Journal of Chromatography, 426 (1988) 417–424
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4090

Note

Enantiomeric analysis of a new anti-inflammatory agent in rat plasma using a chiral β-cyclodextrin stationary phase

A.M. KRSTULOVIĆ*, J.M. GIANVITI, J.T. BURKE and B. MOMPON

L.E.R.S.-Synthelabo, 58, rue de la Glacière, 75013 Paris (France)

(First received November 9th, 1987; revised manuscript received December 21st, 1987)

In the past several years there has been a tremendous impetus to develop efficient analytical and preparative separations for a wide variety of enantiomeric compounds. The search for suitable methods of analysis has been fuelled by the demands on analytical chemists, particularly in pharmaceutical companies, since it is known that enantiomers may have different biological activities, different action and/or toxicities, transport mechanisms and routes of metabolism. It is also possible that the inactive or less active enantiomer may compete for receptor binding sites with the more active isomer and thus hinder the activity of the latter.

It was estimated in 1982 that 60% of the top 200 prescribed drugs contained at least one asymmetric centre [1]. For economic reasons many bioactive agents are marketed as racemates. Since the aim of drug synthesis is to produce as pure a chemical as possible with the desired action, during the initial stages of drug testing it is important to develop methods capable of separating the enantiomers in order to determine their relative potencies, toxicities and side-effects. Traditionally, racemic mixtures are resolved by fractional recrystallization of diastereomeric salts or by reaction of a racemate with an optically active reagent to form diastereomeric esters, amides, carbamates, etc., which are separated by conventional means. However, these methods are often tedious, not always efficient and certainly limited in their applicability. By now it is clear that the more elaborate approach through chiral high-performance liquid chromatography (HPLC) or gas chromatography (GC) is the method of choice for the determination of enantiomeric purity, since these techniques offer both speed of analysis and separation efficiency. However, GC is notoriously plagued by the limitations of vol-

0378-4347/88/$03.50 © 1988 Elsevier Science Publishers B.V.

atility and thermal stability of the solutes. Thus HPLC on the analytical or semi-preparative scale appears of more general use, and interesting results have been obtained by using chiral chemically bonded stationary phases and achiral stationary phases with chiral mobile phases [2,3].

A widely accepted rationale for chiral recognition is that of Dalgliesh [4]. Several strategies are available for the provision of the three molecular interaction sites: charge transfer, π–π interactions, dipole interactions, hydrogen bonding and steric interactions.

The direct separation of isomeric compounds through formation of cyclodextrin inclusion complexes (CD) was first exploited in thin-layer chromatography (TLC) by Armstrong [5] and by Hinze and Armstrong [6]. Since then, CDs have been used extensively in HPLC either as mobile phase additives or chemically bonded to the stationary phase. This topic has been recently reviewed [7]. The factors reportedly responsible for the enantioselectivity have been discussed in the literature [8–10].

trans-6,6a,7,10,10a,11-Hexahydro-8,9-dimethyl-11-oxodibenz[*b,e*]oxepin-3-acetic acid, a compound recently developed by our company [11], is a promising anti-inflammatory agent. In the course of a study of the bioavailability of this molecule, it was important to devise a method capable of measuring the two enantiomers. This paper describes the analysis of their levels in rat plasma via HPLC with a chiral β-cyclodextrin-bonded stationary phase.

EXPERIMENTAL

Chemicals

HPLC-grade methanol and dichloromethane were purchased from Merck (Darmstadt, F.R.G.). Water was deionized using the Millipore Milli-Q water system (Millipore s.a., Saint Quentin-en-Yvelines, France). The remaining chemicals used were of the best reagent grade available.

Apparatus

The HPLC analyses were performed with the following equipment: WISP 710 B automatic sampler (Waters Assoc., Milford, MA., U.S.A.), ConstaMetric II G pump (LDC/Milton Roy, Paris, France), SpectroMonitor III D (LDC) set at 254 nm and a Spectra-Physics SP-4200 integrator (Spectra-Physics France, Les Ulis, France).

Chromatography

The column was a Cyclobond I (10 μm, 250 mm $\times$ 4.6 mm I.D.) from Advanced Separation Technologies (Whippany, NY, U.S.A.). The mobile phase was a mixture of 0.05 *M* potassium dihydrogenphosphate (pH 5.3) and methanol (35:65, v/v). The flow-rate was 1.0 ml/min and the temperature was 22 $\pm$ 1 °C. The mobile phase components were separately filtered through Millipore membrane filters (0.45 μm) prior to use.

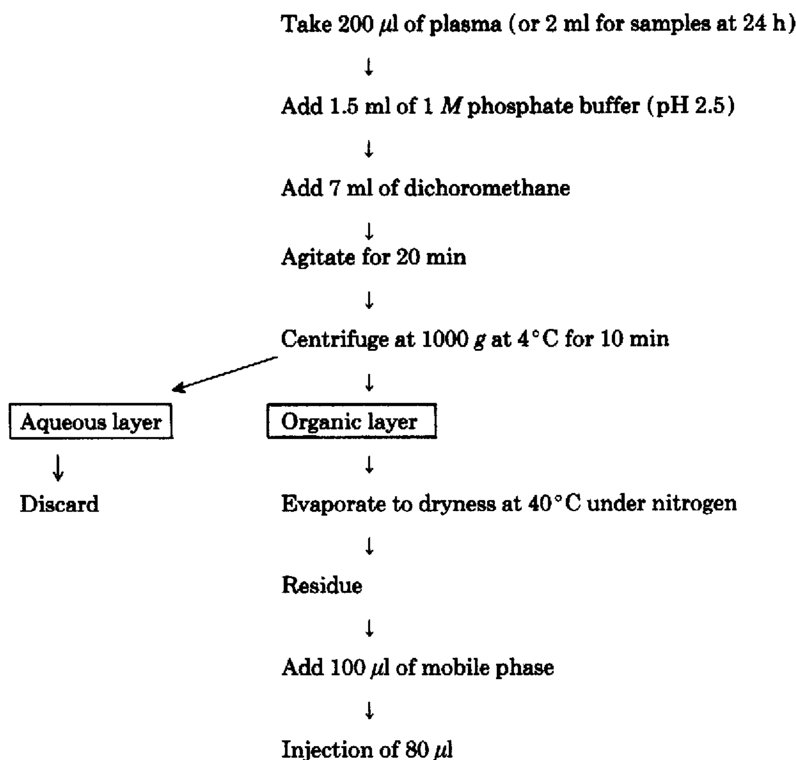

Fig. 1. Schematic representation of the extraction procedure.

Samples

Preparation. Male Sprague–Dawley rats, strain OFA (Iffa Credo, France) weighing ca. 200 g were used. The product was administered orally in the form of a suspension (1% Tween 80) at a dose of 10 mg/kg (4 ml/kg) to rats fasted for 16 h. The animals were anesthesized under light ether anesthesia and the blood was withdrawn into heparinized syringes from the inferior vena cava. Plasma was separated and stored at −20°C until analysis.

*Extraction.*The drug was extracted from rat plasma according to the scheme outlined in Fig. 1.

RESULTS AND DISCUSSION

Stereochemistry of the drug

The compound has two chiral centres, each of which can have the absolute configuration *R* or *S*, according to the scheme shown in Fig. 2. In the absolute configurations *R*-*R* or *S*-*S*, the two hydrogens on the two asymmetric carbons are *trans*, while in the configurations *R*-*S* or *S*-*R* they are *cis*.

The synthesis of drug substance leads to the *trans* configuration, which has been confirmed by NMR spectroscopy. The two enantiomers were separated by fractional crystallization of their diastereomeric quinidine salts and character-

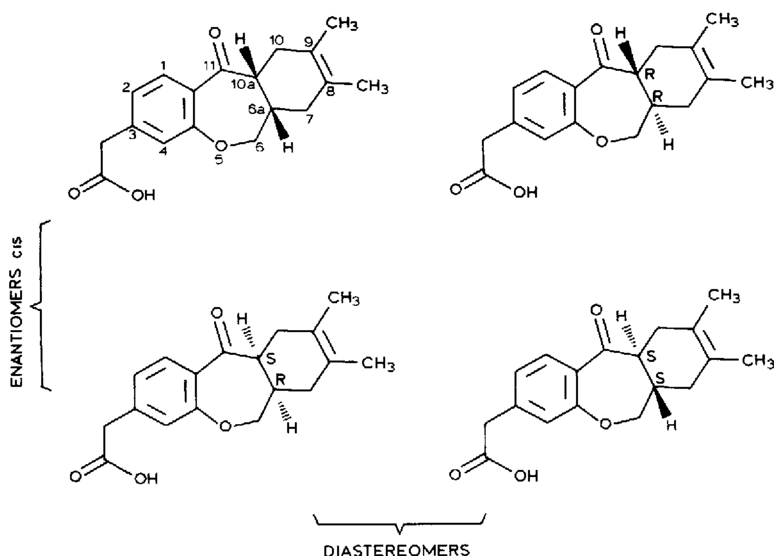

Fig. 2. Stereochemistry of the molecule under study.

ized, in addition to all usual physicochemical properties, by their optical activities, $[\alpha]_D$. The circular dichroism spectra of the enantiomers (+) and (−) are of opposite sign.

Proof of structure

As expected, the ^{1}H NMR spectra (200 MHz, in C^2HCl_3) of the two enantiomers are identical with that of the racemic mixture (δ H-6 4.3 ppm, 4.0 ppm; δ H-10a 2.96 ppm; δ H-6a 2 ppm). The *trans* configuration of the protons in positions 10a and 6a was deduced from the analogy with the spectra of the corresponding methyl ester (for the numbering see Fig. 2).

Both the *cis* and the *trans* isomers of the methyl ester were available. The chemical shifts (δ, ppm) and the coupling constants (J) for the two methyl ester isomers are given in Table I. The *cis* isomer was characterized by the nuclear Overhauser effect (NOE): the irradiation of H-3 gave a positive NOE on H-8.

TABLE I

^{1}H NMR DATA FOR THE *cis* AND *trans* ISOMERS OF THE METHYL ESTER DERIVATIVE OF THE COMPOUND UNDER STUDY

Hydrogen atom	*cis*-Isomer		*trans*-Isomer	
	δ-Value (ppm)	Coupling constants (Hz)	δ-Value (ppm)	Coupling constants (Hz)
H-6	4.65	${}^2J_{2,2'}=12$; ${}^3J_{2,3}=7$	4.3	${}^2J_{2,2'}=12$; ${}^3J_{2,3}=0$
	3.55	${}^3J_{2',3}=11$	4.0	${}^3J_{2',3}=4$
H-10a	ca. 3.45	${}^3J_{8,3}=7$; ${}^3J_{8,7}=7$ and 2	ca. 2.95	${}^3J_{8,3}=11$
H-6a	ca. 2.8		ca 2	

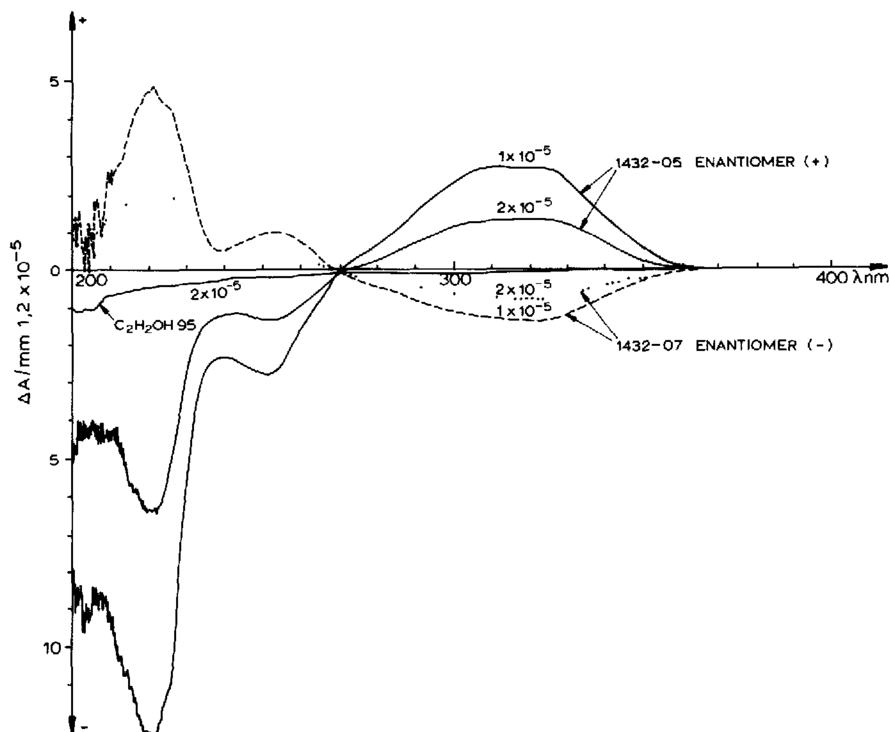

Fig. 3. Circular dischroism spectra of the enantiomers under study and the solvent (ethanol); wavelength range, 200–400 nm.

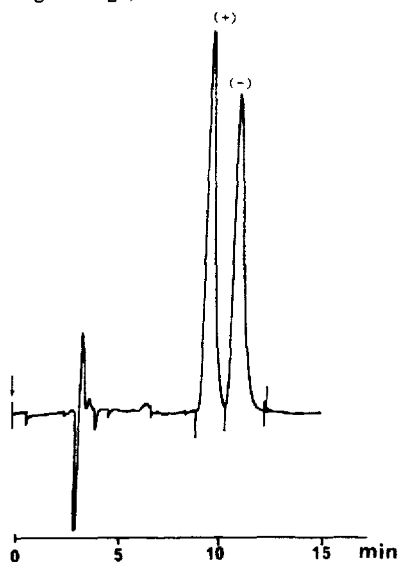

Fig. 4. HPLC separation of the enantiomers of 6,6a,7,10,10a,11-hexahydro-8,9-dimethyl-11-oxodibenz[*b,e*]oxepin-3-acetic acid. Chromatographic conditions as in Experimental.

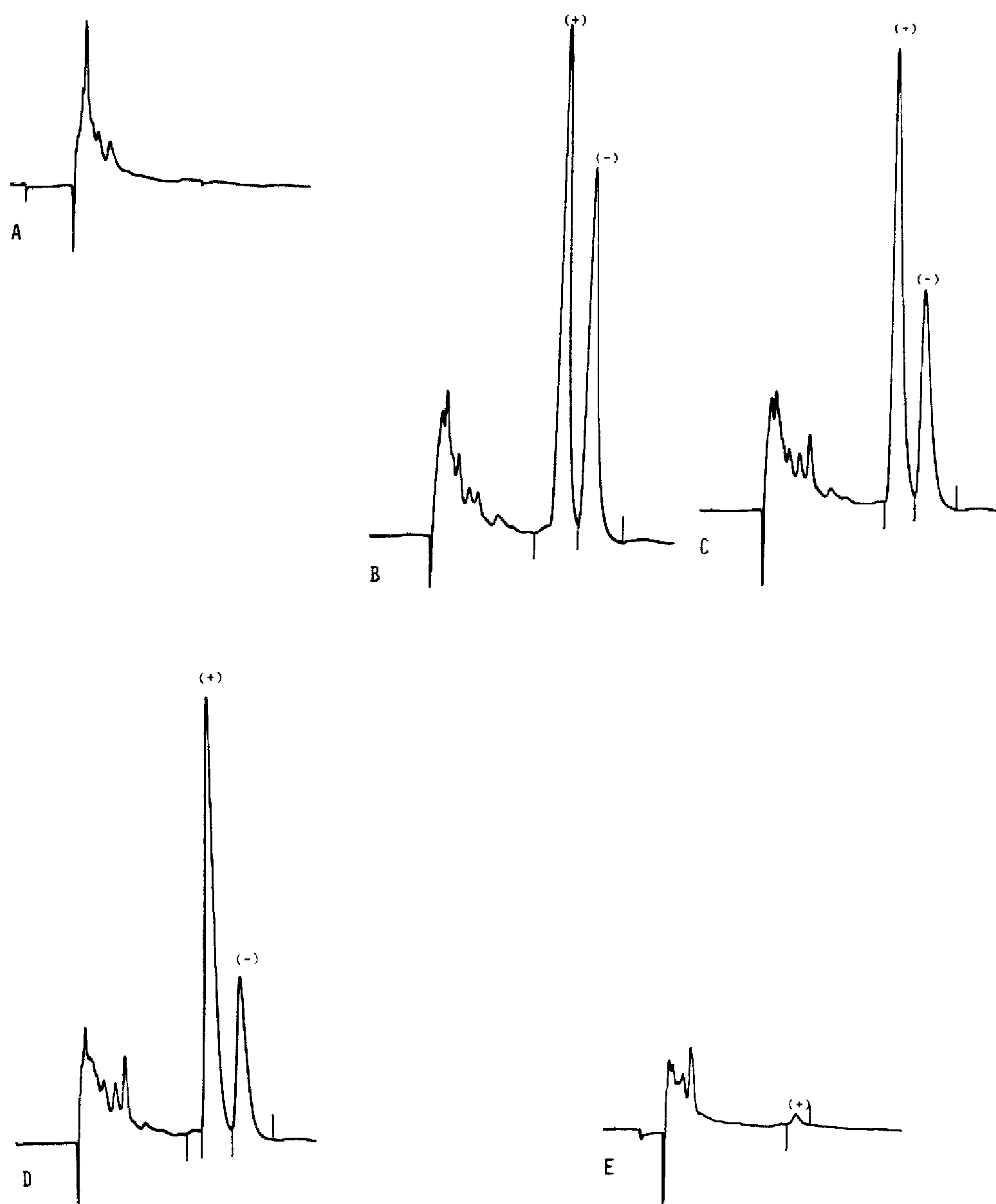

Fig. 5. (A) HPLC of the blank extract of rat plasma: volume injected, 20 μl (corresponding to 200 μl of plasma). Chromatographic conditions as in Fig. 4. (B) HPLC separation of the enantiomers of the drug in rat plasma after 1 h. The (+)-isomer represents 53.5% of the mixture. (C) HPLC separation of the enantiomers of the drug in rat plasma after 3 h. The (+)-isomer represents 64.1% of the mixture. (D) HPLC separation of the enantiomers of the drug in rat plasma after 6 h. The (+)-isomer represents 71.5% of the mixture. (E) HPLC separation of the enantiomers of the drug in rat plasma after 24 h. The (+)-isomer represents 100% of the mixture.

TABLE II

VARIATION OF THE PERCENTAGE OF THE (−)-ISOMER IN RAT PLASMA SAMPLES AS A FUNCTION OF TIME

Rat. No	Percentage (−)-isomer			
	1 h	3 h	6 h	24 h
1	53.9	68.4	71.5	72.3
2	54.2	64.1	64.0	74.7
3	55.0	61.0	70.9	100.0

The circular dichroism spectra of the two enantiomers are shown in Fig. 3. The spectra are of the opposite sign. However, they are not perfectly symmetrical because of the insufficient purity of the (−) enantiomer.

Column liquid chromatography

Prior to the establishment of protocol conditions, the roles of the mobile phase constituents were systematically investigated. The pH of the buffer and the methanol content play an important role. The best resolution of the two enantiomers was achieved by using a mobile phase consisting of 65% methanol and 35% 0.05 *M* potassium dihydrogenphosphate (pH 5.3). The asymmetry factors for the two enantiomers were 1.4 under these conditions. A typical chromatogram of the separation of the racemic mixture is shown in Fig. 4.

The extraction of the drug substance from rat plasma was quantitative for both enantiomers (more than 90%) in the concentration range of interest (0.5–5 μg/ml), and the calibration curve was linear (peak area = 34 225; slope = +1873; coefficient of correlation = 0.99971). The detection limit in plasma samples was of the order of 0.085 μg/ml, with a coefficient of variation of 5%. The plasma constituents extracted into dichloromethane did not interfere with the analysis of the drug (Fig. 5A).

In order to improve the precision of the assay, it would have been desirable to find an internal standard. Several compounds belonging to the same series were assayed. However, under the protocol conditions, all of them gave two distinct peaks on chromatography and thus interfered with one or both of the enantiomers under study. Therefore only the ratios of enantiomers were calculated. The chromatograms showing the plasma levels of the two enantiomers after 1, 3, 6 and 24 h are given in Fig. 5B–E. It is evident that the inactive isomer (−) is eliminated more rapidly than the active isomer (+).

It is interesting to note that, while there is little variation between the plasma levels of the three rats at 1, 3, and 6 h, the values at 24 h differ considerably (74–100%) (Table II). The assays at 24 h were performed using 2-ml plasma samples because of the low concentration of enantiomers at this time interval.

CONCLUSIONS

The aim of this work was to demonstrate the importance of studying single enantiomers in the development of racemic therapeutic agents. Neglect of this

factor may produce misleading conclusions to pharmacokinetic and ultimately clinical results. One should be aware that the acceptance of a racemate implies the acceptance of 50% or more of a possibly harmful enantiomer. Exceptions to this include the few cases where the two enantiomers are equally active or when one enantiomer contributes positively to the efficacy of the other. The decision to synthesize stereoselectively or to isolate pure enantiomers depends on a number of scientific and economic factors, and it may change during the development of the drug substance.

ACKNOWLEDGEMENTS

We thank Dr. A. Wick for valuable discussions, Drs. T. Purcell and L. Zard for the synthetic work, and E. Kauffmann for the structural analysis.

REFERENCES

1 R. Dennis, Pharm. Int., (1986) 246.
2 S. Cacchi, G. Gargano, F. Gasparrini and D. Misiti, Chimica Oggi, 12 (1984) 11.
3 J. Debowski, G. Grassini-Strazza and D. Sybilska, J. Chromatogr., 349 (1985) 131.
4 C.E. Dalgliesh, J. Chem. Soc., 137 (1952) 3940.
5 D.W. Armstrong, J. Liq. Chromatogr., 3 (1980) 895.
6 W.L. Hinze and D.W. Armstrong, Anal. Lett., 13 (1980) 1903.
7 T.J. Ward and D.W. Armstrong, J. Liq. Chromatogr., 9 (1986) 407.
8 D.W. Armstrong and W. De Mond, J. Chromatogr. Sci., 22 (1984) 411.
9 J. Debowski, D. Sybilska and J. Jurczak, Chromatographia, 16 (1982) 198.
10 W.L. Hinze, T.E. Riehl, D.W. Armstrong, W. De Mond, A. Alak and T. Ward, Anal. Chem., 57 (1985) 237.
11 T. Purcell and L. Zard, Fr. Pat. No. 2580280 (1985).