Journal of Chromatography, 424 (1988) 424–429
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3961

Note

Simultaneous assay of disopyramide and monodesisopropyldisopyramide enantiomers in biological samples by liquid chromatography

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(First received July 2nd, 1987; revised manuscript received September 1st, 1987)

Disopyramide (DP), a widely prescribed antiarrythmic agent, is marketed as a racemate of two enantiomers (Rythmodan, Lab. Roussel, Paris, France). In humans, separate administration of DP enantiomers displays stereoselectivity in the metabolism [1]. The plasma protein binding, which involves essentially the α_1-acid glycoprotein (α_1-AGP), is stereoselective [1,2] and concentration-dependent [3–6]. Thus it is important to study how the enantiomers are processed in the body when the drug is administered as a racemate.

Several approaches allow stereospecific chromatographic analysis of drug enantiomers either by optical resolution with or without diastereoisomer formation or by isotopic derivatization.

Optical resolution with diastereoisomer formation may be achieved (a) indirectly by formation of covalent diastereomeric compounds prior to the chromatographic analysis or (b) directly with the use of a chiral agent (present in either of the two phases), which generates in situ diastereomeric complexes with the compound during chromatography.

Optical resolution without diastereoisomer formation consists of using stereoselective stationary phases, which are obtained by binding on a chromatographic support a macromolecule (natural or synthetic) that has a stereoselective behaviour towards enantiomers. A stereoselective liquid chromatographic method for the determination of DP enantiomers has been described by Hermansson et al. [7]; the stationary phase was human α_1-AGP.

Isotopic derivatization requires the use of a pseudoracemate, one of the enantiomers being labelled with a stable isotope on a molecular site that is not involved

0378-4347/88/$03.50

in the metabolism. Determination of the relative amounts of each enantiomer in biological samples may be performed by two different techniques. The first involves a single-step analysis of the compounds by gas chromatography (GC) or high-performance liquid chromatography (HPLC) coupled directly with mass spectrometry (MS); differentiation between enantiomers is based on the mass difference between labelled and unlabelled forms. In the second, an indirect procedure, the compounds of interest are isolated from biological samples with HPLC by collection of a fraction of the mobile phase. Subsequent analysis by MS allows determination of the enantiomeric ratio of labelled to unlabelled compound. Concurrently, the concentration of the pseudoracemate in the samples is determined. Thus the concentrations of each enantiomer are calculated from the enantiomeric ratio and the concentration of the pseudoracemate. Such a multi-step technique was used by Giacomini et al. [8] in a pharmacokinetic study of DP enantiomers when co-administered.

Unlike optical resolution without diastereoisomer formation, isotopic derivatization and optical resolution with diastereoisomer formation are time-consuming and costly. In addition, other drawbacks should be taken into account: (a) for optical resolution with diastereoisomer formation, the risk of different reaction rates of the enantiomers with the chiral reagent; (b) for isotopic derivatization, the necessity of demonstrating the lack of an in vivo isotopic effect in the metabolism, especially if deuterated compounds are used [9].

Furthermore, although stable-isotope-labelling techniques may be entirely suitable for pharmacokinetic studies in healthy volunteers, it is not the same for clinical pharmacokinetic studies when a therapeutic effect is required. Since the pseudoracemate is not an active drug and its pharmacological properties may be different on account of the isotopic labelling, ethical problems may arise.

Up to now, two methods allowing a stereospecific evaluation of DP enantiomers have been published [7,8]; these methods, however, did not evaluate the enantiomers of the monodesisopropyldisopyramide (MND).

In humans, mono-N-dealkylation is the only known metabolic pathway of DP [10]. This metabolic pathway is stereoselective [1], and because it does not affect the chiral carbon centre [10], the metabolite formed should be stereochemically related to the parent DP enantiomer. The stereoselective biotransformation of DP, when administered as a racemate, has not yet been studied.

The advantage of the method herein described, referring to a method published by Hermansson et al. [7], is the simultaneous assay of $R(-)$ and $S(+)$-DP and $R(-)$- and $S(+)$-MND in biological samples (plasma or urine). The present method was applied to a study of the stereoselective metabolism and pharmacokinetics of DP enantiomers after co-administration as a racemate as part of a clinical pharmacokinetic study as well as to the determination of the purity of DP enantiomer standards.

EXPERIMENTAL

Chemicals and reagents

DP and MND were supplied, as racemic bases, by Roussel UCLAF (Paris, France). Ethyl acetate, formic acid, sodium hydroxide, monosodium phosphate

(Merck, Darmstadt, F.R.G.) and 2-propanol (Farmitalia Carlo Erba, Milan, Italy) were of analytical grade.

Apparatus

Chromatography was performed via a Waters Model 6000 B pump (Waters Assoc., Milford, MA, U.S.A.) equipped with a Waters Model WISP 710 B automatic injector, a Waters Model 440 detector set at 254 nm and a Delsi Model Enica 21 integrator (Delsi, Suresnes, France).

The columns, coupled in series, were a Supelco Model Supelcosil LC-8-DB reversed-phase (50×4.6 mm I.D.) (Supelco, Bellefonte, PA, U.S.A.) and an LKB Model Enantiopac (100×4 mm I.D.) (LKB, Bromma, Sweden).

Extraction procedure

To each plasma sample (250 μl) were added distilled water (500 μl) and 1 *M* sodium hydroxide (100 μl). DP and MND enantiomers were then extracted with ethyl acetate (4 ml) by shaking for 2 min. The tubes were centrifuged at 1500 *g*, and the whole organic phase was transferred to a conical tube that contained 0.1 *M* formic acid (100 μl). The conical tube was shaken for 1 min and centrifuged at 1500 *g*, then the organic phase was discarded. The formic phase was evaporated to dryness under a gentle stream of nitrogen at 20°C. The residue was dissolved in 50 μl of the mobile phase, and 20-μl samples were injected.

Since urine concentrations of DP and of its metabolite are generally higher than plasma concentrations, urine samples (10 μl) were diluted with drug-free plasma (240 μl) and then handled according to the procedure described.

Chromatographic conditions

The mobile phase contained phosphate buffer (8 m*M*, pH 6.20)–2-propanol (92:8, v/v). Isocratic chromatography was carried out at a flow-rate of 0.3 ml/min.

RESULTS AND DISCUSSION

Chromatographic studies

Since the affinity constants of *R*(−)-DP and of *S*(+)-MND for human α_1-AGP are quite similar, the simultaneous resolution of the four optical species with the chiral column alone was not possible, despite modifications to the mobile phase. Thus, a previous separation of the two chemical species on an hydrophobic packed column was necessary. Such a technique was employed by Hermansson et al. [7] in the plasma determination of DP enantiomers; however, quantitation of MND enantiomers was not carried out.

The chromatographic data are reported in Table I; the resolution factor (R_s) was determined by

$$R_s = \frac{2(t_R^{S(+)} - t_R^{R(-)})}{w_t^{R(-)} + w_t^{S(+)}}$$

where t_R is the retention time and w_t is the base width.

TABLE I

CHROMATOGRAPHIC DATA

Conditions as described under Experimental.

Compound	$k'_{R(-)}$	$k'_{S(+)}$	α	R_s
Disopyramide	2.67	3.93	1.47	1.81
Monodesisopropyldisopyramide	1.40	1.87	1.34	1.27

Enantiomeric purity of standards

The optical resolution of racemic disopyramide base was performed according to the method described by Burke et al. [11]. Enantiomeric purity was evaluated by the chiral chromatographic method. Reversed-phase chromatography was not necessary for this application, and the α_1-AGP column was used alone; all other chromatographic conditions were as described under Experimental. Solutions of both DP enantiomer standards (20 μg/ml) and racemic DP (10 μg/ml) were prepared in the mobile phase and then directly injected ($n=10$) onto the column.

The relative amounts of $R(-)$-DP and $S(+)$-DP in the two standards were determined by comparison with the racemic standard [50:50 mixture of $R(-)$-DP and $S(+)$-DP]. Thus this method allows direct determination of enantiomeric purity. The results indicate that the purity of $R(-)$- and $S(+)$-DP standards were 97.7 ± 0.1 and $96.2 \pm 0.3\%$, respectively. This level of purity is suitable for administration in human pharmacokinetic studies.

Quality control

It has been demonstrated for DP that the binding of $S(+)$-DP to human α_1-AGP was stronger than that of $R(-)$-DP [2]. Accordingly, the extents of extraction of $R(-)$-DP and $S(+)$-DP and of $R(-)$-MND and $S(+)$-MND were compared.

A human plasma sample was spiked with 0.01 *M* hydrochloric acid aqueous standard solutions of DP and MND as racemic bases at 1 and 0.5 μg/ml, respectively, and then divided into ten aliquots. Each sample was handled according to the procedure described and compared with a standard solution.

The data reported in Table II show, for each enantiomer, the extraction yield and the coefficient of variation. The extraction yields were 79.5 and 79.9% for

TABLE II

EXTRACTION YIELD

Compound	Concentration (μg/ml)	Yield (%)	Coefficient of variation (%)	*n*
$R(-)$-DP	1	79.5	5.5	10
$S(+)$-DP	1	79.9	5.7	10
$R(-)$-MND	0.5	82.3	5.3	8
$S(+)$-MND	0.5	82.1	6.6	8

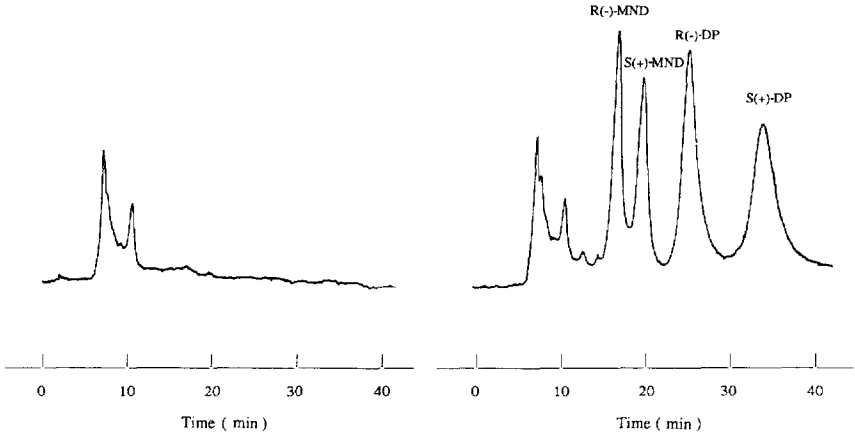

Fig. 1. Chromatograms of extracted plasma. (Left) Blank plasma sample. (Right) Plasma containing $R(-)$-DP and $S(+)$-DP at 1 μg/ml and $R(-)$-MND and $S(+)$-MND at 0.5 μg/ml. Chromatographic conditions as described under Experimental.

$R(-)$-DP and $S(+)$-DP ($n=10$) and 82.3 and 82.1% for $R(-)$-MND and $S(+)$-MND ($n=8$), respectively. The lack of significant difference ($p<0.05$) shows that the extraction procedure described is not stereoselective. The coefficients of variation for the extraction procedure were 5.5 and 5.7% for $R(-)$-DP and $S(+)$-DP and 5.3 and 6.6% for $R(-)$-MND and $S(+)$-MND, respectively.

The specificity of the simultaneous analysis of DP and MND enantiomers is illustrated in Fig. 1.

The linearity of the present method was studied in the concentration range 0.5–4 μg/ml for DP enantiomers and 0.125–1 μg/ml for metabolite enantiomers. Standard curves were constructed by plotting the peak heights (y) versus the total plasma concentration (x), in μg/ml. The correlation coefficients (r^2) were 0.9989 for $R(-)$-DP, 0.9998 for $S(+)$-DP, 0.9996 for $R(-)$-MND and 0.9999 for $S(+)$-MND. The equations of the standard curves, fitted by linear regression, and their correlation coefficients are reported in Table III.

The reproducibility was checked daily ($n=12$) at 1.5 μg/ml for $R(-)$-DP and $S(+)$-DP and at 0.375 μg/ml for $R(-)$-MND and $S(+)$-MND. The coefficients of variation, 6.7% for $R(-)$-DP and $S(+)$-DP and 8.5% for $R(-)$-MND

TABLE III

CALIBRATION CURVE DATA

Compound	Concentration range (μg/ml)	Correlation coefficient (r^2)	Slope	y-Intercept
$R(-)$-DP	0.5–4	0.9989	111.7	−32.7
$S(+)$-DP	0.5–4	0.9998	66.7	−17.0
$R(-)$-MND	0.125–1	0.9996	242.1	−10.2
$S(+)$-MND	0.125–1	0.9999	184.8	−5.9

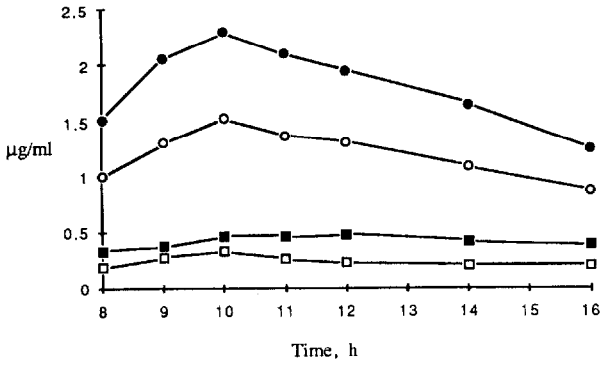

Fig. 2. Total plasma concentration-time curves of $R(-)$-DP (○), $S(+)$-DP (●), $R(-)$-MND (□) and $S(+)$-MND (■) in a subject receiving racemic disopyramide at a dose of 200 mg three times a day.

and 7.7% for $S(+)$-MND, indicate a suitable reproducibility of the present method.

Pharmacokinetic application

Fig. 2 presents a typical concentration–time curve for DP and MND enantiomers extracted from six subjects who received racemic DP (200 mg three times a day) for treatment of arrythmias. Blood samples were drawn on the fifth day after administration. It can be seen that levels of $S(+)$-DP are always higher than those of $R(-)$-DP, with a $S(+)$-DP/$R(-)$-DP plasma peak ratio of 1.5. These results indicate an interaction between DP enantiomers, when co-administered, since when they are administered separately, their total plasma concentrations are quite similar [8,12]. Such results agree with those obtained by Giacomini et al. [8] in a pharmacokinetic study involving healthy subjects after single parental administration of DP as a pseudoracemate. Work is in progress to determine the source of this interaction, which may involve biotransformation and/or distribution.

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