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Quantitative determination of disopyramide, verapamil and flecainide enantiomers in rat plasma and tissues by high-performance liquid chromatography

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Abstract

Enantiomers of disopyramide (DP), flecainide (FLC) and verapamil (VP) were extracted from rat plasma and tissues (brain, lung, heart, liver, kidney and muscle), followed by quantitative determination using enantioselective high-performance liquid chromatography with chiral stationary-phase columns. The recoveries of *S*-(+)- and *R*-(−)-DP from tissues were higher than 69%, and the within- and between-day coefficients of variation were very low (0.5 – 5.7%). The lower limits of detection in each tissue were less than 289 ng/g tissue. The recoveries of *S*-(+)- and *R*-(−)-FLC from tissues were higher than 88%, and the within- and between-day coefficients of variation were 1.2–6.0%. The lower limits of detection in each tissue were less than 37 ng/g tissue. The recoveries of *S*-(−)- and *R*-(+)-VP from tissues were higher than 80%, and the within- and between-day coefficients of variation were 0.5–6.2%. The lower limits of detection in each tissue were less than 51 ng/g tissue. The analytical methods established in this study will be suitable for determining the concentrations of the enantiomers of these anti-arrhythmic agents in rat plasma and tissues.

Keywords: Enantiomer separation; Disopyramide; Verapamil; Flecainide

1. Introduction

Many commercially available drugs have chiral centers in their structures and are supplied as racemates. Racemic drugs may show stereoselective pharmacokinetic behavior, with respect to their plasma protein binding, hepatic metabolism, renal excretion and tissue distribution and stereoselective pharmacological effects [1,2].

In this study, in order to study the stereoselective behavior of three anti-arrhythmic agents, disopyramide (DP, class Ia), flecainide (FLC, class Ic) and verapamil (VP, class IV), enantioselective analytical methods to determine the concentrations of their enantiomers in plasma and various tissues were developed. DP and FLC are used widely for the treatment of ventricular and supraventricular arrhythmias [3,4] and VP is used for supraventricular tachyarrhythmias, hypertension and angina pectoris [5]. All three drugs are available racemic mixtures and stereoselective differences in their pharmacokinetics, such as hepatic metabolism, the clearance of unbound drug and plasma protein binding, and pharmacodynamics have been reported by a

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number of workers [6–10]. However, little is known about the stereoselectivity of their tissue distribution.

Some analytical methods for determining tissue concentrations of racemic drugs have been reported [11–14], but none of them involved the separation of the enantiomers. We have reported methods for the simultaneous determination of the concentrations of DP enantiomers in human plasma and urine, and of propranolol enantiomers in human plasma and urine and rat tissues using a chiral stationary-phase column consisting of cellulose triphenylcarbamate derivatives [17,22].

In this study, we developed enantioselective analytical methods to determine the concentrations of the enantiomers in rat plasma and tissues using high-performance liquid chromatography (HPLC) with chiral stationary-phase columns.

2. Experimental

2.1. Chemicals and reagents

Racemic DP and clonidine hydrochloride were gifts from Nippon Roussel K.K. (Tokyo, Japan) and Boehringer Mannheim (Germany), respectively. Racemic FLC acetate and VP hydrochloride were gifts from Eisai (Tokyo, Japan). The (+)- and (−)-enantiomers of these drugs were separated by HPLC as described in Section 2.2, and the chemical and stereochemical purities (both greater than 98.0%) of each enantiomer were ascertained by the optical rotation determination and stereospecific HPLC resolution and elemental analysis, as reported previously [17]. Metoprolol succinate was obtained from Sigma (St. Louis, MO, USA), (+)-propranolol hydrochloride was obtained from Aldrich (Milwaukee, WI, USA) and all the other reagents used were of analytical grade, unless stated otherwise.

2.2. HPLC apparatus and analytical conditions

The HPLC system consisted of a Shimadzu HPLC apparatus (Kyoto, Japan), a LC-6A or 10A HPLC pump and a C-R6A Chromatopac integrator. Samples were introduced into a 50-μl loop (Rheodyne, Cotati, CA, USA). DP was detected by a SPD-6A spectrometric detector operated at a wavelength of 260 nm with a range of 0.02 AUFS. FLC was detected by a RF-550 fluorescence detector operated at respective excitation and emission wavelengths of 295 and 347 nm with a range of low X 4 (plasma, brain heart, liver, kidney and muscle) or X 8 (lung). VP was detected by a RF-535 fluorescence detector operated at respective excitation and emission wavelengths of 272 and 312 nm with a range of low X 1 (plasma, brain and muscle), X 4 (heart, liver and kidney) or X 8 (lung).

Separation of DP enantiomers with a Chiralcel OF column (50×4.6 mm I.D., Lot No. 50118, Daicel Chemical Industries, Tokyo, Japan) and FLC and VP enantiomers with Chiralpak AD columns (250×4.6 mm I.D., Lot No. 13-03-10509, Daicel) were performed at ambient temperature.

The mobile phases comprised hexane–isopropanol–diethylamine [82:18:0.1, 96:4:0.1 and 94:6:0.1 (v/v) for DP, FLC and VP, respectively], at constant flow-rates of 0.6, 1.5 and 1.2 ml/min, respectively. Guard-Pak™ Inserts Resolve C_{18} and C_8 (Waters, Milford, MA, USA) were used as guard columns for DP and both FLC and VP, respectively.

2.3. Sample preparation

Male Wistar rats (230–270 g body weight) were used. Under light anesthesia (ethyl ether), blood was withdrawn from the abdominal artery and ice-cold 0.9% (w/v) sodium chloride was infused into the heart to clear the blood from the other organs. Freshly-isolated tissues (brain, lung, heart, liver, kidney and muscle) were flushed with ice-cold 0.9% (w/v) sodium chloride to remove the blood, blotted on filter paper, weighed and homogenized (1 g/10 ml) in 0.25 *M* sucrose–0.05 *M* phosphate buffer, pH 7.4 using a Potter–Elvehjem apparatus with a PTFE pestle (Iuchi, Tokyo, Japan). The blood was centrifuged at 1000 *g* for 10 min at ambient temperature and the plasma and tissue homogenates were stored at −20°C until assayed.

2.4. Extraction procedures

DP was extracted from plasma as follows: 0.1 ml of 2.0 *M* sodium hydroxide, 0.1 ml clonidine (1.0 μg/ml in mobile phase) as an internal standard and 5 ml benzene were added to 0.1 ml plasma. The

mixture was shaken vigorously for 10 min and then centrifuged at 1000 *g* for 10 min at ambient temperature. A 4-ml aliquot of the organic layer to which 50 μl of 0.2% (w/v) Tween 80 in absolute ethanol had been added was evaporated to dryness under a stream of nitrogen gas in a water-bath at 50°C, the residue was reconstituted with 0.1 ml mobile phase and a 50-μl aliquot was injected into the HPLC system. Peaks corresponding to *S*-(+)- and *R*-(−)-DP were identified by comparing their retention times with those of the authentic enantiomer standards.

DP was extracted from 0.5-ml aliquots of brain, heart and muscle homogenates, as described above for plasma, except carbon tetrachloride was used as the extraction solvent.

A different procedure was used to extract DP from the other tissues (lung, liver and kidney), in order to eliminate interference from the tissue blanks. A 0.1-ml aliquot of 2.0 *M* sodium hydroxide, 0.1 ml clonidine (1.0 μg/ml) and 6 ml carbon tetrachloride were added to 0.5 ml tissue homogenate. The mixture was shaken vigorously for 10 min and then centrifuged at 1000 *g* for 10 min. A 5-ml aliquot of the organic layer was shaken with 0.2 ml 1.0 *M* hydrochloric acid for 10 min, followed by centrifugation at 1000 *g* for 10 min. A 0.2-ml aliquot of the aqueous layer was mixed with 0.1 ml 2.0 *M* sodium hydroxide and 6 ml carbon tetrachloride, shaken for 10 min and centrifuged at 1000 *g* for 10 min. A mixture of 5 ml organic layer and 50 μl 0.2% (w/v) Tween 80 in ethanol evaporated to dryness under a stream of nitrogen gas in a water-bath at 50°C, the residue was reconstituted with 0.1 ml mobile phase and a 50-μl aliquot was injected into the HPLC system.

FLC was extracted as described for DP, except that metoprolol (50 μg/ml in mobile phase) was used as an internal standard and ethyl ether was used as the extraction solvent.

VP was extracted as described for DP, except that (+)-propranolol (0.25 μg/ml in mobile phase) was used as an internal standard and ethyl ether (plasma, heart, lung, liver and kidney) or *n*-hexane (brain and muscle) were used as the extraction solvents.

The within and between-day coefficients of variation (C.V.s) under the above conditions were determined by analyzing five samples once a day and one sample on four different days, respectively. The detection limit in all biological fluids was determined as the signal-to-noise ratio of 3.

2.5. Standard curves

We prepared standards by adding known amounts of racemic DP, FLC or VP and internal standard to drug free rat plasma and tissue homogenates to give final concentrations described in Table 1. The assay of each sample was performed as described above. Calibration curves were constructed for each sample assay using the peak-area of analyte to internal standard.

2.6. Application of the HPLC analytical method

Male Wistar rats (250–300 g) were anesthetized with ethyl ether and polyethylene cannulae (Type SP-10, 0.28 mm I.D.×0.61 mm O.D., Natume, Tokyo, Japan) were inserted in both the femoral vein and artery of one leg just before drug administration.

A loading dose of racemic DP, FLC or VP was infused constantly (13.8, 5.23 or 0.60 mg/h/kg, respectively) through the femoral venous cannula for 30 (DP and FLC) or 20 (VP) min. Thirty min after starting the loading infusion, a maintenance dose was infused at 3.60, 2.62 or 0.30 mg/h/kg, respectively. Blood (0.5 ml) was withdrawn from the femoral artery 120, 150 and 180 min after starting drug administration. The rats were sacrificed by withdrawing whole blood from the abdominal artery 180 min after starting drug administration. Following the procedures described above, the concentrations of the enantiomers in plasma and tissues were determined using the HPLC method established in this study.

The values are expressed as means and the tissue drug concentrations are expressed as μg per g wet tissue weight (μg/g tissue).

3. Results and discussion

We used our HPLC method to determine the concentrations of DP, FLC and VP enantiomers in rat plasma and tissues (brain, lung, heart, liver, kidney and muscle).

Table 1
Precisions and recoveries of analytical methods for DP, FLC and VP enantiomers in rat plasma and tissue homogenates

	Concentration range (μg/ml)	C.V. (%)				Recovery (%)		Detection limit (ng/ml or g tissue)	
		Within-day		Between-day					
		S	*R*	*S*	*R*	*S*	*R*	*S*	*R*
DP									
Plasma	1.1–5.3	0.6–1.1	0.5–2.8	1.8–3.4	3.8–4.2	95–101	95–101	33	83
Brain	1.1–5.3	2.6–4.5	3.8–4.5	2.9–3.1	3.3–4.8	92–95	87–92	87	228
Lung	1.1–5.3	0.7–3.0	1.8–3.4	2.0–3.7	1.9–4.7	69–80	70–79	103	289
Heart	1.1–5.3	1.6–2.7	1.6–5.7	3.6–4.4	3.0–3.8	92–101	92–100	53	146
Liver	1.1–5.3	1.5–5.2	1.8–5.5	2.6–4.3	4.1–5.0	71–78	71–77	100	285
Kidney	1.1–5.3	3.0–4.9	2.9–4.8	1.9–3.6	1.9–3.6	76–77	72–77	79	201
Muscle	1.1–5.3	1.7–3.0	1.4–2.8	2.9–4.7	3.6–4.9	91–94	87–90	70	152
FLC									
Plasma	0.1–1.0	2.5–3.6	3.7–5.4	4.0–4.9	3.6–5.0	93–103	93–103	21	20
Brain	0.2–2.0	4.0–4.4	2.0–3.3	4.5–5.9	4.6–5.8	93–105	93–104	30	33
Lung	2.0–20	1.6–4.0	1.5–3.8	2.9–5.9	3.0–5.6	93–100	88–104	27	27
Heart	0.5–5.0	3.8–5.1	2.9–5.9	5.3–6.0	5.1–6.0	94–102	99–100	30	36
Liver	0.5–5.0	2.5–5.0	2.5–4.3	2.7–5.8	4.4–5.3	95–102	92–101	20	28
Kidney	0.5–5.0	3.4–5.5	1.2–6.0	4.6–5.7	5.0–5.9	100–104	99–104	31	37
Muscle	0.2–2.0	1.8–4.9	2.8–5.3	5.3–6.0	5.1–6.0	94–106	98–108	23	28
VP									
Plasma	0.1–0.5	1.7–3.7	1.4–2.9	1.0–4.5	3.3–6.2	93–102	92–102	2	2
Brain	0.1–1.0	3.2–4.7	2.4–2.8	3.4–5.1	3.6–5.2	80–83	80–86	47	51
Lung	2.0–20	1.5–2.5	0.8–3.0	3.8–4.9	1.7–4.8	91–102	92–100	13	14
Heart	0.2–1.0	1.3–4.3	1.7–3.7	3.2–4.0	3.1–4.7	93–98	91–97	13	16
Liver	0.2–1.0	1.1–2.5	0.5–3.1	4.3–5.2	4.7–5.9	101–102	98–102	20	23
Kidney	0.5–5.0	2.7–3.9	1.7–4.0	2.5–5.8	3.5–5.8	98–101	91–98	15	15
Muscle	0.1–1.0	1.4–3.8	1.9–4.7	2.0–4.3	2.9–4.1	85–94	81–82	34	36

Recovery and within-day C.V. were obtained using five different sample preparations.
C.V. of between-day variation was obtained using four different sample preparations on four days.

3.1. Disopyramide

A method for analyzing tissue concentrations of racemic DP has been reported [9], but it involved the use of gas chromatography–mass spectrometry (GC–MS) and the enantiomers were not separated. In a previous study, we succeeded in determining the concentrations of DP enantiomers in human plasma and urine simultaneously using a chiral stationary-phase column consisting of cellulose triphenylcarbamate derivatives [17]. We tried to use this method with a minor modification, to determine the concentration of each DP enantiomer in rat tissues. None of the chromatograms showed any interfering peaks with the same retention times as the DP enantiomers [*S*-(+)-DP, 7 min; *R*-(−)-DP, 14 min] or internal standard (5 min) (see Fig. 1 for typical chromatogram). Carbon tetrachloride was used to extract DP from brain, heart and muscle, because interfering peaks with the same retention times as DP occurred when benzene was used as extraction solvent. The extraction procedure was modified further in order to eliminate the interfering peaks observed with lung, liver and kidney tissues. After extraction with carbon tetrachloride, DP in the organic layer was reextracted into an aqueous layer under acidic conditions and then the drug in the aqueous layer was reextracted with carbon tetrachloride under alkaline conditions. Using these procedures, the substances deriving from lung, liver and kidney could be eliminated from the chromatograms.

The recovery of each enantiomer from brain, heart

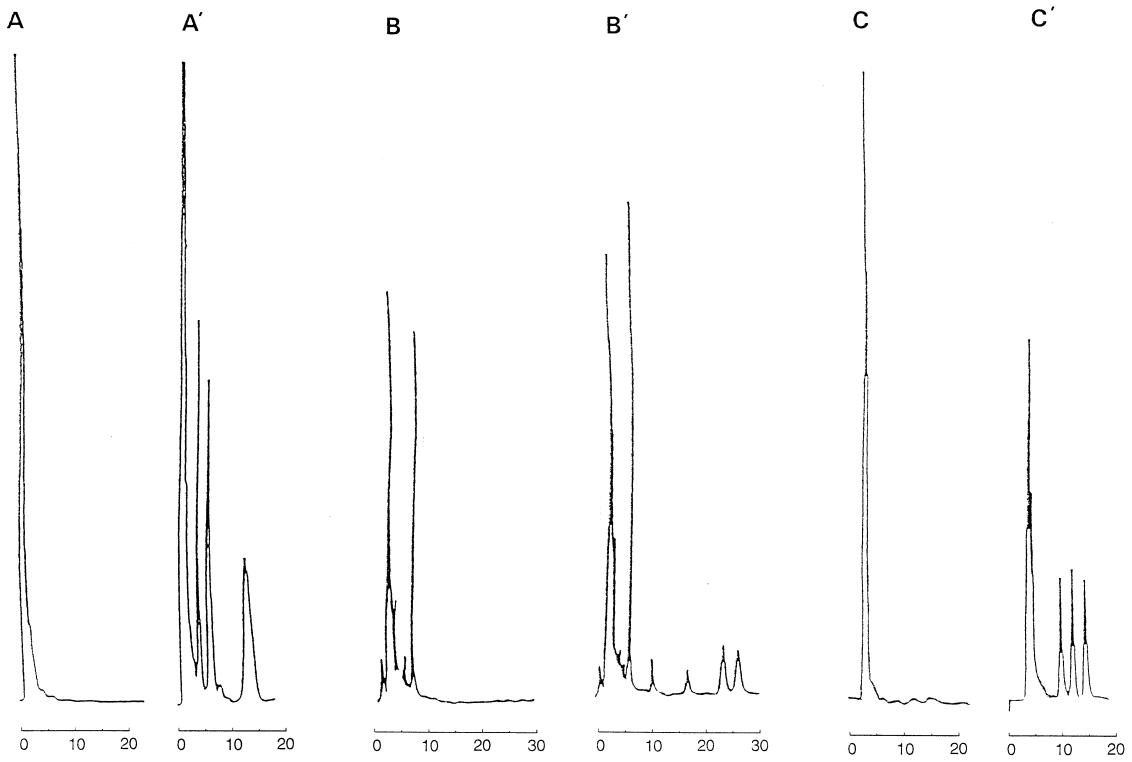

Fig. 1. Typical chromatograms of DP, FLC and VP racemate used to spike rat blank plasma. (A–C) represent chromatograms of blank plasma and (A′–C′) represent chromatograms of DP (5.3 μg/ml), FLC (0.5 μg/ml) and VP (0.2 μg/ml) spiked in the same blank, respectively.

and muscle was higher than 87% with C.V.s below 5.7% (Table 1). The recoveries from lung, liver and kidney were slightly lower due to the more extensive extraction procedure, but did not vary much (C.V.s were 0.7–5.5%).

3.2. Flecainide

Several analytical methods for determining serum concentrations of FLC enantiomers have been reported [15,18,19]. Oda et al. [18] developed a direct-injectable on-line HPLC system, which used an avidin column coupled with an ovomucoid column. This method needs no sample pretreatment, but it is expensive. Alessi-Severini et al. [19] and Turgeon et al. [15] developed HPLC methods in which the drug was derivatized after and before loading the sample onto the analytical column, respectively. This derivatization process is time-consuming. We tried to carry out HPLC using a Chiralpak AD column. None of the chromatograms showed any interfering peaks at the same retention times as the FLC enantiomers [*S*-(+)-FLC, 21 min; *R*-(−)-FLC, 25 min] or internal standard (16 min) when ethyl ether was used as the extraction solvent (Fig. 1).

The recovery of each enantiomer from each tissue was higher than 88% and all the C.V.s were below 6.0% (Table 1).

3.3. Verapamil

Some methods for analyzing racemic VP in tissues involving the use GC–MS after a two-step extraction procedure have been reported [13,14], but analysis of each enantiomer was not performed in these studies. However, enantioselective determination of VP in plasma and urine has been reported: Shibukawa and Wainer [16] reported an enantioselective analytical method using a chiral stationary-phase column and Robinson and Mehvar [20] applied this method to

the determination of enantiomer concentrations in human and rat erythrocytes.

Recently, Hashiguchi et al. [21] analyzed VP enantiomers in plasma using enantioselective HPLC methods developed by Shibukawa and Wainer in order to study the effect of high-protein food on the stereoselective bioavailability and pharmacokinetics of VP in healthy volunteers. We used this assay method with a minor modification to determine VP enantiomer concentrations in rat tissues. None of the chromatograms showed any interfering peaks at the same retention times as the VP enantiomers [(−)-VP, 11 min; (+)-VP, 14 min] or the internal standard (8 min) (see Fig. 1). Hexane was used as the extraction solvent for brain and muscle in order to eliminate the interfering peaks observed when ethyl ether was used as the extraction solvent.

The recovery of each enantiomer from brain and muscle was relatively low but not vary variable (each C.V. was below 5.2%) whereas recovery from each of the other four tissues exceeded 90% with low C.V.s (0.5–5.8%).

Under these HPLC conditions, the peaks corresponded to the metabolites, such as mono-*N*-dealkyldisopyramide and norverapamil (which were major metabolites of DP and VP, respectively), did not interfere with those of parent drugs and could be detected at later retention times than those of parent drugs.

The recovery of each internal standard for the three drugs was also higher than 75% with C.V.s below 5.7% (data not shown).

The chiral stationary-phase columns we used in this study showed good resolution even after 500 injections and no variation of retention times of each enantiomer.

Calibration curves used for quantification of these drug enantiomers in rat plasma and tissue homogenates exhibited excellent linearity, with a correlation coefficient $r>0.997$.

3.4. Stereoselective pharmacokinetics of DP, FLC and VP

The concentrations of each enantiomer in rat plasma and tissues after racemic drug administration were determined using the HPLC method established in this study. Fig. 2 shows the time-courses of the mean plasma concentrations after constant infusion of racemic drug in rats ($n=2$). All three drugs were maintained at steady state 180 min after starting the infusions. The plasma concentration of *R*-(−)-DP was about 3-times that of *S*-(+)-DP, the plasma concentrations of the FLC enantiomers did not differ, whereas the plasma concentration of *S*-(−)-VP was about twice that of *R*-(+)-VP.

Fig. 3 shows the mean enantiomer concentrations in tissues after constant infusions of the racemic drugs in rats ($n=2$). The concentrations of *R*-(−)-DP were higher than those of *S*-(+)-DP in all the tissues studied, whereas those of the FLC enantiomers did not differ. The concentration of *R*-(+)-VP, especially in lung tissue was higher than that of *S*-(−)-VP, the converse of the plasma result. These results suggest that stereoselective distribution may occur after administration of racemic, basic anti-arrhythmic drugs.

In conclusion, the results of this study indicate that

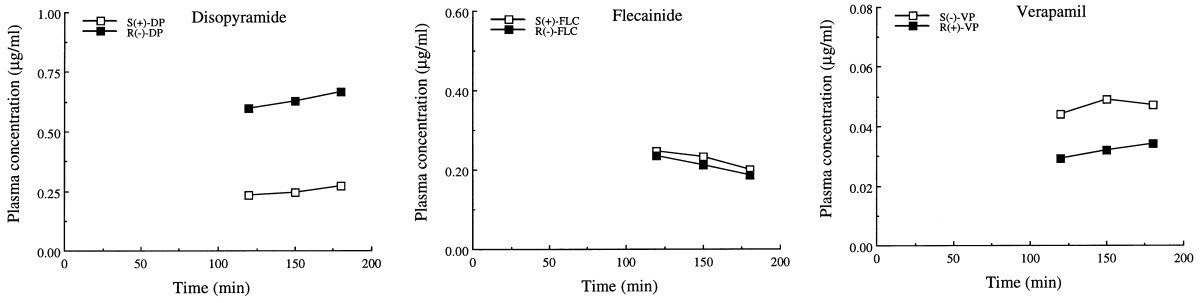

Fig. 2. Time courses of enantiomer concentrations in plasma of DP, FLC and VP after racemic drug administration. Each point represents mean ($n=2$). Doses of these drugs are given in detail in Section 2.6.

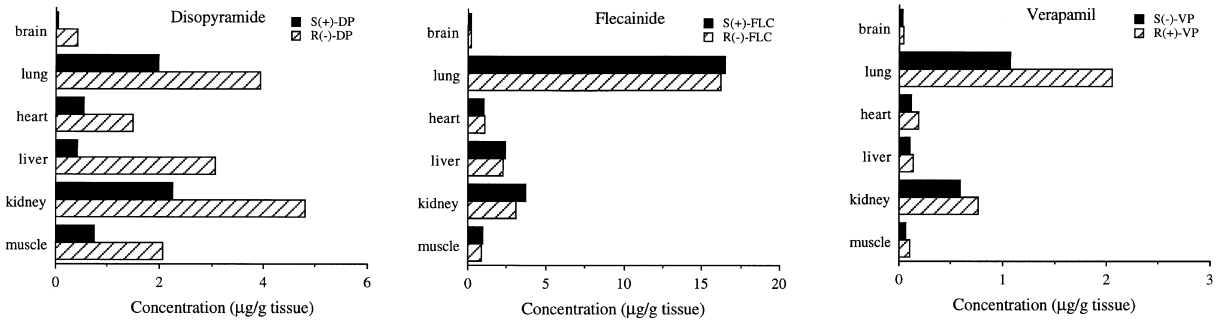

Fig. 3. Concentration of enantiomers in tissues of DP, FLC and VP after racemic drug administration. Each column represents mean ($n=2$). Doses of these drugs are given in detail in Section 2.6.

the methods of resolution and HPLC using a chiral stationary-phase we developed were suitable for the purpose of enantioselective determination of DP, FLC and VP concentrations in rat plasma and tissues, such as brain, heart, lung, liver, kidney and muscle. We have demonstrated that this method is suitable for studying the stereoselective pharmacokinetics of these drugs.

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