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Simultaneous determination of disopyramide and its mono-N-dealkylated metabolite enantiomers in human plasma and urine by enantioselective high-performance liquid chromatography

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ABSTRACT

Enantiomers of disopyramide (DP) and its mono-N-dealkylated metabolite (MND) were determined in human plasma and urine by enantioselective high-performance liquid chromatography using a chiral stationary-phase column. This method was precise and sensitive: the mean recoveries from plasma at a concentration of 0.5 μg/ml were 101.1% for (+)-DP, 98.0% for (−)-DP, 94.4% for (+)-MND and 82.9% for (−)-MND; the within- and between-day coefficients of variation at the same concentration were 4.4 and 3.3% for (+)-DP, 4.7 and 4.1% for (−)-DP, 6.5 and 4.1% for (+)-MND and 7.8 and 2.4% for (−)-MND for plasma; the lower detection limits were 40 ng/ml for (+)-DP, 80 ng/ml for (−)-DP, 100 ng/ml for (−)-MND and 200 ng/ml for (+)-MND, for 0.5 ml of plasma and 0.2 ml of urine. The ultrafiltration technique was used for determination of the unbound concentration of DP enantiomers in plasma. A preliminary study of the determination of DP and MND enantiomers in plasma and urine samples from a healthy subject given racemic DP demonstrated the clinical applicability of the present method for therapeutic monitoring and pharmacokinetic studies.

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INTRODUCTION

Disopyramide (DP), 4-(diisopropylamino)-2-(2-pyridyl)-2-phenylbutyramide, is an effective and widely used agent for treatment of ventricular and supraventricular arrhythmias [1,2]. Although DP is available commercially as a racemic mixture, enantiomeric differences in the pharmacokinetics of DP, such as plasma clearance, renal clearance, half-life, apparent volume of distribution and plasma protein binding, have been reported by a number of workers [3–6]. With regard to the pharmacodynamics, it is suggested that the electrophysiological effects of DP enantiomers are different, only the (+)-isomer prolonging QTc duration and having a less negative inotropic effect than the (−)-isomer [7,8]. On the other hand, the mono-N-dealkylated metabolite (MND), 4-(isopropylamino)-2-(2-pyridyl)-2-phenylbutyramide, which is the only known metabolite in humans [9,10], has a more potent anticholinergic side-effect than DP [11,12]. Therefore, it might be of clinical value to determine the individual enantiomers of DP and MND simultaneously after administration of racemic DP. Furthermore, since the electrophysiological effects show a better correlation with the unbound concentration than with the total concentration of DP [13,14], and the plasma protein binding of DP is not only concentration-dependent but also may involve in vivo interaction between the two enantiomers after racemic DP administration [3,15], we also established a method for determining the unbound concentration of DP enantiomers using the ultrafiltration technique.

Previous methods for the simultaneous determination of DP enantiomers in biological fluids include a high-performance liquid chromatographic (HPLC) assay with mass spectrometric detection [3]. However, this method seems to have some disadvantages for routine analysis, since it requires deuterium-labelled pseudoracemates and also involves rather complicated procedures before the mass spectral analysis. Therefore, several HPLC assays using α_1-acid glycoprotein (AGP) either as the chiral stationary phase or as the chiral complexing agent in the mobile phase have been developed for resolving DP enantiomers [16–18]. However, in our experience, this AGP column has serious problems with regard to durability as well as its cost and lot differences. Recently, various types of column with a chiral stationary phase using cellulose triphenylcarbamate derivatives have become available for resolving many enantiomers [19,20]. Previously, we succeeded in simultaneous determination of propranolol enantiomers using one of these columns [21]. In the present study, we employed a 5-cm column with a different chiral stationary phase for determination of enantiomers of DP and its N-dealkylated metabolite in human plasma and urine. The procedure involves only one solvent-extraction step, followed by HPLC separation with UV detection.

EXPERIMENTAL

Chemicals

Racemic DP, MND and bufetolol, 1-*tert.*-butylamino-3-[*o*-(tetrahydrofurfuryloxy)phenoxy]-2-propanol, were supplied by Nippon Roussel K.K. (Tokyo, Japan) and Yoshitomi Pharmaceutical Industries (Osaka, Japan), respectively. (+)- and (−)-DP were kindly provided by Dr. Hirotoshi Echizen (Division of Clinical Pharmacology, Clinical Research Institute, National Medical Center, Tokyo, Japan). The chemical and stereochemical purities (greater than 99.5%) of (+)- and (−)-DP were ascertained by determining the optical rotation and melting point, and by stereospecific HPLC resolution and elemental analysis. All other chemicals used were of reagent grade.

Apparatus and chromatographic conditions

The chromatographic system consisted of a Shimadzu LC-6A pump (Kyoto, Japan) and a Shimadzu SPD-6AV variable-wavelength UV detector set at 260 nm with a range of 0.01 or 0.02 a.u.f.s. A Chiralcel OF column (5 cm × 4.6 mm, Daicel Chemical Industries, Tokyo, Japan), which shows effective chiral recognition ability towards compounds containing the dihydropyridine group, was used with a mobile phase of 18% (for plasma samples) or 17% (for urine samples) 2-propanol and 0.1% diethylamine in hexane. The flow-rate was 0.6 ml/min, and separation was performed at ambient temperature.

Extraction procedure

A 0.2-ml volume of 2 *M* sodium hydroxide, 0.1 ml of bufetolol (50 μg/ml with methanol) as an internal standard and 5 ml of diethyl ether were added to a 0.5-ml sample of plasma or a 0.2-ml sample of urine. The tube was shaken vigorously for 10 min, and then centrifuged at 1760 *g* for 5 min. A 4-ml aliquot of the organic layer was removed and evaporated to dryness in a 40°C water-bath under a stream of nitrogen gas. The residue was reconstituted with 0.1 ml of mobile phase, and a 20-μl aliquot was then injected into the HPLC system. Peaks corresponding to (+)- and (−)-DP were identified by comparing the retention times of the pure standards of enantiomers, and those of (+)- and (−)-MND were identified using a polarimeter (Jasco DIP-181C) with a 350-μl flow-cell set at a wavelength of 435 nm with a mobile phase of 50% 2-propanol and 0.1% diethylamine in hexane.

Standard curves

We prepared standards by adding known amounts of racemic DP, MND and bufetolol to drug-free human plasma (0.5 ml) and urine (0.2 ml) samples to give final concentrations of 0.5, 2.5 and 5.0 μg/ml for plasma and 1.25, 6.25 and 12.5 μg/ml for urine as each enantiomer of DP and MND. The assay of each sample was performed as described above. Calibration curves were con-

structed for each sample assay using the peak-area ratios of analyte to internal standard.

Determination of plasma protein binding of DP enantiomers

^{3}H-Labelled (±)-DP (specific activity, 95.5 GBq/mmol; New England Nuclear, Boston, MA, U.S.A.) was resolved into (+)- and (−)-[^{3}H]DP by adopting a procedure similar to that used for measuring DP enantiomers in human plasma and urine, and they were purified using Bond Elut (C_{18}) (Analytichem International, Harbor City, CA, U.S.A.). A trace amount of ^{3}H-labelled (+)- or (−)-DP enantiomer was added to the plasma (0.5 ml). Following incubation at 37°C for 5 min, the human plasma was ultrafiltered (Ultrafree C3-LGC, Nihon Millipore, Tokyo, Japan) at 1700 *g* for 10 min at 37°C. Radioactivity in the filtrate (50 μl) and plasma was counted in a liquid scintillation counter (LSC-700, Aloka).

In a preliminary experiment, no significant adsorption of DP onto the membrane and ultrafiltration device was observed [less than 0.8% at 25 ng/ml (+)-DP and 1.7% at 15 ng/ml (−)-DP using 100 μl of pH 7.4 phosphate buffer]. The protein concentration in the filtrate was less than the assay limit (2 μg/ml) using the method of Lowry et al. [22].

To validate the ultrafiltration technique for measuring the unbound fraction of DP in plasma, the unbound fraction of a plasma sample just after the equilibrium dialysis (37°C for 4 h, Spectra/por 2, Spectrum Medical Industries, Los Angeles, CA, U.S.A.) was determined by ultrafiltration, and it was found that the method was comparable with the equilibrium dialysis procedure.

The influence of the column separation of (±)-[^{3}H]DP and evaporation of the effluent on the binding properties of DP was assessed by comparing the binding of racemic [^{3}H]DP with that of a racemate prepared by combining equal amounts of the two resolved radiolabelled enantiomers [5]. The difference between the unbound fraction obtained with racemic [^{3}H]DP and that with the prepared racemate was not statistically significant at concentrations of 1 and 3 μg/ml ($n=3$).

Application

One healthy subject was given a 300-mg oral dose of DP (Rythmodan® capsule, Roussel Japan K.K.). Blood samples were collected into heparinized tubes at 0.5, 1, 2, 3, 5, 8, 12 and 24.5 h post-dose, and the plasma was separated immediately after collection. Urine was collected into plastic containers at intervals 0–0.25, 0.25–0.75, 0.75–1.5, 1.5–2.5, 2.5–4.0, 4.0–6.0, 6.0–10.0, 10.0–14.0 and 14.0–37.5 h after DP administration. Plasma and urine samples were stored at −20°C until analysis. The plasma unbound fraction was obtained as described above.

Pharmacokinetic analysis

The total and unbound plasma concentration versus time curves for (+)- and (−)-DP were fitted to a two-compartment model with a first-order absorption, whereas the total concentration versus time curves for (+)- and (−)-MND were fitted to a one-compartment model with a first-order absorption, with the aid of a non-linear least-squares regression computer program [23]. From these fittings, the terminal elimination rate constant (K_{el} or β) and half-life ($T_{1/2}$ or $T_{1/2\beta}$) were determined. The area under the concentration–time curve to infinite time (AUC_∞) was calculated by the trapezoidal rule and extrapolation to infinity (the last measured concentration/K_{el} or β). The oral clearances of total (Cl_t/F) and unbound (Cl_u/F) enantiomer in plasma were determined as $Cl/F = \text{dose}/AUC_\infty$ for either total (AUC) or unbound enantiomer (AUC_u). The renal clearance of total (Cl_r) and unbound ($Cl_{r,u}$) DP enantiomer and total (Cl_r) MND enantiomer, and the partial metabolic clearance through MND (Cl_m) were calculated from the following equations:

$$Cl_r = A_{e(\infty)}/AUC_\infty \quad (1)$$

$$Cl_{r,u} = A_{e(\infty)}/AUC_{u(\infty)} \quad (2)$$

$$Cl_m = A_{em(\infty)}/AUC_\infty \quad (3)$$

where $A_{e(\infty)}$ and $A_{em(\infty)}$ are the cumulative amounts of DP and MND excreted in the urine as DP equivalent until infinite time, respectively.

Differences at p values of less than 0.05 were considered to be statistically significant. Throughout the study, values were expressed as means with standard deviation (S.D.).

RESULTS AND DISCUSSION

Separation of DP and MND enantiomers using a chiral AGP column coupled with an RP-2 pre-column has been described previously [17]. However, the reported method seemed to have some shortcomings, because the resolution between DP enantiomers had decreased dramatically after only ca. 100 samples had been analysed, and the AGP column is costly. Furthermore, lot variation was sometimes evident in our experience.

The chiral stationary phase column we used here showed good resolution even after 1000 injections, and its cost is reasonable, since only a short column length is required. This short column (Chiralcel OF, length 5 cm) is usually used as a pre-column for the 25-cm separation column. The 25-cm column showed non-linearity in its calibration curve, probably owing to adsorption of DP at low concentrations. Thus, instead of the 25-cm column, the pre-column was applicable for analytical purposes showing a shorter retention time.

In the present method, no peak interfering with those of DP and MND en-

antiomers or the internal standard was observed when blank samples of plasma and urine from a healthy subject were analysed (Fig. 1). The retention times were 5.5 min for (+)-DP, 11.0 min for (−)-DP, 15.1 min for (−)-MND and 33.4 min for (+)-MND in plasma, with high resolution factors [24] (R=2.1 for DP enantiomers and R=3.2 for MND enantiomers). Unlike the DP enantiomers, the (−)-isomer of MND eluted before the (+)-isomer.

Calibration curves used for quantification of DP and MND enantiomers in human plasma and urine exhibited excellent linearity, with a correlation coefficient $r > 0.998$ for human plasma and urine. The values of the lower detection limit for this method, defined as five times the level of baseline noise, were 40 ng/ml for (+)-DP, 80 ng/ml for (−)-DP, 100 ng/ml for (−)-MND and 200 ng/ml for (+)-MND, in 0.5 ml of human plasma.

The accuracy of the method was measured at three concentrations, 0.5, 2.5 and 5 μg/ml for plasma and 1.25, 6.25 and 12.5 μg/ml for urine, and were found to be excellent; the between-day coefficients of variation were less than 5.6% and less than 6.4% for both enantiomers of DP and MND in plasma, respectively (Table I). Analytical recovery data are also given in Table I. The overall recoveries ranged from 98.0 to 109.2% for both enantiomers of DP and from 82.0 to 94.4% for both enantiomers of MND in plasma.

Dichloromethane, diethyl ether and chloroform were examined as possible extraction solvents. Diethyl ether showed the least interference and gave high recoveries from plasma and urine.

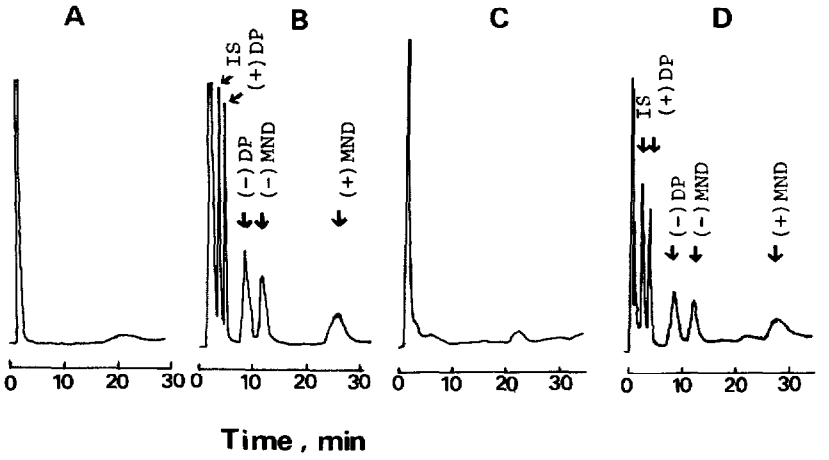

Fig. 1. Chromatograms showing resolution of (+)- and (−)-disopyramide (DP), those of its mono-N-dealkylated metabolite (MND) and the internal standard (IS), bufetolol. Extracts were obtained from (A) blank plasma, (B) plasma sample containing 5 μg/ml each of racemic disopyramide and its mono-N-dealkylated metabolite, (C) blank urine and (D) urine sample containing 12.5 μg/ml each of racemic disopyramide and its mono-N-dealkylated metabolite.

TABLE I

ANALYTICAL PRECISION IN THE DETERMINATION OF DISOPYRAMIDE AND ITS MONO-N-DEALKYLATED METABOLITE ENANTIOMERS IN HUMAN PLASMA

Compound	Concentration (μg/ml)	Coefficient of variation (%)		Recovery (mean ± S.D.)[a] (%)
		Between-day[a]	Within-day[b]	
(+)-DP	0.515	3.3	4.4	101.1 ± 4.5
	2.575	3.0	7.6	106.0 ± 5.3
	5.15	4.5	6.4	109.2 ± 6.4
(−)-DP	0.515	4.1	4.7	98.0 ± 5.2
	2.575	2.6	6.1	103.0 ± 4.7
	5.15	5.6	6.9	108.4 ± 6.1
(+)-MND	0.508	4.1	6.5	94 4 ± 6.3
	2.54	1.3	2.6	82.0 ± 2.3
	5.08	3.8	6.4	93.7 ± 6.9
(−)-MND	0.508	2.4	7.8	82.9 ± 7.1
	2.54	3.4	3.5	85.5 ± 2.9
	5.08	6.4	7.0	85.1 ± 2.3

[a] $n = 5$.
[b] $n = 4$.

A detection wavelength of 260 nm was adopted, at which not only DP but also MND showed maximum absorbance.

We examined possible chromatographic interference from other cardiovascular drugs that may occasionally be coadministered with DP (Table II), including antiarrhythmic drugs (mexiletine and quinidine), calcium channel blockers (verapamil and diltiazem and its four metabolites) and β-blockers (alprenolol, bufetolol, bupranolol, indenolol, penbutolol, pindolol, propranolol and timolol). Although many of the compounds listed in Table II are racemates, none of them except pindolol was optically resolved under the chromatographic condition used. The peaks for mexiletine and diltiazem had similar retention times to those for (−)-DP and (−)-MND, respectively. However, the UV absorbance of mexiletine at 260 nm was so low that it would not be a problem in a clinical situation. None of the other compounds examined showed any interference with the present assay.

The plasma concentration–time curves of the total and unbound DP enantiomers and total MND enantiomers obtained from a healthy subject who received an oral 300-mg dose of DP are illustrated in Fig. 2. The pharmacokinetic parameters are summarized in Table III. There were apparent biexponential declines in the total and unbound concentrations of both DP enantiomers. The plasma concentrations of total (+)-DP were consistently higher than those of (−)-DP after administration of racemic DP, whereas no significant con-

TABLE II

RETENTION TIMES OF SOME CARDIOVASCULAR AGENTS

Drug	Retention time (min)	Drug	Retention time (min)
Alprenolol	1.71	Indenolol	1.99
Bufetolol	3.94	Mexiletine	9.73
Bupranolol	8.28	Penbutolol	1.54
Diltiazem	13.64	Pindolol	3.29 and 4.18[a]
M-1 Diltiazem[b]	9.19	Propranolol	1.98
M-2 Diltiazem[b]	9.41	Quinidine	2.61
M-4 Diltiazem[b]	8.25	Timolol	2.39
M-6 Diltiazem[b]	8.28	Verapamil	8.28

[a]Enantiomers elute at different times.
[b]M-1, M-2, M-4 and M-6 diltiazem are the four metabolites of diltiazem.

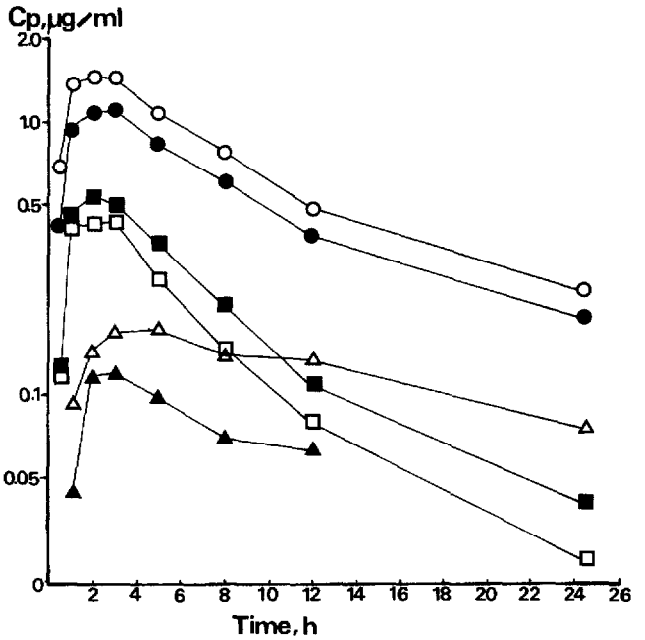

Fig. 2. Plasma concentration-time curves of (+)-disopyramide (○), (−)-disopyramide (●), unbound (+)-disopyramide (□), unbound (−)-disopyramide (■), (+)-mono-N-dealkylated metabolite (△) and (−)-mono-N-dealkylated metabolite (▲) in a normal subject administered a 300-mg oral dose of disopyramide.

centration differences in DP enantiomers have been reported after administration of DP enantiomers separately [3]. As a possible explanation for these distinct pharmacokinetic properties following racemic DP dosing, in vivo in-

TABLE III

PHARMACOKINETIC PARAMETERS OF DISOPYRAMIDE AND ITS MONO-N-DEALKYLATED METABOLITE ENANTIOMERS AFTER ORAL ADMINISTRATION OF THE RACEMATE TO A HEALTHY SUBJECT

Dose, 300 mg.

Parameter	Value			
	(+)-DP	(−)-DP	(+)-MND	(−)-MND
AUC_{∞} (μg h/ml)	20.4	15.7	4.58	3.41
$AUC_{u(\infty)}$ (μg h/ml)	3.67	4.92		
$A_{e(\infty)}$ (mg)	72.5	82.0	37.3 (42.5)[b]	42.5 (58.4)[b]
Cl/F (ml/min)	122.5	159.2		
Cl_u/F (ml/min)	681.2	508 1		
Cl_m (ml/min)			34.7	51.5
Cl_r (ml/min)	59.2	87.0	135.7	207.7
$Cl_{r,u}$ (ml/min)	329.2	277.8		
$T_{1/2,h}$	12.4[a]	13.6[a]	15.4	8.66
$T_{1/2,u,h}$	4.56[a]	6.86[a]		

[a]Values represent $T_{1/2\beta}$ or $T_{1/2\beta,u}$ respectively.
[b]Values in parentheses are mg DP equivalent.

teraction of plasma protein binding between the two enantiomers has been suggested [3,15]. In order to confirm the existence of such an in vivo binding interaction, determination of plasma protein binding of the two enantiomers after administration of the racemate will be necessary. For the present, however, no data are available on this aspect, to our knowledge. Although our data obtained from only one subject are highly preliminary, our results showed that the unbound concentration of (−)-DP was consistently higher than those of (+)-DP, and that the unbound fraction of (−)-DP seems to be higher than that obtained after separate administration of the DP enantiomers [3]. As DP is a low extraction drug, the higher unbound fraction of (−)-DP after administration of the DP racemate may result in a higher total clearance, and hence a lower total concentration of (−)-DP. Therefore, our data may support the possible binding interaction between the two enantiomers after administration of the DP racemate. The partial metabolic clearance (Cl_m) through MND appears to be stereoselective [34.7 ml/min through (+)-MND and 51.5 ml/min through (−)-MND]. Although significance was not observed, dependence of total Cl_r upon the unbound fraction was suggested, as reported previously [3].

Fig. 3 shows the cumulative urinary excretion–time curves of DP and MND enantiomers after administration of 300 mg of racemic DP to a healthy subject. Urinary excretion of DP and MND almost reached a plateau within 37.5 h,

and the percentage recoveries of the dose in urine, (±)-DP 51.5% and (±)-MND 30.3%, were very similar to the results of the previous study [25,26].

Fig. 4 shows the relationship between the unbound fraction and the concentration of (+)- and (−)-DP, respectively. Both DP enantiomers showed non-

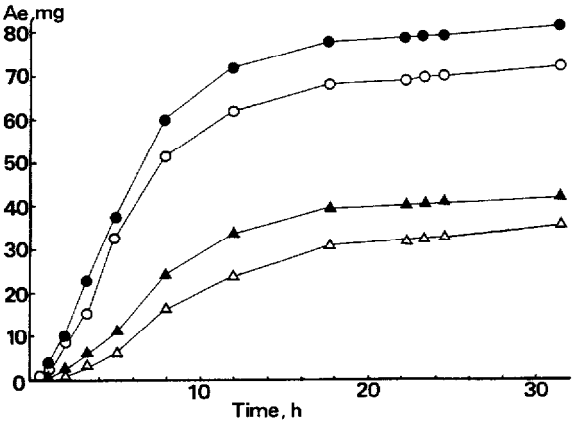

Fig. 3. Cumulative urinary excretion-time curves of (+)-disopyramide (○), (−)-disopyramide (●), (+)-mono-N-dealkylated metabolite (△) and (−)-mono-N-dealkylated metabolite (▲) in a normal subject administered a 300-mg oral dose of disopyramide.

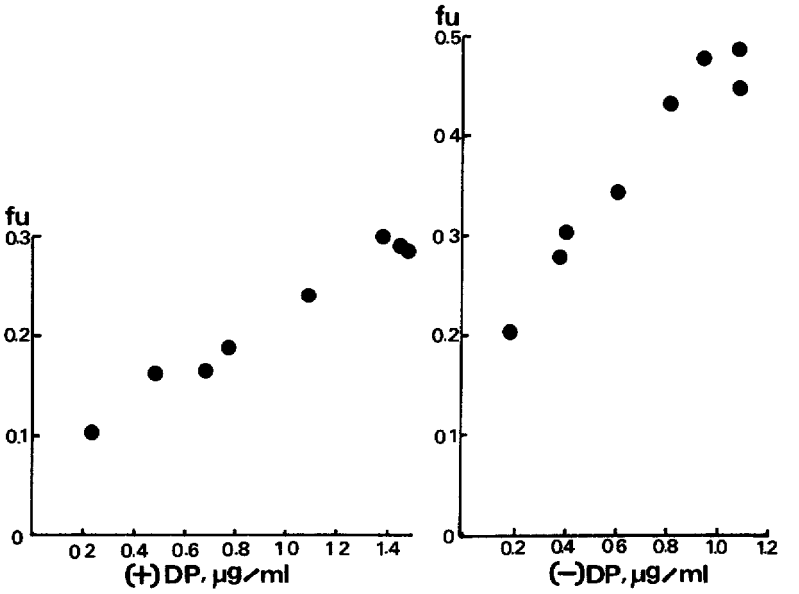

Fig. 4. Unbound fraction (fu) versus concentration in plasma of (+)-disopyramide (left) and (−)-disopyramide (right) in a normal subject administered a 300-mg oral dose of disopyramide.

linear plasma protein binding within this concentration range. The percentage binding of (+)-DP to plasma protein was significantly higher than that of (−)-DP ($p<0.001$). The time-course of the unbound fraction was similar to that of the total concentration of DP enantiomers, suggesting concentration-dependent plasma protein binding.

In conclusion, the results of this study indicate that the method of resolution using chiral stationary phase liquid chromatography is useful not only for therapeutic monitoring but also for pharmacokinetic studies of DP, unbound DP and MND enantiomers in plasma and urine after administration of racemic DP.

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REFERENCES

1 L.A. Siddoway and R.L. Woosley, Clin. Pharmacokin., 11 (1986) 214.
2 R.N. Brogden and P.A. Todd, Drugs, 34 (1987) 151.
3 K.M. Giacomini, W.L. Nelson, R.A. Pershe, L. Valdivieso, K.T. Tamiyasu and T.F. Blaschke, J. Pharmacokin. Biopharm., 14 (1986) 335.
4 P.L. Corre, D. Gibassier, P. Sado and R. LeVerge, Drug Metab. Dispos., 16 (1988) 858.
5 J.J. Lima, Life Sci., 41 (1987) 2807.
6 L. Valdivieso, K.M. Giacomini, W.L. Nelson, R. Pershe and T.F. Blaschke, Pharm. Res., 5 (1988) 316.
7 C. Pollick, K.M. Giacomini, T.F. Blaschke, W.L. Nelson, K.T. Tamiyasu, V. Briskin and R.L. Popp, Circulation, 66 (1982) 447.
8 J.J. Lima and H. Boudoulas, J. Cardiovasc. Pharmacol., 9 (1987) 594.
9 A. Karim, R.E. Ranney and S. Kraychy, J. Pharm. Sci., 61 (1972) 888.
10 C.S. Cook, A. Karim and P. Sollman, Drug Metab. Dispos., 13 (1982) 116.
11 G.W. Holt, R.L.G. Norris, P.J. Ravenscroft, J.H.N. Bett, L.G. Dryburgh and C.M. Boyle, J. Cardiovasc. Pharmacol., 5 (1983) 51.
12 W.T. Chiang, C. von Bahr, B. Calissendorff, R. Dahlqvist, H. Emilsson, A. Magnusson and K.S. Gustafsson, Clin. Pharmacol. Ther., 38 (1985) 37.
13 M. Thibonnier, N.H.G. Holford, R.A. Upton, C.D. Blume and R.L. Williams, J. Pharmacokin. Biopharm., 12 (1984) 559.
14 H.J. Duff, L.B. Mitchell, C.F. Nath, D.E. Manyari, R. Baynton and D.G. Wyse, Clin. Pharmacol. Ther., 45 (1989) 542.
15 K.M. Giacomini and T.F. Blaschke, Clin. Pharmacokin., 9 (1984) 42.
16 J. Hermansson, J. Chromatogr., 269 (1983) 71.
17 J. Hermansson, M. Eriksson and O. Nyquist, J. Chromatogr., 336 (1984) 321.
18 J. Hermansson, J. Chromatogr., 316 (1984) 537.
19 Y. Okamoto, M. Kawashima and K. Hatada, J. Chromatogr., 363 (1986) 173.
20 Y. Okamoto, M. Kawashima, R. Aburatani, K. Hatada, T. Nishiyama and M. Masuda, Chem. Lett., (1986) 1237.

21 H. Takahashi, S. Kanno, H. Ogata, K. Kashiwada, M. Ohira and K. Someya, J. Pharm. Sci., 77 (1988) 993.
22 O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, J. Biol. Chem., 193 (1951) 265.
23 K. Yamaoka, Y. Tanigawara, T. Nakagawa and T. Uno, J. Pharmacobio-Dyn., 4 (1981) 879.
24 B. Testa, Xenobiotica, 16 (1986) 265.
25 C. Graffner, P.O.G. Lagerström, P.N.G. Lundborg and O.F. Rönn, Int. J. Clin. Pharmacol. Ther. Toxicol., 19 (1981) 414.
26 R.P. Kapil, J.E. Axelson, I.L. Mansfield, D.J. Edwards, B. McErlane, M.A. Mason, D. Lalka and C.R. Kerr, Br. J. Clin. Pharmacol., 24 (1987) 781.