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## Short Communication

# (−)-*R*-FENOPROFEN: FORMATION OF FENOPROFENYL-COENZYME A BY RAT LIVER MICROSOMES

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**Abstract**—The thioesterification of fenoprofen (FPF) by rat liver microsomes has been studied using an HPLC method enabling direct quantification of the FPF-CoA produced. Over the concentration range studied (5–400 μM), studies showed the participation of a single CoA ligase in the formation of FPF-CoA, in contrast with the involvement of several isozymes with different affinities, that has been found with ibuprofen (IPF). The $K_m$ for the reaction was dependent upon the presence of non-ionic detergent, a concentration of 0.05% Triton X-100 reducing the $K_m$ from 397 to 20 μM although the detergent had no effect on $V_{max}$. The microsomal long-chain fatty acid CoA ligase was markedly enantioselective towards (−)-*R*-FPF and the formation of (−)-*R*-FP-CoA was inhibited by both the (+)-*S* enantiomer and palmitic acid.

*Key words:* fenoprofen; non-steroidal anti-inflammatory drug (NSAID); enantiomer; coenzyme A thioester; palmitoyl-coenzyme A ligase; rat liver microsomes

It is now well established that the 2 arylproprionic acid non-steroidal anti-inflammatory drugs (NSAIDs), one of which is FPF, undergo a fortuitous metabolic chiral inversion in which the inactive (−)-*R* enantiomers are converted into the active (+)-*S* forms [for extensive reviews see Ref. 1]. It is now understood that the chiral inversion reaction involves three distinct metabolic steps, as follows:

(1) The (−)-*R* enantiomer undergoes thioesterification with CoA-SH§ catalysed by a microsomal or mitochondrial ligase, specific for the (−)-*R* enantiomers [1–4]. Some of the ligases are inducible by clofibrate [5].

(2) The (−)-*R*-CoA thioester undergoes an epimerization [6, 7] involving cytosolic and mitochondrial enzymes [8].

(3) The racemic CoA thioester so produced is then hydrolysed by a non-stereoselective mechanism [7, 9].

The unidirectional nature of the chiral inversion of the profens is dependent upon the enantiomeric selectivity of the first step of thioesterification. In addition, thioesterification is the first reaction of the incorporation of profens into triacylglycerols [10] and in the formation of amino acid conjugates. In dogs the simplest profen, 2-phenylpropionic acid, undergoes glycine conjugation as a consequence of the formation of 2-phenylpropionyl-CoA [11].

Knights *et al.* [2] have suggested that the hepatic microsomal thioesterification of FPF in rats is carried out by palmitoyl-CoA ligase (EC 6.2.1.3). This enzyme system comprises two isozymes, one with high affinity and low capacity and a second with low affinity, high capacity and low enantioselectivity. The former is competitively inhibited by (−)-*R*-FPF and (−)-*R*-IPF with $K_i$ of 15.4 and 35.6 μM, respectively [12]. Both isozymes are involved in the thioesterification of (−)-*R*-IPF [13] and their mutual involvement in the stereoselective incorporation of FPF into triacylglycerols in rat hepatocytes has been suggested by Sallustio *et al.* [10]. However, these findings are not consistent with those of Knadler and Hall [4] and Tracy *et al.* [14] with regard to the thioesterification of (−)-*R*-IPF; both groups detected only a low affinity system with a $K_m$ in the millimolar range.

The present study was initiated to investigate the enzyme kinetics of the thioesterification of the enantiomers of FPF by rat liver microsomes, using a direct and sensitive HPLC method for the determination of FPF-CoA.

### *Materials and Methods*

FPF and CoA-SH were obtained from Sigma (la Verpilliere, France) and ATP from Boehringer (Meylan, France). The pure enantiomers of α-methylbenzylamine (Fluka, France, St Quentin-Fallavier) underwent reaction with *rac*-FPF, the resulting FPF-α-methylbenzylamine salts were recrystallised from ethylacetate and the free acids liberated by base exchange. (−)-*R*-FPF was obtained in enantiomeric excess (ee) of 94% and *S* with an ee of 92%. A fourth recrystallisation for (+)-*S*-FPF gave an ee of 97%. (+)-*S*- and (−)-*R*- enantiomers were measured after derivatization with L-leucinamide and the diastereoisomers thus produced were resolved by HPLC. FPF-CoA was synthesized as described by Chen *et al.* [6] for IPF-CoA. The concentration of standard solution of FPF-CoA was verified by measurement of both CoA-SH and FPF after alkaline hydrolysis (KOH 2 M, 55°, 45 mn). All other chemicals were obtained from usual commercial sources.

Male Wistar rats (IOPS-OFA; body weight 200–220 g) were purchased from IFFA-Credo (L'Arbresle, France) and hepatic microsomes were prepared according to Benoit *et al.* [15]. *R*- or *S*-FPF (5–400 μM) were incubated with 10–100 μg microsomal protein, 3 mM ATP, 0.3 mM CoA-SH, 15 mM $MgCl_2$ in 150 mM Tris–HCl buffer pH 8.0, final volume 500 μL. After incubation for 5 min at 37°, 100 μL 2 M HCl were added and the reaction mixture extracted

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§ Abbreviations: FPF, fenoprofen; IPF, ibuprofen; CoA-SH, coenzyme-A; ee, enantiomeric excess.

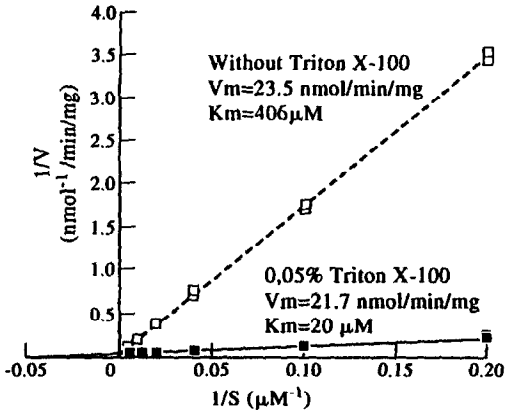


Fig. 1. Double reciprocal plot of thioesterification of (−)-*R*-FPF in the presence or in the absence of non-ionic detergent by liver microsomes from a single rat.

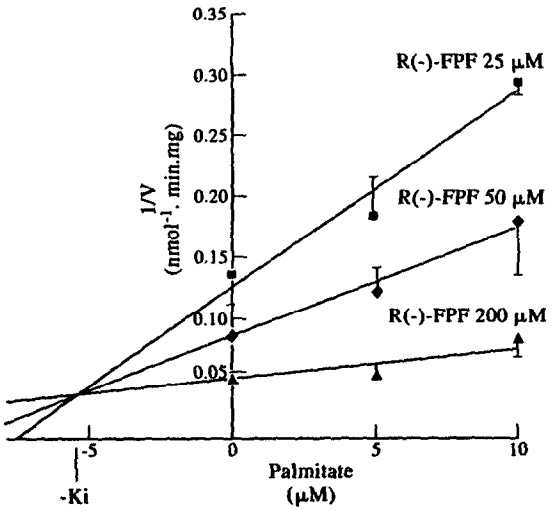


Fig. 2. Dixon plot of the inhibition of (−)-*R*-FPF thioesterification by palmitate.

on an AASP C-18 column (Varian Instruments, Les Ullis, France).

FPF-CoA was assayed using an HPLC gradient system (Beckman model 110 A pump, Model 420 gradient programmer and Model 163 W detector), equipped with a Hewlett-Packard 3390A integrator. The FPF and FPF-CoA, adsorbed onto the AASP C-18 column, was then applied directly (as a precolumn) onto the RP 18 column (150 × 4 mm, 5 μm particle size) with a Varian AASP auto-injector. The column was eluted with solvent A : 10 mM tetrabutylammonium hydrogen sulfate, 50 mM ammonium citrate pH 5 and solvent B : acetonitrile. The mobile phase was adjusted in a 10 minute linear gradient to contain from 45 to 55% of solvent B. The flow rate was 1.25 mL/min throughout and the UV detector was set at 262 nm. The retention times observed for FPF-CoA and FPF were 8.9 and 12.9 min, respectively.

*Results*

The effect of the concentration of *R*-FPF on the rate of thioesterification is presented as a Lineweaver–Burk plot in Fig. 1. The $V_{max}$ and $K_m$ were determined on four separate liver preparations, giving a mean $V_{max}$ of 20.5 nmol/min/mg and a mean $K_m$ of 397 μM. In the presence of 0.05% v/v Triton X-100, the $K_m$ decreased to 23 μM but the $V_{max}$ remained essentially unaltered (19.7 nmol/min/mg). The inhibition of *R*-FPF thioesterification by palmitate in the presence of 0.05% Triton X-100 is shown in the Dixon plot in Fig. 2. The inhibition of the reaction by palmitate is competitive, with a $K_i$ of 5.7 μM. The $K_m$ for palmitate thioesterification under the same conditions was 1.9 μM.

In contrast to these findings, the *S*-enantiomer of FPF was a much less preferred substrate. The $V_{max}$ for thioesterification of (+)-*S*-FPF in the presence of 0.05% Triton X-100 was 3.7 nmol/min/mg with $K_m$ 476 μM. When (+)-*S*-FPF of higher ee (97%) was used, the $V_{max}$ was 2.0 nmol/min/mg and the $K_m$ was approximately 1200 μM (Fig. 3).

*Discussion*

To our knowledge, the present report is only the second concerning the direct quantification of the formation of a profen-CoA, the only other report in the literature being that of Tracy and Hall [7] concerning IPF. The limit of detection of the present method is approx. 0.05 nmol per incubation, which, under the conditions employed, allows the measurement of FPF-CoA production as low as 25 pmol/min/mg. The formation of FPF-CoA was demonstrated by the appearance of an HPLC peak with a retention time identical to that of synthetic FPF-CoA. Alkaline (KOH) hydrolysis of the HPLC eluate containing FPF-CoA resulted in the formation of FPF.

Using this analytical method, the linearity of the formation of FPF-CoA has been established as a function of incubation time and protein concentration at 5 μM (−)-*R*-FPF. The high sensitivity of the present analytical method makes possible the demonstration of the involvement of a low capacity–high affinity isoenzyme in the formation of FPF-CoA. The direct measurement of the formation of FPF-CoA is inherently superior to methods relying on determination of CoA consumption by colorimetric methods [13].

Over the concentration range 5–400 μM (−)-*R*-FPF, linear Lineweaver–Burk plots were obtained both in the presence and absence of detergent. Within this range of concentrations, there was evidence for the involvement of only one single enzyme, or of different isoforms with an identical $K_m$, in the formation of FPF-CoA. The involvement of one isoform with low affinity and high capacity for substrate concentrations higher than 400 μM cannot be excluded. The affinity of the enzyme system for *R*-FPF was considerably increased by the addition of non-ionic detergent Triton X-100. The apparent latency of the microsomal CoA ligase could explain, at least in part, the wide range of affinities for FPF-CoA formation reported in the literature. Thus, the $K_m$ values for *R*-FPF in the absence of Triton X-100 (397 μM) were similar to those obtained by Knadler and Hall [4] and Tracy *et al.* [14]. Similarly, in the presence of Triton X-100, the $K_m$ for *R*-FPF (23 μM) is similar to the values obtained by authors who included non-ionic detergents in their incubations. Interestingly, the $K_m$ for FPF-CoA formation in the presence of Triton X-100 is close to the $K_i$ for the inhibition of palmitoyl CoA ligase in liver microsomes by FPF and IPF [12]. Furthermore, these $K_m$ and $K_i$ values are similar to those reported by Sallustio *et al.* [10] for the incorporation of FPF into the triacylglycerols in isolated rat hepatocytes.

The thioesterification of (−)-*R*-FPF is competitively inhibited by palmitic acid with a $K_i$ of 5.7 μM, a value close to that reported by Tracy *et al.* [14] for (−)-*R*-IPF and

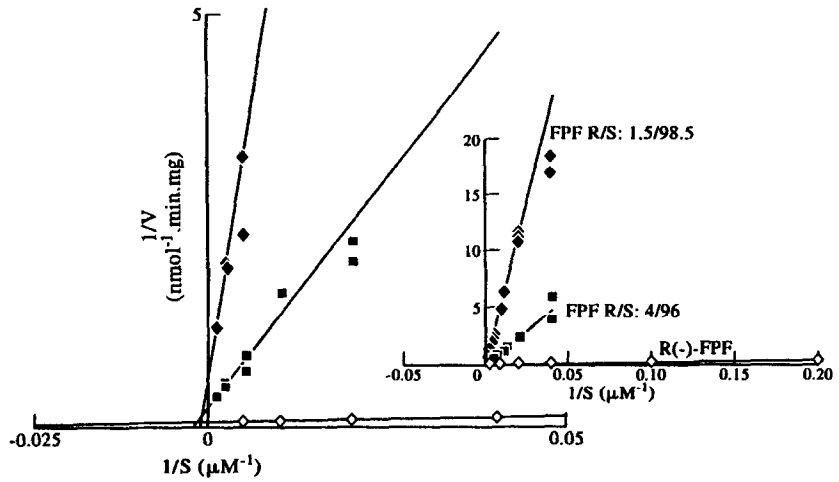


Fig. 3. Double reciprocal plot of thioesterification of (−)-*R*-FPF or (+)-*S*-FPF in the presence of 0.05% Triton X-100 by liver microsomes from a single rat.

close to the $K_m$ for the thioesterification of palmitate itself under the same conditions (1.9 μM). These data provide yet further support for the involvement of the microsomal long chain fatty acid-CoA ligase in the activation of the *R*-enantiomers of profens such as FPF and IPF.

As has already been stated, the enantiospecificity of the thioesterification of profens is the basis of their metabolic chiral inversion, although the magnitude of selectivity remains, at present, unknown. Knadler and Hall [4] have shown that while (−)-*R*-FPF is a better substrate for thioesterification than the *S*-antipode, the latter enantiomer nevertheless gives rise to (+)-*S*-FPF-CoA. This low production of (+)-*S*-FPF-CoA may be accounted for on the basis of contamination of the *S*-enantiomer by (−)-*R*-FPF. Knights *et al.* [13] have pointed out that the enantioselectivity of the thioesterification of IPF is lost at high concentrations; this is also suggested by the data of Sallustio *et al.* [10] concerning the incorporation of FPF into the triacylglycerols in isolated rat hepatocytes after incubation of either the (+)-*S*- or the (−)-*R*-enantiomer.

In our studies, the enantiomeric composition of the (+)-*S*-FPF used was *R*/*S* 4/96, and the $K_m$ of FPF CoA formation was 476 μM in the presence of detergent. This value is consistent with the thioesterification being restricted to the small amount of (−)-*R*-FPF present, for which the actual concentration is 19 μM out of a total (+)-*S*-concentration of 476 μM. When a purer batch of (+)-*S*-FPF was used as substrate, *R*/*S* 1.5/98.5, the $K_m$ rose to 1200 μM. These data indicate that the role of the (+)-*S*-enantiomer as substrate is negligible and that the concentration of (−)-*R*-FPF in the substrate accounts for the apparent formation of (+)-*S*-FPF-CoA.

The changes in $V_{max}$ as a function of purity of substrate, decreasing from 3.7 nmol/min/mg for the *R*/*S* 4/96 to 2.0 nmol/min/mg for *R*/*S* 1.5/98.5, indicate that (+)-*S*-FPF inhibits the thioesterification of the (−)-*R*-enantiomer as has been found for the thioesterification of IPF by Tracy *et al.* [14], but not, as discussed above, by acting as an alternative substrate.

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