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Chiral Inversion of R-(-)-Fenoprofen Enantiomer in Cats with Toxic Hepatic Disease

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Abstract: The 2-arylpropionic acids (2-APA) or profens, is a family of Non-Steroidal Anti-Inflamatory Drug (NSAIDS), widely used in human and veterinary medicine for the treatment of the arthritis, musculoskeletal disorders and hyperthermia. The molecule of fenoprofen (FPF), a member of the familiy of 2-APA, contains an asymetric carbon atom and exists as two enantiomeric forms, R-(-) fenoprofen and S-(+) fenoprofen . The R-(-) FPF enantiomer is metabolically inverted to their optic antipode, the S-(+) FPF enantiomer as result of the action of a metabolic pathway known as chiral inversion. The liver is the principal site for the 2-APA biotransformation. Severe hepatic disease should alter the percentage of chiral inversion obtained for R-(-) FPF. To test this hypothesis we studied the chiral inversion of R-(-) FPF in cats with toxic hepatic disease (THD) induced by carbon tethrachloride (CCl_4). The percentage of chiral inversion in animals with THD was 90.5±21.1 (mean±sd) and the difference with healthy animals was not statistically significant.

Key words: Fenoprofen, cats, enantioselective, chiral inversion, carbon tethrachloride

INTRODUCTION

Fenoprofen (FPF) ([±]-2-[3-Phenoxyphenyl]propionic acid) is a Non-Steroidal Anti-Inflamatory Drug (NSAIDS), widely used in human and veterinary medicine in the treatment of the arthritis, musculoskeletal disorders and hyperthermia. This drug possesses a chiral centre located at C_2 of the propionic moiety and, therefore, exists in two enantiomeric forms, S-(+) fenoprofen and R-(-) fenoprofen. Studies in animals have shown that fenoprofen inhibition of prostaglandin synthetase is highly stereoselective with predominant activity residing in the S antipode[1,2]. However, it is marketed as a racemate (50:50 of each enantiomer). The enantiomers may differ not only in their pharmacodynamic properties, but also in their kinetic disposition[3-5]. The degree of kinetic disposition has been shown to be stereoselective for fenoprofen enantiomers[6,7]. This stereoselectivity can be generally explained on the basis of a metabolic pathway known as chiral inversion process[8-10]. This process of biotransformation corresponds to a selective unidirectional transformation from the inactive R-(-) to the active S-(+) enantiomer. This process has been documented in cats[1], dogs and horses[4], sheep[11], rats[12] and humans[13], with a considerable variation in extension according to the species[10]. The stereoconversion mechanism has been described in different organs such as liver, intestine, kidney and lungs[14-17]. However, the liver seems to have the predominant role in the chiral inversion process[12].

Therefore, it is expected that hepatic lesions of certain degree might affect the general behavior of these xenobiotics by reducing their therapeutic effect, or inducing unwanted, acute or chronic, toxic effects.

As far as we know, no studies have been performed on the effects of experimental THD on the metabolic pathway of chiral inversion of FPF in cats .To evaluate those possible effects, we used carbon tetrachloride to induce a experimental THD. Carbon tetrachloride (CCl_4) is well known as a highly hepatotoxic compound due to its activation to reactive metabolites which induce centrilobular necrosis[18,19,20].

The knowledge that changes might occur in the metabolic behavior of a xenobiotic in animals with hepatic dysfunction will contribute to the adoption of more rational therapeutic criteria in patients with hepatic dysfunction.

The aim of the present study was to study changes that might occur in the chiral inversion of FPF under toxic hepatic disease induced by CCl_4.

MATERIALS AND METHODS

Drugs and reagents: FPF racemic was obtained from Sigma (fenoprofen calcium salt; hidrate. Sigma, Saint Louis, USA). The R-(-) and S -(+) enantiomers of FPF were

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obtained by stereospecific crystallizations, using α-methylbenzylamine as a chiral inducer[21]. After completing the crystallization process, the final purities determined by high precision liquid chromatography were 98.1 and 97.2% for R-(-) and S-(+) FPF, respectively. Reactives for determining enzymatic activity were provided by Biosystem, S.A., Barcelona, Spain (ALP), Wiener, S.A., Rosario, Argentina, (ALT y AST). For albumin determination, reagents were provided by Merck, Darmstadt, Germany. The remaining compounds and reagents were obtained through usual commercial channels.

Animals and experimental protocol: Biochemical determinations prior to treatment with CCl_4-Animal preparation and compound administration (Ccl_4 and FPF enantiomers).

Eight healthy adult cats weighing between 3.2 and 4.4 kg were placed in individual cages for three weeks. Blood samples for biochemical determination were obtained from each cat two days before CCl_4 administration. Determinations included hemogram, serum creatinine, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and serum alkaline phosphatase (ALP). All Cats were considered apt for the test and divided in two groups of four animals each. They were anesthetized and a K33 Teflon catheter was introduced in jugular vein according to the technique previously described[22].

After recovery from anesthesia, each cat received a single dose of 0.15 mL kg^{-1} BW of CCl_4 subcutaneously. In order to assess the progress of acute exposure to the drug, blood samples of 2 mL were collected from the jugular vein every six hours for a period of twenty four hours after CCl_4 administration to determine serum enzyme concentration. Increases of twenty, ten and fivefold in serum enzyme activities of ALT, AST and ALP, respectively were considered as indicative of hepatic damage. Normal reference values were ALT: 28-91, AST: 9-46 and ALP: 10-77 IU/L[23,24]. Previous studies carried out with CCl_4 in cats showed, that induction of toxic hepatic disease with enzymatic activity values equal to the ones found in this test, were reached 24 h after subcutaneous administration of 0.15 to 0.3 mL kg^{-1} of CCl_4[25-26]. These references are similar to the maximum hepatonecrogenic effect (24h) of CCl_4 induction in rats carried out in other experimental works[27]. Based on these references, we chose 24 hrs for FPF enantiomer administration .

Four animals were administered R-(-) FPF and the other four, S-(+) FPF, at a dose of 1 mg/kg intravenously. The enantiomers were dissolved in a mixture of 200 μL DMSO and 800 μL physiological solution. Blood samples (2 mL) were collected at 5, 10, 20, 30 min and 2, 6, 8 and 12 h after FPF enantiomer administration. Plasma was obtained by centrifugation and then stored at-20°C for future analysis.

Histopathology: In order to assess induced hepatic damage by CCl_4, the cats were euthanized with pentobarbital and liver sections from different lobes were examined by light microscopy.

Analytical methods

Biochemical determinations: ALP, ALT and AST activities were determined by kinetic method, at 37°C, using a Lisa 200 measuring device Hycel Autoanalyser, Lisabio Group, France[28,29]. Serum albumin concentrations were determined by colorimetric techniques[30-31]. Determination of serum creatinine concentration was made by the Jaffe method[32]. Protein electrophoresis was made using cellulose acetate in alkaline medium (sodic veronal buffer 0.4 molar). Band development was made using amide black coloring[33].

Histopathologic technique : Liver samples were fixed in 10% buffer formaldehyde, processed by routine methods and colored with hematoxilin-eosin[34].

Physical-chemical extraction, chromatographic analysis and determination of R-(-) and S-(+) FPF concentrations: The enantiomer (500 μL) was extracted from plasma using Sep-Pak(R) cartridges C18 (Waters Associated, Milford, MA, USA). The Sep-Pak(R) cartridges were prepared with 4 mL methanol followed by 1 mL of PO_4H_3 (1 % in water). The plasma (500 μL) was passed through a C18 cartridge and was rinsed with 1 mL of water-methanol (80:20) and 1 mL hexane. The FPF was eluted with 8 mL methanol (100%). The recovery of R-(-) and S-(+) FPF was 92.6 (C.V: 8.0) and 90.0 % (C .V: 7.10), respectively. After elution, the FPF was concentrated to dryness under nitrogen atmosphere. The dried residue was derivatized with L-leucinamide (Fluka,.S.A., Saint-Quentin Fallavier, France), in accordance with a method adapted from Foster and Jamali[35]. This procedure converts enantiomers into diastereoisomers, which can be analysed using an achiral High Performance Liquid Chromatography (HPLC)[36]. To accomplish the diastereoisomeric conversion, 100 μL triethylamine (50 mM), 50 μL ethylchloroformate, 50 μL L-leucinamide hydrochloride (1 M) and 50 μL water were successively added to the dry extract. The derivatised extract solution was analysed by high performance liquid chromatography system (LKB, Pharmacia), Pump model 2949, UV variable detector model 2141 and software HPLC

Table 1: Pharmacokinetic parameters of FPF enantiomers in animals with toxic hepatic disease and normal animals (Castro et al., 2000). (Values in the same line with equal superindexes are different; Student *t*, test, p<005)

	Toxic hepatic disease		Normal animals	
Kinetic parameter	R-(-) FPF	S-(+) FPF	R-(-) FPF	S-(+) FPF
AUC (O-t) (ug mL^{-1})	1.30	8.05	1.12	6.45
CL (mL/h kg^{-1})	1368.93$^{(1)}$	78.62$^{(2)}$	978.6$^{(1)}$	117.7$^{(2)}$
t 1/2 el h.	0.76	2.58	0.53	2.85
Vd*ss* (l kg^{-1})	0.730$^{(3)}$	0.267$^{(4)}$	0.651$^{(3)}$	0.423$^{(4)}$
AUC (O-T)(ug mL^{-1}) S (+) after R(-)		7.45		5.62

manager. The flow rate was 1.5 mL min^{-1}. The UV detection was set at 232 nm. The diastereoisomer forms were eluted from RP 18 column (0.4 x 15 cm, 5 μm particles size) with a binary gradient, A : K_2PO_4 10 mM, B : acetonitrile. The retention times for R-(-) and S-(+) FPF were 9.7 and 10.6 min, respectively. The linear regression lines (plasma) for each enantiomer, in the range of the assay (0.2-15 μg mL^{-1}), showed correlation coefficients of r: 0.9990 and r: 0.9988 for R-(-) and S-(+) FPF, respectively. The limit of detection was 0.2 μg mL^{-1} (C.V: 5.0 %). The solvents and reagents (Baker Inc. Phillipsburg, NJ, USA) used during the extraction and drug analysis were HPLC grade.

Analysis of data: Pharmacokinetic parameters were estimated using model-independent methods and were fitted for each animal after intravenous administration of FPF enantiomers using a computer program (PK Solution ®). The area under the concentration-time curve (AUC) of FPF enantiomers up to the last plasma sampling time was determined using the linear trapezoidal method. The enantiomeric conversion percentage of R-(-) into S-(+) FPF was calculated using the formula of Pang and Kwang[37].

Inversion rate: $AUC_{(S)\ after\ (R)}$ x dose (S) / $AUC_{(S)\ after\ (S)}$ x Dose (R)

This formula considers the plasma concentration of the S-(+) FPF after the individual administration of the same dose (1mg kg^{-1}) of R-(-) and S-(+) FPF.

Differences between the pharmacokinetic parameters for R-(-) and S-(+) FPF were evaluated using a t test[38]. A p-value<0.05 was considered significant.

RESULTS

Biochemical determinations prior to administration of Ccl_4: Serum enzyme activity determined 48h before CCl_4 administration were 38±11, 41±21 y 15±11 IU/L for ALT, AST and ALP, respectively. Creatinine, albumin concentration and blood cell count were normal.

Biochemical determinations after administration of Ccl_4: Twentyfour hours after CCl_4 administration (a single dose of 0.15 mL kg^{-1} BW) , enzymatic activity values were

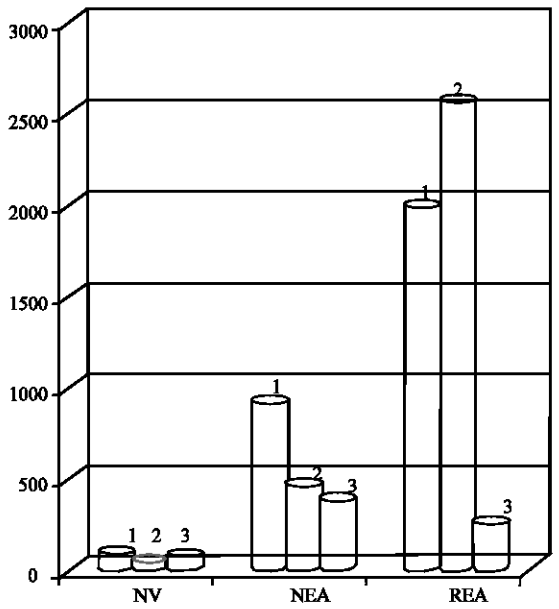

Fig. 1: Enzymatic activity values of (1) : ALT, (2) : AST, y (3) : FAS, after CCl_4 administration. Abbreviations: NV: Normal values. NEA: necessary enzymatic activity for R-(-) FPF administration. REA: reached enzymatic activity after subcutaneous administration of 0.15 mL kg^{-1} BW of CCl

1991±807, 2563±655 and 256±79 IU/L for ALT, AST and ALP, respectively (Fig. 1). Serum albumin concentration was 1.78±0.34 gr dl^{-1} (Normal value : 3.0-4.6 gr dl^{-1})[26]. Values are presented as the mean±standard deviation. Creatinine concentration was normal. Protein electrophoresis showed a reduction in albumin but an increase in γ - globulins.

Histopathology: Severe hemorrhagic centro lobular necrosis was observed. Different levels of hepatic degeneration with predominant polymorphic nuclear leucocytes was also observed.

Plasma concentration of FPF enantiomers: Mean plasma concentrations of R-(-) and S-(+) FPF after intravenous administration of R-(-) FPF (dose : 1 mg kg^{-1} BW), are shown in Fig. 2A. No trace of R-(-) FPF enantiomers in

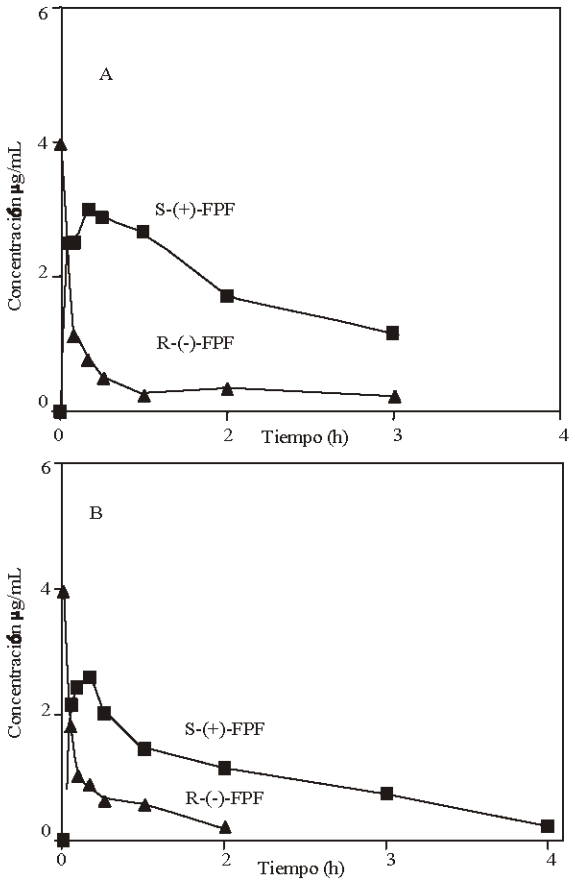

Fig. 2: Mean plasma concentrations of R-(-) FPF and S-(+) FPF after intravenous administration of 1mg kg^{-1} BW of R-(-) FPF in (A): animals with toxic hepatic disease cause by CCl_4, (B) normal animals

plasma could be detected after administration of the same dose of S-(+) FPF. Ten minutes after R-(-) FPF administration its concentration was surpassed by that of S-(+) FPF. The chiral inversion rate was 90.5±21.1 %. Values for $AUC_{(0-T)}$, CL and $t_{½el}$ (half life elimination), for R-(-) and S-(+) FPF were 1.30 and 8.05 µg.h mL^{-1}, 1368.93 and 78.62 mL h^{-1} per kg and 0.76 and 2.58 h, respectively. For these parameters, differences between enantiomers were statistically significant ($p<0.05$). Pharmacokinetic parameter values and plasma concentrations, of animals with THD are compared with those observed in normal animals, after the administration of the same dose of FPF enantiomers (Table 1) (Fig. 2)[39].

DISCUSSION

Fenoprofen is a Non-steroidal Anti-inflammatory Drug (NSAID) which belongs to the main group of available NSAIDs with a high degree of chemical homogeneity, the 2-arylpropionic acid derivatives or profens[1,40]. This group contains an asymmetric carbon atom, a chiral centre located at the C-2 of the propionic moiety and therefore exists in two enantiomeric forms, R-(-) and S-(+). In vitro studies on the relative anti-inflammatory activity of individual FPF enantiomers have shown that their effect on cyclo-oxygenase is due to the S -(+) enantiomer[2]. The structural characteristic of FPF can influence its biological fate; for example, the chiral inversion process transforms selectively the inactive (R)-FPF enantiomer into the pharmacologically active (S)-form[9]. The key molecular basis for this mechanism involves the enantioselective formation of the coenzyme A (CoA) thioester by long chain CoA ligase[41]. The extent of the inversion is different for each profen and is species dependent and usually unidirectional[4].

The liver seems to have a predominant role in the chiral inversion process (6). In previous studies with individual enantiomers of FPF in healthy cats, the percentage of chiral inversion was 93.2±13.7[44].

Carbon tetrachloride is a highly hepatotoxic compound capable of inducing severe hepatic damage[21,44]. Carbon tetrachloride induced liver damage has been a very useful experimental model to explain how free radicals produce cell damage and one of the most thoroughly studied[18-20]. Enzymatic studies have shown the capacity of this compound to induce hepatic necrosis in the cat[25,27]. These studies demonstrated a clear association between hepatic damage induced by CCl_4 and blood increase of biochemical markers accepted as indicative of cellular damage[26, 43-45]. These markers have been specifically validated in the cat. Serum activity values above 1900 and 2500 IU L^{-1} for ALT and AST, respectively, mean severe cell and mitochondria damage[26,28,46]. For ALP, any increase in activity is considered significant in he cat because of its low cellular concentration and its short half life due to its quick renal elimination[24]. Thus, ALP (256 IU L^{-1}. Normal value: 10-77 IU L^{-1}), ALT and AST values measured in this study show the severity of the lesion produced by CCl_4 (Fig. 1). Moreover, histopathologic lesions found in this study are identical to the ones described for CCl_4[19,20] and the necrosis observed matched the highest grade (90 to 100% necrosis) in the scale proposed by Bernacchi *et al.*[28].

According to the above description, we would expect a decrease in the chiral inversion of R-(-) FPF in animals pretreated with CCl_4. We would also expect both enantiomers to accumulate in the body when administered to animals with liver damage by CCl_4[5,47].

However, chiral inversion in animals with THD induced by CCl_4 was 90.5±21.1 % and the difference with that observed in a previous study carried out in our

laboratory with normal animals administered with the individual enantiomers of FPF (93.2±12.7 %) was not statistically significant ($p>0.05$) (Fig. 2A and B)[41].

Two mechanisms could account for the similarity of chiral inversion observed between control and CCl_4 treated cats. First, the 2-APA has been shown to suffer extrahepatic and presistemic chiral inversion in the lumen, mucous membrane and the wall of the small intestine[48,49]. This process has also been found in lungs, kidney and brain[16,50,51]. Hall *et al*., concluded that, the anatomic site of the lungs allows them to exert a first passage effect in intravenous administration of the compound. Although ten times smaller than that detected in the liver, R-(-) FPF activation in its coenzyme A thioester takes place in the rat brain microsomes[52]. Studies *In vitro* performed in our laboratory with microsomes from cat kidney showed acyl-CoA-ligase activity with kinetic parameters similar to those observed in liver microsomes of the same species (unpublished data). Complementary tests *In vitro* in extrahepatic tissues of animals with THD such as epimerization and hydrolysis, would help to evaluate whether fenoprofenil-CoA: 1) is derived to lipid metabolism, 2) is conjugated with amino acids, or 3) contributes significantly to keep a high rate of R-(-) to S-(+) FPF conversion *in vivo*.

Second, acyl-CoA ligase is mostly periportal, since fatty acids metabolism can only occur in the presence of oxygen and the periportal zone has 13% more oxygen than the center of the lobule, which is hypoxic if compared with other tissues[54]. In this study, the liver sections showed a greater structural preservation of the periportal space as compared to the center lobular zone, which could contribute to the chiral inversion value obtained[55].

The understanding of the mechanisms responsible for a compound behavior is closely related to the stereo chemistry of its binding to plasma proteins[56-58]. The 2-APA circulate highly bound to these proteins (99%), mostly albumin. Such binding is favorable for the S-(+) enantiomer and dependent on albumin concentration. Inflammatory states show an increase of acute phase proteins and a decrease in albumin levels. All the animals used in this experiment showed lower than normal serum albumin values for the species and an increase in the fraction related to the γ-globulins. Due to its greater affinity, the S-(+) enantiomer may saturate the albumine sites for both enantiomers, increasing the whole body clearance and the distribution volume of the displaced enantiomer[56-59].

This could explain why both clearance and distribution volume of R-(-) FPF, were greater in animals treated with Ccl_4 than in normal animals ($p<0.05$); (Table 1). In the same way and although the differences were not ststifically siginificant, the AUCs S-(+) FPF is mostly eliminated by glucuronic acid conjugation[60,61]. Conjugation metabolism seems to be less affected than oxidative metabolism in the case of both acute and chronic hepatic disease[62-65]. Enantiomer S-(+) clearance was lower in animals with toxic hepatic disease as compared to normal ones (statistically significant ; $p<0.05$) (Table 1). In the same way and although the differences were not statistically significant, the AUCs of both S-(+) FPF and S-(+) FPF after R-(-) FPF, were greater in intoxicated animals than in normal ones.

In conclusion, our results suggest that THD does not alter the chiral inversion process of FPF. This is in agreement with other reports which show a poor correlation between hepatic lesion tests and metabolism alteration of certain compounds *in vivo*[66-68]. Moreover the effect of a supposed hepatic dysfunction on the metabolism of a xenobiotic is not always consistent, or predictable, even f or drugs sharing the same enzymatic pathway[24,69-72].

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