Chiral inversion of fenoprofen in horses and dogs: an in vivo-in vitro study
A Soraci, P Jaussaud, E. Benoit, P Delatour

To cite this version:
Chiral inversion of fenoprofen in horses and dogs: an in vivo-in vitro study

A Soraci, P Jaussaud *, E Benoit, P Delatour

Unité associée INRA-DGER de toxicologie métabolique, École vétérinaire de Lyon, 1, avenue Bourgelat, BP 83, 69280 Marcy-l’Étoile, France

(Received 22 March 1995; accepted 28 June 1995)

Summary — Fenoprofen (FPF) is a chiral non-steroid anti-inflammatory drug, marketed as a racemic mixture of its R(−) and S(+) enantiomers. Its stereoselective disposition in humans and animals is due to a chiral inversion converting R(−)FPF into S(+)FPF. The first step of this reaction, which produces an acyl-CoA thioester, is catalysed by an acyl-CoA ligase. A stereospecific high performance liquid chromatography assay was used to study the disposition of FPF enantiomers in four geldings and three male beagle dogs, following intravenous doses of racemic FPF (1 mg/kg in horses), R(−)FPF (0.5 mg/kg in horses, 1 mg/kg in dogs), and S(+)FPF (0.5 mg/kg in horses, 1 mg/kg in dogs). A unidirectional stereoinversion of the R(−) enantiomer into its optical antipode (38% in horses, 90% in dogs) was demonstrated. This explained the clear enantioselective behaviour of FPF in both species. Acyl-CoA ligase activity (K_m = 473.2 ± 92.5 μM; V_max = 23 ± 3.3 nmol/min/mg) has also been quantified in vitro on equine hepatic microsomes, using a high performance liquid chromatography method to measure thioester formation. The present study showed that, in horses and dogs, as previously demonstrated in rats and sheep, the R(−)FPF clearance was better correlated with ligase activity than with inversion rate. A highly significant linear relationship was demonstrated between these variables.

fenoprofen / chiral inversion / dog / horse


* Correspondence and reprints
chiens Beagle mâles, après administration intraveineuse de FPF racémique (1 mg/kg chez les chevaux),
de R(-)FPF (0,5 mg/kg chez les chevaux, 1 mg/kg chez les chiens), et de S(+)FPF (0,5 mg/kg chez les
chevaux, 1 mg/kg chez les chiens). Une stéréo-inversion unidirectionnelle de l’énantiomère R(−) en sa
antipode optique (à raison de 38 % chez les chevaux et de 90 % chez les chiens) a été mise en évi-
dence. Celle-ci explique le comportement nettement enantio-sélectif du FPF dans les deux espèces
étudiées. Par ailleurs, l’activité de l’acyl-CoA ligase (Km = 473,2 ± 92,5 μM ; Vmax = 23 ± 3,3 nmol/min/mg)
a été quantifiée in vitro sur des microsomes hépatiques de cheval, en utilisant une méthode de chro-
matographie liquide haute performance pour mesurer la formation du thioester. L’étude montre que,
chez le cheval et le chien comme chez le rat et le mouton, la clairance du R(−)FPF est mieux corrélée
avec l’activité de la ligase qu’avec le taux d’inversion chirale. Une relation linéaire caractéristique a été
établie entre ces deux paramètres.

fénoprofène / inversion chirale / chien / cheval

INTRODUCTION

The two enantiomers of racemic drugs often have different pharmacological potencies. This is demonstrated in 2-arylpropionates (profens) (Caldwell et al, 1988; Muller et al, 1990). These non-steroid antinflammatory drugs (NSAID), due to the presence of a centre of asymmetry (C2), are chiral compounds, which, with the exception of naproxen, are marketed as racemates. The profens usually show a stereoselective disposition, which varies widely with the animal species and the structure of the drug, and is frequently explained by chiral inversion. This process, which corresponds to a selective unidirectional transformation, from the pharmacologically inactive R(−) enantiomer into its active S(+) optical antipode (Hutt and Caldwell, 1983), takes place in several organs (liver, intestine, kidney and lung) and tissues (muscle and fat) (Cox et al, 1985; Jeffrey et al, 1991; Hall et al, 1992). Berry and Jamali (1991) have confirmed however the predominant role of the liver in chiral inversion. It is for this reason that a number of authors use the hepatocyte preparation model to study the process in vitro.

A hypothesis for the molecular mechanism of chiral inversion of profens was proposed, then confirmed by Wechter et al (1974), Nakamura et al (1981), Knhinicki et al (1989) and Menzel et al (1994). The process involves three steps: (i) stereoselec-
tive activation of the R(−)-2-arylpropionic acid through the formation of the acyl-CoA thioester, the reaction being catalysed by acyl-CoA synthetase(s); (ii) enzymatic epimerization of the R(−)-thioester to the S(+) thioester (Sheih and Chen 1993); and (iii) hydrolysis of the thioester to release the free drug.

Fenoprofen (FPF) or (±)-2-(3-phenoxypyphenyl)propionic acid (fig 1), is a profen used in human medicine as a racemic mixture (rac-FPF) of the 2 enantiomers R(−)FPF and S(+)FPF. Stereoselective disposition and chiral inversion of the drug have been studied in man (Rubin et al, 1985), rabbit (Hayball and Meffin, 1987), and rat (Berry and Jamali, 1991), but no data are available to date for dogs. Moreover, no chiral inversion of any profen has yet been studied in the equine species. For these reasons, the present in vivo and in vitro study was undertaken to investigate possible chiral inversion of fenoprofen in horses and dogs.

MATERIALS AND METHODS

In vivo studies

Animals

Three adult, male beagle dogs weighing 10, 11 and 12 kg, and four geldings aged between 10 and 15 years and weighing 440, 520, 520 and
560 kg, with free access to food and water, were used in this study.

**Drug administration and sampling**

$R(-)$ and $S(+) FPF$ were obtained by resolving FPF (calcium salt, Sigma Chemicals, Saint Louis, USA) enantiomers using their $\alpha$-methylbenzylamine diastereomeric salts, a method previously described for fenoprofen (Hayball and Meffin, 1987). The enantiomeric purity of $S(+) FPF$ was 98%, and that of $R(-) FPF$ 96%.

Each horse received a single dose of 1 mg/kg rac-FPF in the right jugular vein. The drug was administered as a calcium salt (Sigma Chemicals, Saint Louis, USA) placed in solution with 2 mL pure dimethylsulfoxide. Blood samples were collected into heparinized tubes by venipuncture from the left jugular vein at standardized time intervals, up to 120 min after the drug administration. After a 2 month interval, the same horses received intravenously a single dose of 0.5 mg/kg $R(-) FPF$, and 2 months later, a single dose of 0.5 mg/kg $S(+) FPF$. The acidic form of each drug was placed in solution with 2 mL dimethylsulfoxide. Blood samples were collected in heparinized tubes at standardized intervals, up to 50 min after the drug administration.

Three dogs were given a single dose of 1 mg/kg $R(-) FPF$ and $S(+) FPF$ individually (each compound being administered as a solution in 1 mL dimethylsulfoxide), into the right jugular vein. Blood samples were taken from the left jugular vein at standardized intervals, up to 8 h after the $S(+) FPF$ and $R(-) FPF$ administration, and placed into heparinized tubes. All samples were immediately centrifuged for 10 min at 900 g, and then the plasma was separated and stored at $-20 \, ^\circ C$ until analysis.

**Analytical method**

**Extraction and derivatization**

The plasma (1 mL) was acidified with 500 $\mu$L HCl (1 N) and extracted twice with 6 mL diethyloxide, which was subsequently evaporated to dryness under nitrogen stream at 60 °C. The dry residue was derivatized with l-leucinamide, in accordance with a method adapted from Foster and Jamali (1987). This procedure converts enantiomers into diastereomers, which can be analysed on classic high performance liquid chromatography (HPLC) reversed-phase columns. To accomplish the conversion, 100 $\mu$L triethylamine 50 mM (in acetonitrile), 50 $\mu$L ethylchloroformate 60 mM (in acetonitrile), 50 $\mu$L L-leucinamide hydrochloride 1 M (in water), 50 $\mu$L triethylamine 1 M (in methanol) and 50 $\mu$L water were successively added to the dry extract.

**HPLC analysis**

The derivatized extract solution (20 $\mu$L) was injected into an HPLC system (Beckman 110 A pump, coupled to a Varian 2050 UV detector and a 3393 A Hewlett-Packard integrator). The chromatograph was equipped with a 25 cm x 4 mm ID column (Kromasil RP18, 5 $\mu$m particle diameter, Interchim, Montlucon, France). The product was eluted with a binary gradient A, acetonitrile (50 mM), and B, $H_3PO_4$ (50 mM) in water, with a flow rate of 2 mL/min, and the UV detection wavelength was 232 nm. Under these conditions, retention times for $R(-) FPF$ and $S(+) FPF$ were 9.06 and 9.40 min, respectively. The selectivity factor, $\alpha$, was found to be 1.04 and the resolution factor, $Rs$, was 1.52. The extraction efficiency of $S(+) FPF$ was 88%, and that of $R(-) FPF$ 86%. The limit of quantification of each enantiomer was 0.25 pg/mL. Detection was linear for the two antipodes between 0.25 pg/mL and 10 pg/mL, and the repeatability was 3% for $S(+) FPF$ and 0.9% for $R(-) FPF$.
Data analysis

The $R(-)$FPF and $S(+)$FPF plasma concentration–time curves were plotted, and the areas under the curves (AUCs) were measured by the linear trapezoidal rule without extrapolation to infinity. Intercepts were obtained using a linear regression method, based upon the two first experimental points. The concentration at zero time was fixed at zero for the $S(+)FPF$ kinetics, following $R(-)$FPF administration. The enantiomeric conversion of $R(-)$FPF into $S(+)FPF$ was calculated using the following formula (Pang and Kwan, 1983; Beck et al, 1991):

$$\text{inversion rate} = \frac{\text{AUC (S) after } R \times \text{dose } S}{\text{AUC (S) after } S \times \text{dose } R}$$

In this equation, AUC (S) after $R$ and AUC (S) after $S$ are the AUCs calculated for $S(+)FPF$ after $R(-)$FPF and $S(+)FPF$ administration, respectively, at dosages dose$_R$ and dose$_S$.

Clearances (CI) were calculated using the formula:

$$\text{CI} = \frac{D}{\text{AUC (D: dose administered to the animal)}}$$

In vitro study

Thioesterification

Horse microsomes were prepared in accordance with a method adapted from a previous work (Benoit et al, 1992). A range of concentrations from 25 to 400 $\mu M$ of $R(-)$FPF was incubated with 100 $\mu g$ of microsomal protein, 2.5 mM ATP, 0.3 mM CoA, 15 mM MgCl$_2$ in 150 mM Tris-HCl buffer, pH 8.0 (final volume 500 $\mu L$). After a 10 min incubation period at 37 °C, 100 $\mu L$ 2 N HCl was introduced, and the product of the reaction was extracted on a small C18 column (AASP system, Varian, Les Ulis, France).

Analytical method

The $R(-)$FPF-CoA analyses were performed using an HPLC gradient system (Beckman 110A pump, Beckman 163 UV detector, 3390 A Hewlett-Packard integrator, and automatic Varian AASP injector). Products were eluted from a Kromasil RP18 column (0.4 x 15 cm, 5 $\mu m$ particle size, Interchim) with a binary gradient A, tetrabutylammonium hydrogen sulfate 10 mM, ammonium citrate 50 mM, pH adjusted to 5, and B, acetonitrile. The flow rate was 1.25 mL/min, and detection was monitored by UV absorption at 232 nm. Under these experimental conditions, the retention times were 8.9 min for $R(-)$FPF-CoA and 12.9 min for $R(-)$FPF. The extraction efficiency of the thioester, and the limit of its quantification were 95% and 25 pmol/min/mg, respectively.

DATA ANALYSIS

A Lineweaver–Burk graph was plotted, and values for the Michaelis–Menten parameters ($V_{max}$ and $K_m$) were estimated by extended least-square nonlinear regression (Pononniin®, version 3.0 SCI Software, Statistical consultants, Inc).

RESULTS

In vivo studies

The arithmetic plot of the mean plasma $R(-)$FPF and $S(+)FPF$ concentrations versus time after intravenous rac-FPF administration to horses is shown in figure 2a. At all sampling times, the concentration of $S(+)FPF$ exceeded that of $R(-)$FPF, and the $S(+)/R(-)$ plasma concentration ratio increased rapidly. This last parameter reached the value of 9, 20 min post-dosing. Therefore, in horse plasma, $S(+)FPF$ became predominant very rapidly, with an AUC value for the total duration of measurements (2 h) of 1.62 $\mu g \cdot h/mL$.

The mean $R(-)$FPF and $S(+)FPF$ plasma profiles, plotted after an intravenous administration of 0.5 mg/kg $R(-)$FPF in horses and 1 mg/kg $R(-)$FPF in dogs, are shown in figures 2b and 3a. The plasma level of the $R(-)$ enantiomer decreased in dogs more rapidly than it did in horses and could
not be quantified in plasma of both animals for longer than 18 and 40 min after injection, respectively. Concurrently $S(+)$FPF appeared, and had increasing plasma levels up to 10 min in horses and 30 min in dogs, where it reached the maximal values of 0.75 and 5.46 μg/mL. The $S(+)$FPF concentration then slowly decreased. The $S(+)$FPF AUC value in horses, for the total duration of measurements (50 min), reached 0.47 μg·h/mL, and the corresponding AUC obtained in dogs was 18.5 μg·h/mL. The $R(-)$FPF AUC values after $R(-)$FPF intravenous administration were 0.53 and 0.55 μg·h/mL in horses and dogs, respectively.

After an intravenous administration of 0.5 mg/kg (in horses) and 1 mg/kg (in dogs) of $S(+)$FPF, the mean plasma profiles indicated the presence of the $S(+)$ enantiomer only, since no trace of $R(-)$FPF could be measured throughout the duration of the experiments (figs 2c and 3b). The $S(+)$FPF AUC values were 1.23 and 20.40 μg·h/mL in horses and dogs, respectively.

The inversion rate of $R(-)$FPF into $S(+)$FPF was found to be 38% in horses and 90% in dogs. The clearance values were 15.1 and 33.3 mL/min·kg, respectively.

**In vitro study**

The effect of in vitro $R(-)$FPF concentrations upon thioesterification with CoA in horses, according to the Lineweaver–Burk graph, is shown in figure 4. Mean values ($n = 4$) of $K_m$ and $V_{max}$ were 473.2 ± 92.5 μM and 23 ± 3.3 nmol/min/mg, respectively.

**DISCUSSION**

The experimental data clearly demonstrated that FPF had an enantioselective pharmacoc-
kinetic behaviour pattern in horses and dogs, with a large predominance of the S(+) form. The chiral behaviour of FPF in these animals is likely to involve unidirectional stereoinversion from R(-)FPF into S(+)FPF, as previously described in other species. Two arguments support this hypothesis: (i) an administration of R(-)FPF generated S(+)FPF; (ii) conversely, an intravenous injection of S(+)FPF did not induce the appearance of R(-)FPF, and the drug plasma concentration–time profile in horses was different from that observed after administration of the racemate, as shown by comparison of the AUC values (1.21 and 1.62 µg·h/mL, respectively). In dogs, 90% of the R(-)FPF dose was found to be inverted towards the S(+)FPF antipode. Similar per-
centages have been established for sheep (80%) (Soraci et al, 1995), rabbit (73%) (Hayball and Meffin, 1987), and man (60%) (Rubin et al, 1985). Nevertheless, in the horse, only 38% of the \( R(-) \)FPF dose was inverted, a value similar to the ratio of 42% determined in rats (Berry and Jamali, 1991). These results indicate that, as with flurbiprofen (Menzel et al, 1992), the chiral inversion of FPF is strongly species-dependent. Such interspecies variation could be explained by interspecies variation in the activity of the enzymes involved in chiral inversion.

The first step of \( R(-) \)FPF stereoinversion in the rat probably involves palmitoyl CoA ligase, as suggested by Knight et al (1988, 1992) and Benoit et al (1995). In hepatocytes, this enzyme is present in endoplasmic reticulum membranes (microsomes), mitochondria and peroxisomes (Tanaka et al, 1979; Krisans et al, 1980; Miyazawa et al, 1985), but there is no evidence of any differences in the enzymes taken from the different cell fractions. As previously described for sheep, rats and dogs (Soraci et al, 1995; Soraci and Benoit, 1995), liver microsomes catalysed fenoprofenyl-CoA formation, and large interspecies variations in this first step of the chiral inversion are now documented. \( V_{\text{max}} \) values for the thioesterification of fenoprofen actually range from 2.1 (in sheep) (Soraci et al, 1995) to 60.6 nmol/min/mg (in dogs) (Soraci and Benoit, 1995); this value is 22.2 nmol/min/kg in rats (Berry and Jamali, 1991). No correlation between the kinetic parameters of ligase activity (\( V_{\text{max}} \)) and the extent of \( R(-) \)FPF chiral inversion, appreciated by the inversion rate, was observed. For example, in sheep a low enzyme activity (\( V_{\text{max}} = 2.1 \) nmol/min/mg) contrasts with a large inversion rate (80%) (Soraci et al, 1995), whereas in rats or horses a high ligase activity (22 and 23 nmol/min/mg) contrasts with a low inversion rate (38 and 42%) (Berry and Jamali, 1991). This could be probably explained in rats and horses by the variety of the pathways which use \( R(-) \)FPF, independently of the CoA thioester formation: aryl oxidation, glucuronidation and urinary elimination. One of these processes might occur to a significant extent and remove \( R(-) \)FPF from the chiral inversion process. However, because of the high \( V_{\text{max}} \) in rats and horses, the probability of such a hypothesis is very low in these species. None of the three evoked possibilities, which use \( R(-) \)FPF, could compete with the ligase.

Moreover, the highly significant \((r = 0.980)\) correlation between the enzyme activity and the plasma \( R(-) \)FPF clearance confirms the involvement of thioesterification in the metabolism of \( R(-) \)FPF, in all the species investigated. Figure 5 has been plotted with the following data: \( R(-) \)FPF clearance in sheep, and ligase activity in sheep and rats which were previously published by our group. The \( R(-) \)FPF clearance in rats was estimated from the half-life (5 min) (Berry and Jamali, 1991) and from an approximate mean volume of distribution for profens of 0.14 L/kg bwt. We have also previously measured ligase activity in dogs (Soraci and Benoit, 1995).

The acyl-CoA thioester, a pivotal substrate in the metabolism of aryl-2-propionic acids (Caldwell, 1984), is the obligatory intermediate in chiral inversion (Wechter et al, 1974; Nakamura et al, 1981; Menzel et al, 1994). The low inversion rate observed in horses and other species could therefore be explained principally by the existence of other competitive metabolic pathways originating with the FPF-CoA thioester. In particular, activated profens can be taken up by lipid biochemistry pathways. Using glycerol and palmitate as precursors, Fears et al (1978) showed that ibuprofen, ketoprofen and fenoprofen were incorporated into hybrid triglycerides. In the same way, thioesterification also leads to the incorporation of fenoprofen and ibuprofen into triacylglycerols in rats (Williams et al, 1986; Sal-
Another possible way of consuming thioesters could be conjugation with the amino acids glycine and taurine (Hutt and Caldwell, 1990). Tanaka et al (1992) and Shirley et al (1994) have observed conjugation with the amino acids of hydratropic acid and ibuprofen. The differences observed in horses and dogs in chiral inversion processes could therefore be explained by competition with other metabolic pathways that consume FPF-CoA (fig 6).

**ACKNOWLEDGMENTS**

The authors gratefully acknowledge R Sechet, L Olivier (INRA) and S Besse (DGER), who performed the HPLC analysis. They also wish to thank P Berny for his reading of the final manuscript.
REFERENCES


Miyazawa S, Hashimoto T, Yokata S (1985) Identity of long chain acyl coenzyme A. A synthetase of microsomes, mitochondria and peroxisomes in rat liver. J Biochem (Tokyo) 204, 723-733


Tanaka T, Hosaka K, Hoshimaru M, Numa S (1979)
Purification and properties of long-chain acyl-coenzyme-A synthetase from liver. Eur J Biochem 98, 165-172

Tanaka Y, Shimomura T, Hirota T (1992) Formation of glycine conjugate and (−)-(R)-enantiomer from (+)-(S)-2-phenylpropionic acid suggesting the formation of the CoA thioester intermediate of (+)-(S)-enantiomer in dog. Chirality 4, 342-348
