



Effect of Etofibrate on Bile Production in the Normolipidemic Rat

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Abstract—1. The effect of etofibrate, the ethandiol-1,2 diester of nicotinic and clofibrac acids on bile production was studied in male rats that received a daily dose of 300 mg of etofibrate/kg body weight by stomach tube for 10 days and were compared with control rats receiving the medium.

2. The bile duct was cannulated, animals were intravenously given 1 μ Ci (4-¹⁴C)-cholesterol/100 b.w. and bile was collected at different intervals for a total of 4 hr.

3. Etofibrate treatment decreased plasma cholesterol and triglyceride concentrations and increased the bile flow. The cumulative amount of both bile volume and total bile radioactivity secreted increased linearly in all the animals; the respective slopes being higher in etofibrate treated rats than in controls.

4. The main labelled component found in the bile was always bile acids rather than cholesterol and the proportion of each of these compounds was similar in both groups. Neither was any difference between the groups found in the concentration of bile acids, cholesterol and phospholipids nor in the cholesterol/(bile + phospholipid) ratio.

5. Besides other factors, the present results indicate that an increase in bile flow and biliary cholesterol excretion in its free form and after its conversion into bile acids should contribute to the hypocholesterolemic effect of etofibrate.

Key Words: Hypolipidemic drugs, bile production, etofibrate, cholesterol excretion, lithogenic risk

INTRODUCTION

Etofibrate (Lipo-Merz retard, Merz & Co., Frankfurt/Main, Germany) is the ethandiol-1,2 diester of the nicotinic and clofibrac acids, and it has been shown to be a potent hypolipidemic agent in animals (Priego *et al.*, 1979a; Fabiani *et al.*, 1989; Herrera *et al.*, 1988; Bocos *et al.*, 1993) and humans (Knuchel, 1974; Series *et al.*, 1988). The hypotriglyceridemic effect of etofibrate is reasonably well understood. In rats it decreases adipose tissue lipolysis and liver VLDL (very low density lipoproteins) synthesis and secretion which together with facilitating the clearance of circulating triacylglycerols as result of increasing lipoprotein lipase activity in adipose tissue (Herrera *et al.*, 1988; Bocos *et al.*, 1993) causes its hypotriglyceridemic effect. The hypocholesterolemic effect of etofibrate is, however, less understood. It may be a secondary consequence of the reduced liver VLDL production found after acute treatment, but after prolonged treatment the drug has

been shown to inhibit HMG-CoA reductase activity in the rat (Bocos *et al.*, 1993) and the hamster (Fabiani *et al.*, 1989), something which would reduce cholesterol synthesis. The activity of cholesterol 7- α -hydroxylase, the enzyme that governs the catabolism of cholesterol to bile acids (Björkhem, 1985), has also been shown to be decreased by etofibrate in both the hamster (Fabiani *et al.*, 1989) and the rat (Bocos *et al.*, 1993). However, whereas etofibrate enhances both bile secretion and cholesterol excretion into hepatic bile in the hamster (Fabiani *et al.*, 1989), in the rat it has been found that it does not affect these parameters (Schatton *et al.*, 1986) even though its two molecular moieties, nicotinic acid and clofibrate or other fibrates, have been found to enhance them in this latter species (Holland *et al.*, 1993; Schatton *et al.*, 1986; Cayen *et al.*, 1982). An increase in the cholesterol content in the bile has been also found in humans after clofibrate (Grundy *et al.*, 1972; Pertsemkidis *et al.*, 1974), bezafibrate, fenofibrate (von Bergmann and Leiss, 1984) and gemfibrozil (Leiss *et al.*, 1985). On the contrary, data on etofibrate are controversial, i.e. in a study in volunteers etofibrate caused a biliary cholesterol increase (Schlierf *et al.*, 1980) whereas in a recently

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published study no effect of etofibrate on biliary cholesterol was found (Raedsch *et al.*, 1992). Since an increase of the cholesterol content is not always paralleled by an effect on the other bile components, phospholipids or bile acids (Okolicsanyi *et al.*, 1986), it may result in a supersaturation of the bile with cholesterol and its consequent lithogenic risk. In the present work we decided to study the effects of etofibrate on bile composition and excretion and biliary ^{14}C -cholesterol metabolism in the rat.

MATERIALS AND METHODS

Male Sprague-Dawley rats weighing 200–220 g, fed *ad libitum* a standard laboratory diet (Panlab, Barcelona, Spain) and subjected to a 12 hr on-off light cycle and 22–24°C were used. Food was removed from the cages at the onset of the light cycle (7.00 a.m.) and 3 hr later, etofibrate freshly suspended in 2% Tween-80 was administered by stomach tube without anesthesia at a dose of 300 mg/kg body weight/day to one group of animals whereas another, control, group was treated with the medium. After treatments, rats were again allowed free access to food. On the 10th day, the animals were treated as above, but 2 hr after receiving the drug or the medium the rats were anesthetized with an intraperitoneal injection (0.30 ml/100 g body weight) of a cocktail composed by ketamine chlorhydrate (50 mg), diazepam (10 mg) and atropine sulphate (1 mg) dissolved in a final volume of 2 ml of distilled water. The bile duct was cannulated by using a PE10 tube (Intramedic, USA), and the cannula was inserted close to the liver to avoid possible contamination with pancreatic juice (Billington *et al.*, 1986). Animals were maintained at 37°C in a thermostatically controlled cabinet. After the first 20 min in which bile production was found stable, animals were intravenously given 1.0 μCi of (4- ^{14}C)-cholesterol/100 g body weight, prepared as indicated below. Bile was further collected over 4 hr at 15 min intervals during the first hour, 30 min during the second hour and 60 min up to the 4th hr. When the last bile collection was ended rats were killed by collecting the blood from the aorta into chilled tubes containing 5 mg $\text{Na}_2\text{-EDTA}$.

(4- ^{14}C)-cholesterol obtained from The Radiochemical Center (Amersham, UK) with a specific activity of 55 mCi/mmol (142 $\mu\text{Ci}/\text{mg}$) was repurified before use by thin layer chromatography in heptane and diethyl-ether (55:45 by vol) medium. Appropriated aliquots of the nitrogen-dried eluates were resuspended in 95% ethanol to the proportion of 50 $\mu\text{Ci}/\text{ml}$ and this solution was dissolved with 0.9% NaCl to obtain a final proportion of 1.0 $\mu\text{Ci}/50 \mu\text{l}$ for administration.

Processing of the bile samples

Lipids were extracted from 100 μl bile aliquots with a mixture of 200 μl 50 mM $\text{KH}_2\text{PO}_4/\text{KOH}$ buffer, pH 8.0 and 500 μl chloroform. After 3 washes with chloroform the radioactivity in the water fraction was counted as ^{14}C -bile acids whereas the chloroform extracts were counted as ^{14}C -cholesterol. Recovery experiments showed that more than 93% of radioactivity initially added as ^{14}C -cholic acid was recovered in the water soluble phase, whereas more than 92% of initially added ^3H -cholesterol appeared in the chloroform phase.

Other bile aliquots were used for the enzymatic determination of bile acids (following the method described by Koss *et al.*, 1974) except that the samples were read against a standard curve of cholic acid processed in the same way as the samples. This modification was considered necessary to minimize the high interassay variability found when calculating the results against the extinction coefficient used in the original method (Koss *et al.*, 1974).

Cholesterol concentration was also measured by high performance liquid chromatography (HPLC) in another bile aliquot following the method recently described (Bocos *et al.*, 1992).

Choline phospholipids were measured in another bile aliquot using a commercial kit (Boehringer Mannheim, Germany).

Processing of plasma samples

Aliquots of plasma were used to measure total radioactivity and to determine the concentration of cholesterol and triacylglycerols by enzymatic procedures (Allain *et al.*, 1974; Buccolo and David, 1973) (commercial kits from Manarini, Italy).

Expression of the results

Radioactivity values were always corrected by the day to day variation in the amount of radioactivity administered to each animal. Results are expressed as means \pm SEM. Statistical comparison between the groups was carried out by the Student's *t*-test, whereas the statistical analysis of the cumulative values in bile was carried out by linear regression analysis following the minimal square method using a Statgraphics v. 5.0 (Maryland, USA) program.

RESULTS

After 10 days of treatment with etofibrate, the rats showed a body weight similar to that found in the control animals but their liver weight was significantly enhanced whereas plasma cholesterol and triacylglycerol levels were decreased (Table 1). As

Table 1. Effect of 10 day etofibrate treatment on body and liver weights and plasma lipidic components in the rat

	Control	Etofibrate-treated	P
Body weight (g)	246 ± 4	255 ± 2	N.S.
Liver weight (g)	8.02 ± 0.21	11.40 ± 5.3	< 0.001
Cholesterol in plasma (mg/dl)	71.0 ± 8.6	43.2 ± 5.3	< 0.05
Triglycerides in plasma (mg/dl)	66.6 ± 5.7	16.3 ± 1.8	< 0.001

Results are mean ± SE of 7 rats/group. Rats received the treatment for 10 days and were killed under ketamine anesthesia after 4 hr of bile collection, approximately corresponding to 7.5 hr of fasting and 4.5 hr after the last drug administration.

P corresponds to the statistical comparison between the two groups. N.S., not significant.

shown in Fig. 1, bile secretion remained rather constant during the 4 hr of collection in the controls. Etofibrate treatment increased the bile flow of the animals, the effect being especially manifest during the first 2 hr after interruption of the enterohepatic circulation and the difference with the controls became statistically significant at 30 and 90 min. The cumulative amount of bile secreted plotted against time gave a highly significant linear correlation for both control and etofibrate treated rats, the slope being significantly higher for the latter group than for controls (Fig. 2). A similar linear correlation was also found in the cumulative total radioactivity found in the bile of both control and etofibrate treated rats after intravenously receiving ($4\text{-}^{14}\text{C}$)-cholesterol, and here, again, the slope was significantly higher for the latter group (Fig. 3). Due to the limited amount of bile, the distribution of radioactivity in bile was only determined in the samples collected at the 120–180 and 180–240 min intervals. As shown in Table 2, at the two times, the main labelled component corresponded to bile acids rather than cholesterol, and this distribution was similar in the two groups studied.

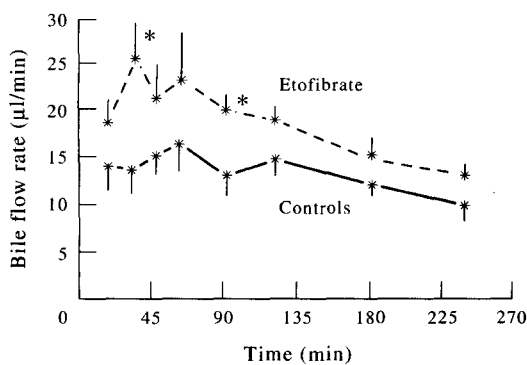


Fig. 1. Bile flow rate in rats receiving etofibrate treatment or the medium (controls) for 10 days. Bile was collected from the 2nd hr after the last treatment. After 20 min of stabilization, bile flow was measured every 15 min during the 1st hr, every 30 min during the 2nd hr and every 60 min during the last 2 hr. Results correspond to mean ± SE of 7 rat/group. * $P < 0.05$.

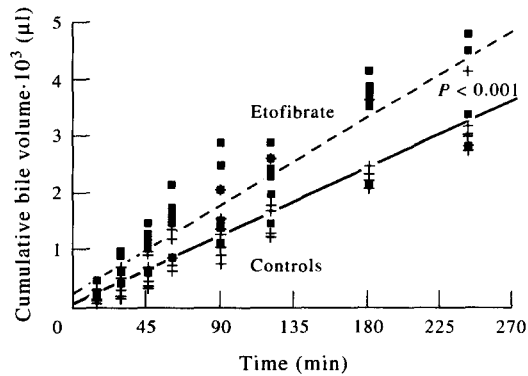


Fig. 2. Cumulative bile volume during the 4 hr of bile collection in the same animals as for Fig. 1. Linear regression for etofibrate treated rats (■---■) was: $y = (213.9 \pm 116.0) + (17.5 \pm 1.0)x$, $r = 0.926$, $n = 57$, and for control rats (+--+): $y = (54.4 \pm 79.2) + (13.4 \pm 0.7)x$, $r = 0.940$, $n = 54$. P value in the figure corresponds to the statistical difference of the slopes between the two lines.

Bile composition was measured at all the time intervals, and since no difference between measurement times could be found within each group, the mean values found for the whole time studied are shown in Table 3. No difference in bile acid, cholesterol and choline phospholipid concentration could be found between the control and etofibrate treated rats. Additionally, there was no difference between the two groups in so far as cholesterol/(bile acid + choline phospholipids) ratio was concerned (Table 3), indicating an unmodified saturation index.

DISCUSSION

In the present study, etofibrate treatment for 10 days to normolipidemic rats decreased both plasma triglycerides and cholesterol, which confirm previous

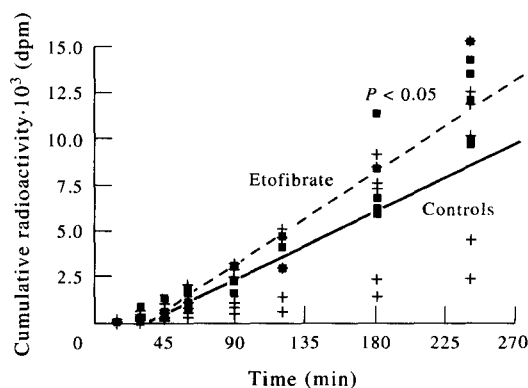


Fig. 3. Cumulative total radioactivity in bile during 4 hr after the intravenous administration of ($4\text{-}^{14}\text{C}$)-cholesterol in the same rats as for Fig. 1. Linear regression for etofibrate treated rats (■---■) was: $y = (-1764 \pm 316) + (55.5 \pm 2.6)x$, $r = 0.953$, $n = 48$, and for control rats (+--+): $y = (-1301 \pm 526) + (42 \pm 4)x$, $r = 0.822$, $n = 48$. P value in the figure corresponds to the statistical difference of the slopes between the two lines.

Table 2. Effect of 10 day etofibrate treatment on the percentual distribution of radioactivity in bile components at the last bile collection periods

	Control	Etofibrate-treated	P
Bile collected between 120–180 min			
Bile acids	77.6 ± 1.0	74.2 ± 3.6	N.S.
Cholesterol	22.4 ± 1.1	25.8 ± 3.5	N.S.
Bile collected between 180–240 min			
Bile acids	79.1 ± 1.3	76.1 ± 2.6	N.S.
Cholesterol	20.9 ± 1.3	23.9 ± 2.6	N.S.

Results are mean ± SE of 7 rats/group. Other experimental details as indicated in legend for Table 1 and in the text.

findings (Priego *et al.*, 1979b; Herrera *et al.*, 1988; Bocos *et al.*, 1993). The mechanism by which etofibrate causes its hypotriglyceridemic effect involves inhibition of lipolysis, inhibition of liver VLDL triacylglycerol synthesis and secretion (Herrera *et al.*, 1988; Bocos *et al.*, 1993) and enhancement in adipose tissue lipoprotein lipase activity (Herrera *et al.*, 1988) which would indicate an increase in the clearance of circulating triacylglycerols, although the latter has never been directly tested.

The hypocholesterolemic effect of etofibrate may also be the result of several factors: (1) a secondary consequence of the hypotriglyceridemic effect, as indicated by the lag in the decrease in plasma cholesterol as compared to the hypotriacylglycerolemic effect found after acute treatment (Bocos *et al.*, 1993); (2) a decrease in the cholesterol synthesis (Priego *et al.*, 1979b) as a result of an inhibiting effect on hepatic HMG-CoA reductase as shown in the rat (Bocos *et al.*, 1993) and in the hamster (Fabiani *et al.*, 1989); (3) an activation of the LDL (low density lipoproteins) receptor pathway as proposed by studies in hypercholesterolemic subjects (Series *et al.*, 1988); and (4) an increase in the bile flow and in the biliary excretion of cholesterol either in its free form or after its conversion into bile acids. Present findings show that this fourth possibility is what is actually occurring after 10 days of treatment in the rat and agrees with similar findings previously found in the hamster (Fabiani *et al.*, 1989). However they contrast with the unchanged bile flow reported by Schatton *et al.* in rats receiving the etofibrate treatment for 2

or 4 weeks (Schatton *et al.*, 1986), although the drug doses used by these authors were some 3–4 times lower than the one used here. In that study, however, the concentration of cholesterol in the bile was enhanced by 2 weeks of etofibrate treatment in the rats, and no change could be detected in the lithogenic index (Schatton *et al.*, 1986) as was also the case in the present study and in hamster gallbladder bile (Fabiani *et al.*, 1989). These findings therefore indicate that the risk of gallstone formation by treatment with etofibrate appears to be low. Moreover, data of a recently published study in hyperlipidemic patients (Raedsch *et al.*, 1992) give even stronger evidence that etofibrate has a low risk. However, it must be kept in mind that the cholesterol saturation index has been shown to be augmented in the hepatic bile of treated hamsters (Fabiani *et al.*, 1989) and in that of healthy volunteers (Schlierf *et al.*, 1980).

Present findings showing that etofibrate enhances bile production contrast with the decreased cholesterol 7 alpha-hydroxylase activity found previously by us in rats receiving the same treatment (Bocos *et al.*, 1993). This enzyme is the rate-limiting one in the bile acid biosynthesis pathway (Björkhem, 1985; Myant and Mitropoulos, 1977), and it is known to change in parallel with the activity of HMG-CoA-reductase activity (Pandak *et al.*, 1990), which we know is also decreased by etofibrate treatment (Bocos *et al.*, 1993). The cholesterol-7-alpha-hydroxylase is not fully saturated *in vivo* (Pandak *et al.*, 1990) and other conditions exist besides the one found here for etofibrate such as bezafibrate treatments in the rat where decreased cholesterol-7-alpha-hydroxylase activities (Stahlberg *et al.*, 1989) appear in spite of enhanced bile acid concentration in the bile (Schatton *et al.*, 1986). Biliary cholesterol secretion in the rat is largely independent of newly synthesized cholesterol (Turley and Dietschy, 1981), and cholesterol and phospholipids are packaged and secreted in vesicles that are brought to the canalicular membrane of the hepatocytes via a microtubular system (Crawford *et al.*, 1989). Although bile acids are known to be mainly originated from newly synthesized hepatic cholesterol (Schwartz *et al.*, 1975, 1977; Staple and Gurin, 1954; Cronholm *et al.*, 1974; Norman and Norum, 1974), our findings show the rapid conversion of administered labelled cholesterol into bile acids, a result which confirms the observations of others (Siperstein *et al.*, 1952; Siperstein and Chaikoff, 1952; Nilsson and Zilversmit, 1972) and which indicates that particulate cholesterol is transferred directly from Kupffer cells to liver parenchyma for bile acid synthesis. In any case, bile acids are transported across the hepatocytes as discrete molecules (Barnwell *et al.*, 1984) but all of these

Table 3. Effect of 10 day etofibrate treatment on bile components in the rat

	Control	Etofibrate-treated	P
Bile acids (mmol/l)	11.7 ± 0.5	12.1 ± 0.5	N.S.
Cholesterol (mmol/l)	0.209 ± 0.011	0.192 ± 0.006	N.S.
Choline phospholipids (mmol/l)	1.89 ± 0.04	1.95 ± 0.03	N.S.
100 × cholesterol/(bile acids + choline phospholipids) ratio	1.53 ± 0.05	1.44 ± 0.04	N.S.

Results are mean ± SE of 7 rats/group. Other experimental details as indicated in legend for Table 1 and in the text.

molecules (bile acids, cholesterol and phospholipids) are transported to the canalicular membrane via vesicle-mediated transport (Rigotti *et al.*, 1993). Since etofibrate did not modify the bile composition, we may therefore suppose that it enhanced the movement of secretory vesicles within the hepatocytes through a mechanism that still needs to be elucidated.

The effect of etofibrate enhancing bile production could be the result of the action of its constitutive moieties. Both nicotinic acid (Holland *et al.*, 1993) and clofibrate (Cayen *et al.*, 1992) have been found to enhance bile flow rates in the rat. However both of these molecules increased the biliary output of cholesterol to a much greater extent than that of either phospholipid or bile acids (Holland *et al.*, 1993; Cayen *et al.*, 1982) and we did not find such an effect with etofibrate. It seems then that a compensatory action occurs in the combination molecule of nicotinic acid and clofibrate which allows them to maintain their respective choleteretic effect but balances bile composition. Further studies are needed to understand the mechanism for such an effect.

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