dose of racemic cisapride.

Table 1
Human plasma extraction ratios for enantiomers of cisapride

EL (ng/ml)	Extraction ratio (%)	ELL (ng/ml)	Extraction ratio (%)
5	79.1	5	86.7
7.5	86.4	7.5	84.4
12.5	86.7	12.5	99.7
25	85.2	25	85.5
37.5	77.4	37.5	76.7
50	83.4	50	84.9
125	93.0	125	88.4
Mean±SD	84±5.2	Mean±SD	86.6±6.9

3.4. Assay precision and accuracy

Triplicate standards ($n=4$) in human plasma were extracted and analyzed to assess the intra-day variability of the assay method. Accuracy and precision (C.V.) throughout the standard curve are summarized in Table 2 for both enantiomers of cisapride. The largest variation observed was 20.3% at 12.5 ng/ml of EII. C.V.s at all other standard concentrations were $<15\%$ for both enantiomers (range: 3.6–13.5 for EI and 5.2–12.7 for EII).

Similarly, plasma samples containing 10–250 ng/ml of racemic cisapride (5–125 ng/ml of each enantiomer) were extracted and analyzed daily to determine inter-day variability. As shown in Table 3, the inter-day variability of the method for both enantiomers was below 10% (range: 1–9.7% for EI and 2.4–9.2% for EII) over all concentrations tested except at 12.5 ng/ml for EII, which was ~19%.

3.5. Limit of detection and quantification

The lower limit of quantification (LLOQ) was 5 ng/ml for both EI and EII and was used to calculate peak area. The intra- and inter-day C.V.s for EI (10.3 and 6.7%) and EII (12.7 and 9.2) at these concentrations were close to 10%. The lower limit of

Table 2
Intra-day reproducibility data for cisapride enantiomers in human plasma

Nominal concentrations (ng/ml)	EI			EII		
	Mean analyzed (ng/ml)	Accuracy (%)	C.V.s (%)	Mean analyzed (ng/ml)	Accuracy (%)	C.V. (%)
5	5.8	116.97	10.3	6.0	120	12.7
7.5	7.9	104.97	13	8.2	108.6	9.6
12.5	11.5	92.1	13.5	11.1	88.5	20.3
25	22.9	91.7	7.3	23.1	92.4	11.4
37.5	36.5	97.4	4.4	35.4	94.3	10.7
50	50.1	100.2	6.7	50.0	100.1	7.8
125	125.7	100.6	3.6	125.9	100.7	5.2

Table 3
Inter-day reproducibility data for cisapride enantiomers in human plasma

Nominal concentrations (ng/ml)	EI			EII		
	Mean Analyzed (ng/ml)	Accuracy (%)	C.V. (%)	Mean analyzed (ng/ml)	Accuracy (%)	C.V. (%)
5	5.4	107.8	6.7	5.2	103.4	9.2
7.5	7.6	101.7	5.9	7.5	99.8	4.3
12.5	11.1	89.0	9.7	10.5	83.8	18.8
25	23.1	92.5	1.0	23.1	92.5	6.8
37.5	37.6	101.1	2.0	36.2	96.5	6.9
50	51.6	103.2	2.5	52.0	103.9	2.4
125	130.4	104.3	5.9	132.8	106.3	8.3

detection (LLOD), defined as the concentration for which signal-to-noise ratio = 3, in 1 ml of plasma was 1 ng/ml for both enantiomers. The linearity obtained and the limit of quantification determined (5 ng/ml) are appropriate to allow successful measurement of therapeutic plasma concentrations of cisapride enantiomers in pharmacokinetic studies after a single or multiple therapeutic doses of racemic cisapride [6,13,14].

3.6. Stability of cisapride enantiomers in plasma and ethanol

A concentration of 100ng/ml of cisapride in plasma or ethanol was frozen at −20°C and thawed and assayed on different days. The mean values of the original obtained for the enantiomers were >91% of the original for both the enantiomers up to one month. All the C.V.s were less than 15%. No change was observed with regard to the ratio of EI to EII due to storage.

The chiral column requires equilibration with mobile phase prior to routine use. If this column is stored at an ambient temperature in 100% isopropanol–hexane (1:9), the retention times, peak responses and resolution were similar during this method and during the separation, showing remarkable stability of the chiral stationary phase.

3.7. Pharmacokinetic study

We tested the utility of this method in a pharmacokinetic study in two human volunteers (one male and one female). The mean plasma concentrations of enantiomers of cisapride determined in the healthy volunteers after a single 20 mg oral dose of racemic cisapride are illustrated in Table 4. With the method we describe here, it was possible to detect both enantiomers 24 h after cisapride administration. The plasma concentration–time profiles of both enantiomers of cisapride in the male and female subjects are presented individually in Fig. 4A and Fig. 4B respectively. The plasma concentrations of each enantiomer appear to be consistently lower in the female subject (body weight: 50 kg) compared with that of the male subject (body weight: 80 kg). However, this observation needs to be confirmed in adequate number of subjects, in order to allow conclusions about the effect of gender on the disposition of cisapride. The pharmacokinetic parameters of cisapride enantiomers derived from the plasma concentration–time profile, calculated according to a noncompartmental model after an extravascular input, are shown in Table 5. The AUC and C_{max} of EI are about 2 and 3-fold higher respectively than that of EII. Although these data were obtained from two subjects (one female and one male) which limit our ability to test any statistical comparison between the pharmacokinetic parameters derived from EI and EII or between female and males, they do suggest that cisapride disposition is enantiospecific. This is most probably due to stereoselective intestinal and/or hepatic metabolism of cisapride. Since we did not attempt to measure metabolites of cisapride in plasma and urine, the role of stereoselective transport of cisapride across the intestine by P-glycoproteins cannot be ruled out, but a recent in vitro study that has indicated that cisapride is not a substrate of this transport protein [15] makes it unlikely.

Table 4
Plasma concentrations of cisapride enantiomers ($n = 4$ determinations) in two human healthy volunteers after administration of a 20 mg single oral dose of racemic cisapride tablets

Time (h)	EL (mean±SD ng/ml)	ELL (mean±SD ng/ml)
0	0	0
0.58	94.9±12.9	24.3±11.8
1.2	81.7±7.8	30.7±9.1
1.6	70.6±14.6	26.5±10.5
2.0	62.7±13	25.2±8.4
3.1	45.3±4.5	18.9±2.8
4.1	37.4±5.7	16±4.3
5.1	32.1±6.2	14.1±2.9
6.1	30.2±10.2	11.8±2.6
8.2	21.4±7.5	9.4±3.1
23.8	6.6±0.9	6.0±0.6

4. Conclusions

Because cisapride is a chiral drug, full understanding of the disposition and effect of not only the racemic form but also the enantiomers is required in order to use the drug safely and effectively. The method presented here describes for the first time separation of cisapride enantiomers and a simple,

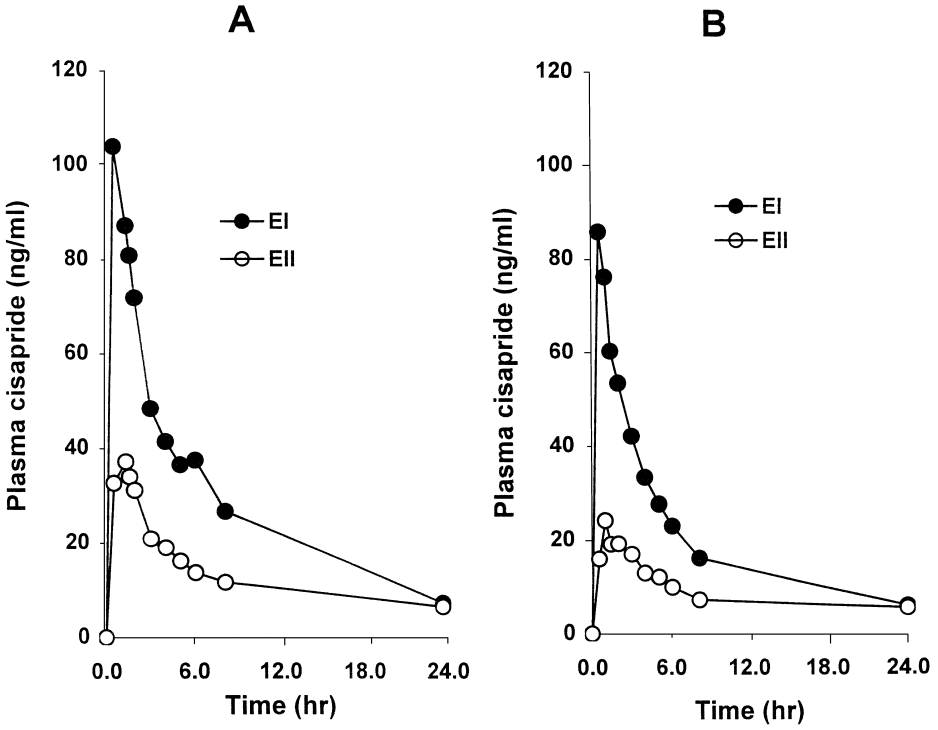

Fig. 4. Plasma concentration time profiles of cisapride enantiomers in healthy male (A) and female (B) healthy volunteers after a 20-mg single oral dose of racemic cisapride.

specific, sensitive and reproducible human plasma assay using chiral HPLC with UV detection. This method should make it possible to conduct detailed studies of the pharmacokinetics and pharmacodynamics of cisapride that will allow better understanding of the properties of each enantiomer of cisapride in various patient populations and to identify factors that through modification of the enantiomer concentrations influence the safety and efficacy of cisapride.

Table 5
Pharmacokinetic parameters of cisapride enantiomers in two human healthy volunteers after administration of a 20 mg single oral dose racemic cisapride tablets

Pharmacokinetic parameters	EI		EII	
	Male subject	Female subject	Male subject	Female subject
T_{max} (h)	1.4	0.6	1.4	1.0
C_{max} (ng/ml)	104	85.8	37.2	24.2
T_{lag} (h)	0	0	0	0
Half-life (h) 25	7.1	7.7	11.0	14.2
MRT∞ (h)	9.4	9.7	18.3	20.6
AUC∞ (h ng ml^{-1})	744	545	400	324
Volume of distribution/F (l)	274	405	804	1315
Clearance/F (ml/h)	26.9	36.7	50	64

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