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Enantioselective analysis of disopyramide and mono-*N*-dealkyldisopyramide in plasma and urine by high-performance liquid chromatography on an amylose-derived chiral stationary phase

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Abstract

An enantioselective high-performance liquid chromatography method was developed for the simultaneous determination of disopyramide (DP) and mono-*N*-dealkyldisopyramide (MND) enantiomers in plasma and urine. The drugs were extracted from plasma samples by liquid–liquid extraction with dichloromethane after protein precipitation with trichloroacetic acid; the urine samples were processed by liquid–liquid extraction with dichloromethane. The enantiomers were resolved on a Chiralpak AD column using hexane–ethanol (91:9, v/v) plus 0.1% diethylamine as the mobile phase and monitored at 270 nm. Under these conditions the enantiomeric fractions of the drug and of its metabolite were analyzed within 20 min. The extraction procedure was efficient in removing endogenous interferents and low values for the relative standard deviations were demonstrated for both within-day and between-day assays. The method described in this paper allows the determination of DP and MND enantiomers at plasma levels as low as 12.5 ng/ml and can be used in clinical pharmacokinetic studies.

Keywords: Chiral stationary phases, LC; Enantiomer separation; Disopyramide; Mono-*N*-dealkyldisopyramide

1. Introduction

Disopyramide (DP) (Fig. 1) is an effective type 1A antiarrhythmic drug used for the treatment and prophylaxis of ventricular and supraventricular arrhythmias and is marketed as a racemate. The antiarrhythimic effect of DP is attributed to the (+)-(*S*)-enantiomer, whereas its major side effect, a negative inotropic effect on the myocardium, is mainly related to (−)-(*R*)-DP [1,2]. The mono-*N*-dealkyldisopyramide (MND) metabolite may significantly contribute not only to the antiarrhythmic effect but also to the anticholinergic side effects of DP [3,4]. The pharmacokinetic disposition and metabolism of DP are also stereoselective, so that the concentration of (+)-(*S*)-DP in plasma after oral administration is higher than that of (−)-(*R*)-DP [5]. Several studies have reported stereoselective plasma protein binding and the interaction between the two

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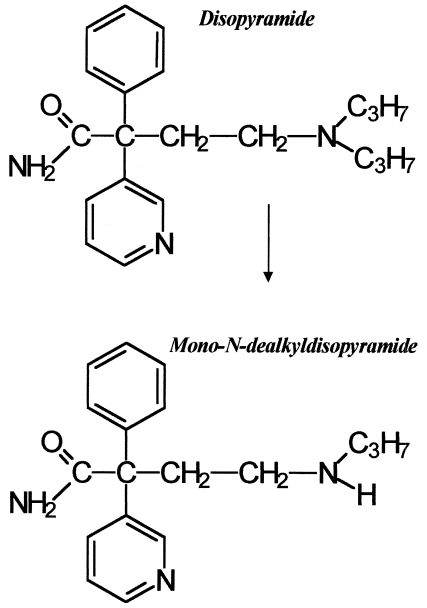

Fig. 1. Metabolism of disopyramide.

DP enantiomers as well as the interaction between DP and its MND metabolite [6–9].

Thus, the determination of the individual enantiomers of DP and MND after administration of racemic DP might be of clinical value. The enantioselective analysis of DP and MND in plasma or urine has been reported in the literature in studies using high-performance liquid chromatography (HPLC) on chiral stationary phases. The determination of DP enantiomers in plasma was first described by Hermansson et al. [10] using a laboratory-made α_1-acid glycoprotein column. In order to avoid the coelution of MND, the authors used a reversed-phase column directly connected to the chiral column. Le Corre et al. [11] and Enquist and Hermansson [12] used similar procedures coupling a reversed-phase or a silica column to the chiral column, but in this case they obtained success with the resolution of DP enantiomers as well as MND enantiomers. Although protein-based chiral stationary phases are claimed to be highly versatile in the resolution of chiral drugs, the short lifetime of this kind of column can impair its application to pharmacokinetic studies due to the high number of analyses required in these studies.

Polysaccharide-based chiral stationary phases have been widely used for the resolution and quantitation of chiral drugs in biological fluids due to their efficiency, versatility and stability. Takahashi et al. [13] and Echizen et al. [14] used a cellulose tris-(4-chlorophenylcarbamate) derived chiral stationary phase (Chiralcel OF) for the determination of DP and MND enantiomers in plasma and urine. Instead of using an analytical column, they used a guard column (5 cm×4.6 mm) to perform the chiral resolution of the drugs. In spite of this, analysis time was more than 30 min. Echizen et al. [14] reported detection limits of 10 ng/ml for (+)-(*S*)-DP and 25 ng/ml for the other compounds. More recently, Hanada et al. [15] reported the use of this column for the enantioselective analysis of DP in rat plasma and tissues.

The Chiralpak AD column, a 3,5-dimethylphenylcarbamate derivative of amylose coated on 10 μm silica gel has been used for the determination of several drugs and metabolites enantiomers [16–19] but not for the resolution of DP and MND enantiomers. Thus, we are reporting the use of this column for the development of an enantioselective method for the analysis of DP and MND in plasma and urine samples suitable for clinical pharmacokinetic studies.

2. Experimental

2.1. Drugs and chemicals

Rac-DP and *rac*-MND were kindly supplied by Laboratórios Silva Araújo Roussel (Roussel UCLAF), Rio de Janeiro, Brazil. Stock standard solutions containing 1 mg/ml as the base of *rac*-DP and *rac*-MND were prepared in methanol acidified with hydrochloric acid (0.01 mol/l). Working solutions of both compounds (1.0–500.0 μg/ml) were prepared by appropriate dilution in methanol–HCl (0.01 mol/l). The internal standard, *rac*-metoprolol, was obtained as the tartrate salt from Biogalênica-Química e Farmacêutica (São Paulo, Brazil). The solution was prepared in methanol at the concentration of 100 μg/ml.

Hexane, dichloromethane (EM Science, Gibbstown, USA) and ethanol (Merck, Darmstadt, Germany) were of HPLC grade. All other chemicals

were analytical-reagent grade and were used without further purification.

Pooled drug-free human plasma and urine were obtained from healthy volunteers, stored at −20°C and allowed to thaw at ambient temperature prior to use.

2.2. Instruments and chromatographic conditions

The HPLC system consisted of an LC10AS solvent pump, an SPD 10A spectrophotometric detector set at 270 nm (0.004 a.u.f.s.), a CR6-A integrator (all from Shimadzu Instruments, Kyoto, Japan) and a 7125 Rheodyne injector with a 50-μl loop (Rheodyne, Cotati, CA, USA). Separations were carried out in a climatized room (22±2°C) on a Chiralpak AD column (250×4.6 mm I.D., 10 μm particle size) purchased from Chiral Technologies (Exton, PA, USA). A CN guard column (4×4 mm I.D., Merck) was used to protect the analytical column. The mobile phase consisted of hexane–ethanol (91:9, v/v) plus 0.1% diethylamine and the flow-rate was 1.2 ml/min with a column inlet pressure of 25 kg/cm^2.

2.3. Extraction procedure

Plasma samples of 1 ml were transferred to 15-ml glass tubes and spiked with 25 μl of metoprolol solution (100 μg/ml). After the addition of 200 μl of a 40% trichloroacetic acid solution, the tubes were vortex-mixed for 1 min, left to stand for 20 min and centrifuged for 5 min at 1800 *g*. Aliquots of 760 μl of the upper phases were transferred to clean tubes and alkalinized with 400 μl of 4 mol/l NaOH solution. After the addition of 5 ml dichloromethane, the tubes were capped, shaken horizontally for 20 min and then centrifuged for 5 min at 1800 *g*. The organic phases were transferred to clean tubes and the solvent was evaporated to dryness. The residues were dissolved in 80 μl mobile phase and 50 μl was chromatographed. The calibration curves were prepared by adding 25 μl of the working solutions to 1 ml of drug-free plasma in order to obtain the concentration range of 25 ng/ml to 625 ng/ml of each enantiomer. The samples were then assayed in duplicate by the described procedure.

Urine samples were similarly prepared except for the omission of the protein precipitation with trichloroacetic acid step. Aliquots of 100 μl of urine were added to 660 μl water, alkalinized with 200 μl of 4 mol/l NaOH solution and extracted with dichloromethane as indicated above. The calibration curves were prepared in the range of 0.625 to 25.0 μg of each enantiomer/ml of urine.

2.4. Recovery and linearity

The analytical recovery of DP and MND enantiomers was determined at plasma concentrations of 25.0, 62.5 and 625.0 ng/ml and urinary concentrations of 0.625, 6.25 and 25.0 μg/ml of each enantiomer ($n=3$). Drug-free plasma (1 ml) or urine (100 μl) was spiked with known amounts of the drug and metabolite to achieve the concentration previously specified. These samples were submitted to the extraction procedure and peak heights were compared with the peak height obtained by the direct injection of the drugs into the mobile phase.

The linearity study was carried out in the range of 25 to 6250 ng/ml and 0.625 to 62.5 μg/ml of each enantiomer in plasma and urine, respectively.

2.5. Precision and accuracy

Precision and accuracy of the assay were obtained by analyzing aliquots of two spiked plasma or urine samples. Within-day precision and accuracy were determined by analyzing 10 aliquots of spiked human plasma or urine and between-day precision and accuracy were determined over a 1-week period ($n=5$). The precision and accuracy of the method were calculated as the relative standard deviation (relative standard deviation, RSD) and the percent deviation of observed concentration from theoretical concentration, respectively.

2.6. Selectivity

Interference of commonly used drugs was evaluated by injecting solutions of the drugs prepared in the mobile phase onto the chromatographic system and recording their retention times. When the retention time obtained was similar to the retention times of DP or MND enantiomers or metoprolol peaks, a plasma sample spiked with the drug in the

upper limit of the therapeutic range was submitted to the extraction procedure and chromatographic analysis.

2.7. Sensitivity

Sensitivity was evaluated using the quantitation limit parameter. The quantitation limit was assessed by analyzing aliquots ($n=5$) of plasma spiked with DP and MND at the concentration of 12.5 ng/ml of each enantiomer.

2.8. Preliminary human experiment

In order to evaluate the applicability of the method, several plasma and urine samples collected from a healthy volunteer after administration of a single p.o. dose of *rac*-DP (Dicorantil, 100 mg) were analyzed under the conditions established in the present study. Blood samples were taken at timed intervals, i.e., 0, 0.5, 1, 1.5, 2, 3, 4, 6, 8 and 10 h after dosing. Blood samples were collected into heparinized tubes and centrifuged at 1800 *g* for 10 min and the plasmas were transferred to clean tubes and stored at −20°C until analysis. Urine samples were collected into plastic containers at intervals of 0–1, 1–3, 3–5, 5–7, 7–9, 9–10 h after DP administration. After measuring the volume, 10-ml aliquots were separated and stored at −20°C until analysis.

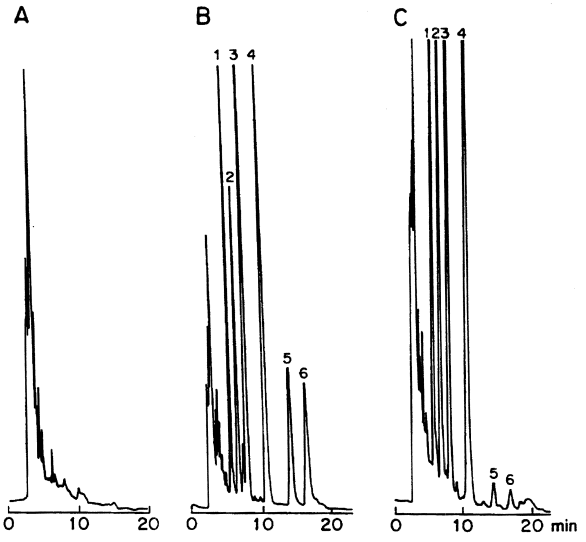

Fig. 2. Chromatograms referring to the analysis of DP and MND enantiomers in plasma. (A) Blank plasma; (B) plasma spiked with 625 ng/ml of DP and MND enantiomers; (C) plasma sample from a healthy volunteer collected 6 h after the administration of 100 mg of Dicorantil. (+)-(*S*)-DP (1); (−)-(*R*)-DP (2); metoprolol (3, 4); (+)-(*S*)-MND (5); (−)-(*R*)-MND (6). Chromatography conditions: Chiralpak AD column (250×4.6 mm I.D., 10 μm particle size); mobile phase: hexane–ethanol (91:9, v/v) plus 0.1% diethylamine; flow-rate was 1.2 ml/min; detection at 270 nm.

3. Results and discussion

Typical chromatograms of drug-free human plasma and urine, standard calibration plasma and urine spiked with DP, MND and the internal standard, and treated subject plasma and urine are shown in Fig. 2 and Fig. 3, respectively. The peaks eluting at retention times closer to 8.0 and 10.5 min. correspond to metoprolol (internal standard) which is also resolved on the Chiralpak AD column. The height of the first eluted peak was arbitrarily chosen for quantitation purposes, but the second one could also be used. No endogenous interfering peaks were observed with drug-free human plasma or urine at the retention times of DP, MND and metoprolol.

The elution order of DP and MND enantiomers was obtained by the analysis of individual enantiomers, previously separated and collected from the Chiralcel OF column according to the method described by Echizen et al. [14].

Tables 1–3 summarize the data obtained in the validation of the method. The calibration curves (Table 1), obtained by least-squares linear regression, were linear up to 6250 ng/ml of plasma and up to 62.5 μg/ml of urine for both enantiomers of DP and MND and the correlation coefficients observed were 0.9839 or better. Table 1 also shows reproducible recovery of the enantiomers using the proposed procedure. Similar results for the recovery were observed by the analysis of plasma samples spiked with the individual enantiomers obtained after separation in the chiral column. In addition, no racemization of the DP and MND was observed during sample work-up. Although the recovery observed for DP and MND enantiomers in plasma samples was lower than that reported in the literature [12–14] due to the inclusion of a protein precipitation step in the extraction procedure, the method reported in this

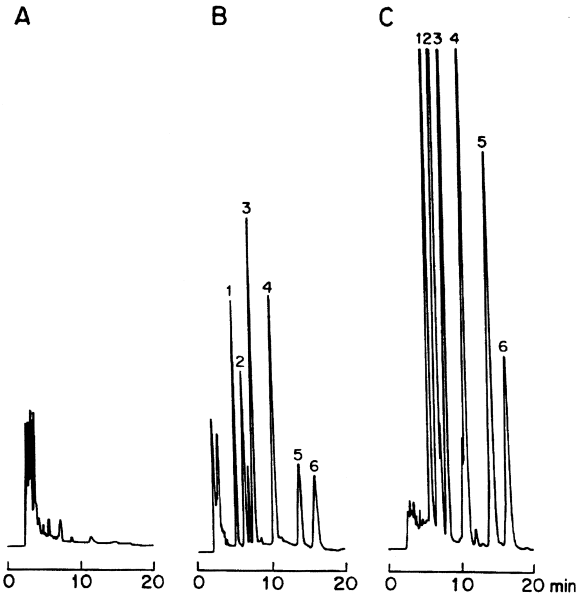

Fig. 3. Chromatograms referring to the analysis of DP and MND enantiomers in urine. (A) Blank urine; (B) urine spiked with 6.25 μg/ml of DP and MND enantiomers; (C) urine sample from a healthy volunteer collected 5–7 h after the administration of 100 mg of Dicorantil. (+)-(*S*)-DP (1); (−)-(*R*)-DP (2); metoprolol (3, 4); (+)-(*S*)-MND (5); (−)-(*R*)-MND (6). Chromatography conditions: Chiralpak AD column (250×4.6 mm I.D., 10 μm particle size); mobile phase: hexane–ethanol (91:9, v/v) plus 0.1% diethylamine; flow-rate was 1.2 ml/min; detection at 270 nm.

paper provides cleaner samples that resulted in lower quantitation limits.

Precision and accuracy were assessed in both plasma and urine by performing replicate analysis of spiked samples against calibration curves. The within-day and between-day precision are reported in Table 2. RSDs of less than 10% were obtained for all samples analyzed. In addition, the method was accurate since the deviation from the theoretical value were also in the range of 10% (Table 2).

In the present study we used the quantitation limit as a parameter for the measurement of the sensitivity of the method, defined as the lowest concentration which can be determined with acceptable accuracy and precision (RSD and systematic error lower than 15% [20]). The quantification limit determined from extracted spiked plasma was 12.5 ng/ml for all enantiomers evaluated (Table 1). This value is similar to the detection limit reported by Echizen et al. [14] for (+)-(*S*)-DP and lower than the detection limit reported for the other compounds (25 ng/ml).The proposed method proved to be highly selective. Among the drugs studied (Table 3), only propiphenazone, carbamazepine, primidone, verapamil, propranolol and bromazepan were likely to show interference.

Figs. 4 and 5 show the plasma concentration–time

Table 1
Recovery, linearity and quantitation limit of the method for analysis of DP and MND enantiomers in plasma and urine

	Recovery		Linearity		Quantitation limit		
	%	RSD (%)	Range[a]	*r*	Concentration (ng/ml)	Precision (RSD, %)	Accuracy (*E*, %)
Plasma							
(+)-(*S*)-DP	71.7	7.3	25–6250	0.9980	12.06	3.8	−3.5
(−)-(*R*)-DP	69.1	8.7	25–6250	0.9975	12.43	5.1	−0.6
(+)-(*S*)-MND	53.7	7.3	25–6250	0.9975	12.57	3.1	0.5
(−)-(*R*)-MND	53.6	5.9	25–6250	0.9977	11.58	4.9	−7.4
Urine							
(+)-(*S*)-DP	97.6	5.8	0.625–62.5	0.9977			
(−)-(*R*)-DP	96.8	7.3	0.625–62.5	0.9995			
(+)-(*S*)-MND	77.5	8.9	0.625–62.5	0.9839			
(−)-(*R*)-MND	78.3	8.8	0.625–62.5	0.9954			

[a] Concentration range in ng/ml for plasma and μg/ml for urine; RSD, relative standard deviation, *r*, correlation coefficient; *E*, systematic error.

Table 2
Analysis of the precision and accuracy of the method for analysis of DP and MND enantiomers in plasma and urine[a]

	Within-day				Between-day			
	Concentration	*n*	RSD (%)	*E* (%)	Concentration	*n*	RSD (%)	*E* (%)
Plasma (40 ng/ml)								
(+)-(*S*)-DP	37.78	10	5.3	−5.5	43.27	5	6.9	8.2
(−)-(*R*)-DP	41.91	10	6.5	4.77	42.49	5	5.8	6.2
(+)-(*S*)-MND	41.95	10	4.8	4.87	41.97	5	9.7	4.9
(−)-(*R*)-MND	39.92	10	4.2	−0.2	40.15	5	6.7	0.4
Plasma (400 ng/ml)								
(+)-(*S*)-DP	408.13	10	6.3	2.0	420.18	5	5.7	5.0
(−)-(*R*)-DP	416.27	10	3.2	4.1	419.89	5	5.5	5.0
(+)-(*S*)-MND	411.62	10	7.5	2.9	409.01	5	8.3	2.2
(−)-(*R*)-MND	378.00	10	7.2	−5.5	400.71	5	8.0	0.2
Urine (1.0 μg/ml)								
(+)-(*S*)-DP	1.01	10	8.5	1.0	1.02	5	5.1	2.0
(−)-(*R*)-DP	1.01	10	8.7	1.0	1.05	5	5.1	5.0
(+)-(*S*)-MND	0.98	10	8.9	−2.0	0.94	5	8.2	−6.4
(−)-(*R*)-MND	0.99	10	9.9	−1.0	0.97	5	8.8	−2.7
Urine (20.0 μg/ml)								
(+)-(*S*)-DP	21.39	10	4.1	6.9	21.28	5	5.3	6.4
(−)-(*R*)-DP	21.51	10	4.5	7.7	21.26	5	5.7	6.3
(+)-(*S*)-MND	22.35	10	5.0	11.7	22.02	5	3.9	10.1
(−)-(*R*)-MND	22.04	10	4.9	10.2	21.43	5	6.6	7.15

[a] *n*, Number of determinations; RSD, relative standard deviation; *E*, systematic error.

curves and the cumulative urinary excretion curves of DP and MND enantiomers obtained from a healthy subject treated with a single dose of *rac*-DP (100 mg). Although our data were obtained from only one subject, our results (Table 4) are in agreement with the literature [9,13]. The preliminary

Table 3
Drugs studied as possible interferents for the determination of DP and MND enantiomers in plasma[a]

Drug	t_R (min)	Concentration (μg/ml)	Drug	t_R (min)	Concentration (μg/ml)
(+)-(*S*)-DP	5.8	0.625	Diazepam	28.0	2.50
(−)-(*R*)-DP	6.9	0.625	(*RS*)-Fenfluramine	3.0	0.15
(+)-(*S*)-MND	14.4	0.625	Flunitrazepam	32.8	0.02
(−)-(*R*)-MND	16.9	0.625	Flurazepam	29.7	0.03
(*RS*)-Metoprolol	8.0; 10.5	1.25	Imipramine	29.0	0.25
Alprazolam	43.3	0.02	(*RS*)-Lorazepam	28.2; 36.0	0.24
Amiodarone	4.6	2.20	(*RS*)-Mexiletine	ND	2.00
(*RS*)-Atenolol	40.1; 42.4	0.40	Phenytoin	ND	20.00
Bromazepam	20.6	0.17	Primidone	12.7; 17.5	12.00
Carbamazepine	17.7	12.00	Propiphenazone	6.6	5.50
Chlorpromazine	31.0	0.12	(*S*,2*R*)-Propoxiphen	35.4; 36.3	0.75
Clobazam	23.4	0.30	(*RS*)-Propranolol	4.4; 5.9	2.5
Clonazepam	23.0	0.07	(*RS*)-Salbutamol	7.2; 7.9	0.20
n-Desmethyl diazepan	12.2	0.48	(*RS*)-Verapamil	6.7; 7.5	0.20

[a] t_R, Retention time; ND, not detected.

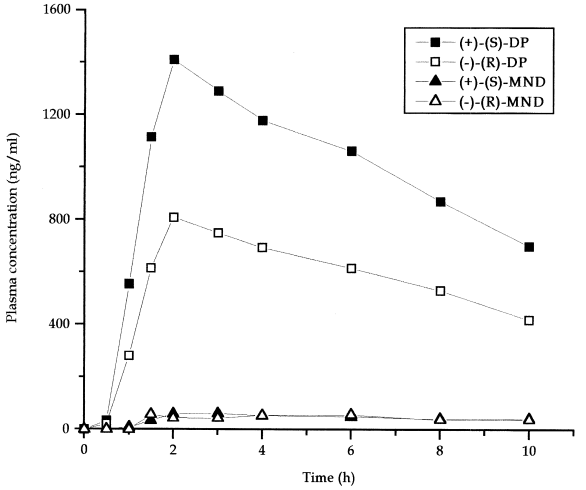

Fig. 4. Time–concentration profiles of DP and MND enantiomers after oral administration of racemic DP to a healthy volunteer.

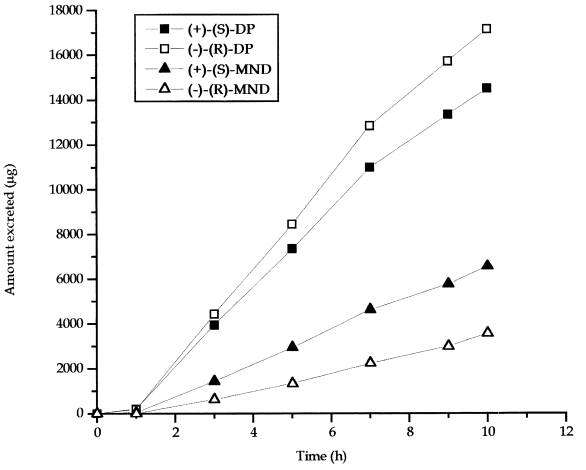

Fig. 5. Cumulative urinary excretion–time curves for DP and MND enantiomers after oral administration of *rac*-DP to a healthy volunteer.

Table 4
Pharmacokinetic parameter of DP and MND enantiomers after oral administration of 100 mg of the racemate to a healthy subject

Parameter	(+)-(*S*)-DP	(−)-(*R*)-DP	(+)-(*S*)-MND	(−)-(*R*)-MND
C_{max} (μg/ml)	1.41	0.81	0.06	0.05
t_{max} (h)	2.00	2.00	3.00	4.00
AUC^{0-10} (μg/h/ml)	9.53	5.55	0.43	0.42
$AUC^{0-10}_{(+)}/AUC^{0-10}_{(-)}$	1.72	–	1.02	–
CL_T/f (ml/min)	58.72	44.58	–	–
$t_{1/2}$ (h)	9.10	12.10	11.00	9.00
V_d/f (l)	35.04	61.43	–	–
Fel/f (%)	29.00	34.30	15.00	8.10
CL_R (ml/min)	25.40	35.31	255.53	142.86

results of this pharmacokinetic investigation suggest that the assay is sensitive enough to be used in pharmacokinetic studies of DP.

4. Conclusion

The method described in the present study is simple and rapid. In the validation step, the method proved to be highly reproducible and accurate, with RSDs and systematic errors in the range of 10%. The quantitation limit of 12.5 ng/ml for all enantiomers permits the use of the method in studies of kinetic disposition.

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