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SEPARATION AND MEASUREMENT OF CLOFIBROYL COENZYME A AND CLOFIBRIC ACID IN RAT LIVER AFTER CLOFIBRATE ADMINISTRATION BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH PHOTODIODE ARRAY DETECTION

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SUMMARY

A method to identify and quantitate clofibric acid and clofibroyl coenzyme A (CoA) products in rat liver was developed using reversed-phase high-performance liquid chromatography. The system was developed with baseline separation of clofibroyl-CoA from clofibric acid using isocratic elution, with a mobile phase consisting of 52% methanol and 28 m*M* potassium phosphate buffer (pH 4.2). With this high methanol concentration, the large amount of UV-absorbing materials present in the liver extracts were eluted earlier than the investigated compounds. Clofibroyl-CoA has a characteristic absorbance spectrum with distinct peaks at 260 and 230 nm, while clofibric acid showed only a distinct peak at 230 nm. Using an on-line photodiode array detector, the spectra could be recorded during analysis without interrupting the flow of the mobile phase. This spectral analysis improves identification possibilities and evaluation of the purity of the chromatographic peaks. In a perchloric extract of rat liver, the recovery of clofibric acid and clofibroyl-CoA added to the liver extract ranged from 70 to 80%. A linear relationship was observed between clofibric acid and clofibroyl-CoA concentration and the area of their peaks in the chromatogram. The detection limit of the method was lower than 5 pmol for both compounds when the absorbance was recorded at 230 nm. The method could be used without modification for the estimation of clofibroyl-CoA and clofibric acid in biological extracts.

INTRODUCTION

In recent years, there has been considerable interest in links between drug metabolism reactions and aspects of lipid biochemistry [1—5]. Attention has focused particularly upon xenobiotics containing carboxylic acid groups.

Clofibrate, one such drug, is the ethyl ester of *p*-chlorophenoxyisobutyric

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acid and has been used for the treatment of hypolipidemics in humans. This drug induces hypertrophy and hyperplasia [6] and in long-term studies produces hepatocellular tumours [7], causing peroxisome proliferation [8] and modulating lipid metabolism [9—10]. In short-term studies, the drug enhances the activities of peroxisomal enzymes catalysing β-oxidation of fatty acids [11], carnitine acetyltransferase [12] and other hepatic enzymes, e.g. palmitoyl coenzyme A (CoA) hydrolase [13—15] and clofibroyl-CoA hydrolase [16]. Furthermore, the levels of free CoASH and long-chain acyl-CoA are increased after clofibrate administration [14—17]. Recently, we have found that clofibrate shows tumour-promoting but not direct-transforming activity in vitro [18].

The active metabolite of clofibrate is thought to be clofibric acid. In rat liver microsomes there are four chemically distinct carboxylesterases, the most abundant of which cleaves clofibrate [19, 20]. Many xenobiotics with carboxyl groups, either ingested or formed in the body, can be activated to CoA-thioester and their potential for disturbing normal lipid metabolism is considerable [1—5]. Thus, one of the active forms of clofibrate, converted and metabolized to carboxylic acid, may be the CoA-ester.

It was the purpose of the present experiments to examine whether clofibrate could be converted to the corresponding acids and CoA derivative. Determination of clofibrate levels in human plasma by high-performance liquid chromatography (HPLC) has been reported [21, 22]. Recently, Paillet et al. [23] have described a rapid determination of clofibric acid in human plasma by HPLC. This paper describes a reversed-phase system suitable for quantitation of clofibric acid and clofibroyl-CoA in biological samples. The method is simple, relatively fast and requires little sample preparation. The results represent the first quantitative determination by reversed-phase HPLC of clofibric acid and its CoA derivative in liver extract of clofibrate-treated rats.

EXPERIMENTAL

Chemicals

HPLC-grade methanol was purchased from Rathburn Chemicals (Walkerburn, Scotland). Potassium phosphate was from Merck. Deionized water was purified by passing it through a Milli-Q water purification system (Millipore, Bedford, OH, U.S.A.). The potassium phosphate buffer was filtered through a Millipore filter, type HA (0.45 μm). Clofibroyl-CoA was a gift from Dr. H. Osmundsen (Department of Biochemistry, The Norwegian College of Veterinary Medicine, Oslo, Norway). Clofibroyl-CoA was essentially synthesized as described by Kawaquchi et al. [24]. The CoA-ester was stored at −20°C and was stable for four months. Clofibric acid, CoASH and dithiothreitol were obtained from Sigma (St. Louis, MO, U.S.A.). All other reagents were of the highest purity commercially available.

Animals

Male Wistar rats weighing about 180 g were used for clofibrate and for control experiments. The drug was added to the commercial rat diet by soaking the food with an acetone solution [13] and evaporating off the organic solvent

to leave pellets containing 0.3% (w/w) clofibrate. Diet with acetone alone was used for the control animals. The rats had free access to water and pellets.

Method of tissue preparation and extraction for HPLC analysis

At the end of the experiments, which lasted for ten days, the rats were stunned, decapitated and the abdominal cavity was quickly opened. The liver was rapidly removed and stored in liquid nitrogen. The liver samples (ca. 0.4 g) were powdered in a mortar cooled with liquid nitrogen. The powdered livers were homogenized in cooled perchloric acid; final concentration 6% (v/v). The denatured protein was removed by centrifugation (8000 *g*) in an Eppendorf centrifuge for 3 min. For studies of pH versus CoA stability, the supernatant of the perchloric acid extract was adjusted to either pH 3.5—4.0 or pH 6.5—7.0 with 1.44 *M* potassium hydroxide—1.2 *M* potassium bicarbonate. Samples of the supernatants were injected directly into the liquid chromatograph.

In some experiments, acid-insoluble material was used. The perchloric acid precipitate (see above) was washed once in 1 ml of perchloric acid (0.6%) containing 10 m*M* dithiothreitol, and once in 1 ml of distilled water. The precipitate was finally resuspended in 200 μl of water containing 10 m*M* dithiothreitol. The pH was adjusted to 12 with 1 *M* potassium hydroxide. The suspension was kept on a thermoplate at 55°C for 1 h and then the pH of the suspension was adjusted to 6.5. After centrifugation, samples of the supernatant were injected directly into the liquid chromatograph.

The recovery of added standards was accomplished as follows: different concentrations of clofibric acid and clofibroyl-CoA, dissolved in 50 m*M* potassium phosphate (pH 6.5), were added to ca. 0.4 g of frozen liver powder. After homogenization and extraction with 1 ml of ice-cold 6% perchloric acid, the denatured protein was removed by centrifugation. The supernatant, adjusted to pH 3.5—4.0 or 6.5—7.0, was used directly for analysis.

Equipment and chromatographic system

A Spectra-Physics SP 8700 solvent delivery system was used for HPLC. The column outlet was connected to a variable-wavelength detector, Model Spectroflow 773 from Kratos. In some experiments, the column was connected to a photodiode array detector, Model HP 1040A (Hewlett-Packard). The chromatographic profiles were routinely recorded and integrated by a Spectra-Physics SP 4270 reporting integrator. The absorbance spectra of the clofibric acid and clofibroyl-CoA peaks were monitored and stored on floppy discs (Model HP 82901M disc drive) from Hewlett-Packard. The spectra and the chromatograms were recorded by a *X*—*Y* plotter from Hewlett-Packard, Model HP 7470A. The chromatographic procedure was as follows: a 5-μm Supelcosil LC-18 column (25 cm × 4.6 mm I.D.) preceded by a 2-cm Supelguard LC-18 column was eluted isocratically at 1.5 ml/min with 52% (v/v) methanol in 28 m*M* potassium phosphate buffer (pH 4.2). Samples of 20 or 50 μl were injected into the column. In the system under these conditions, the pressure was 170 bar. Detection was based on line absorbance at 230 nm.

Clofibrate, clofibric acids and clofibroyl-CoA were dissolved in a 50 m*M* potassium phosphate solution (pH 6.5). The absorbance spectra were recorded between 200 and 400 nm using a diode array spectrophotometer, Model 8450A from Hewlett Packard.

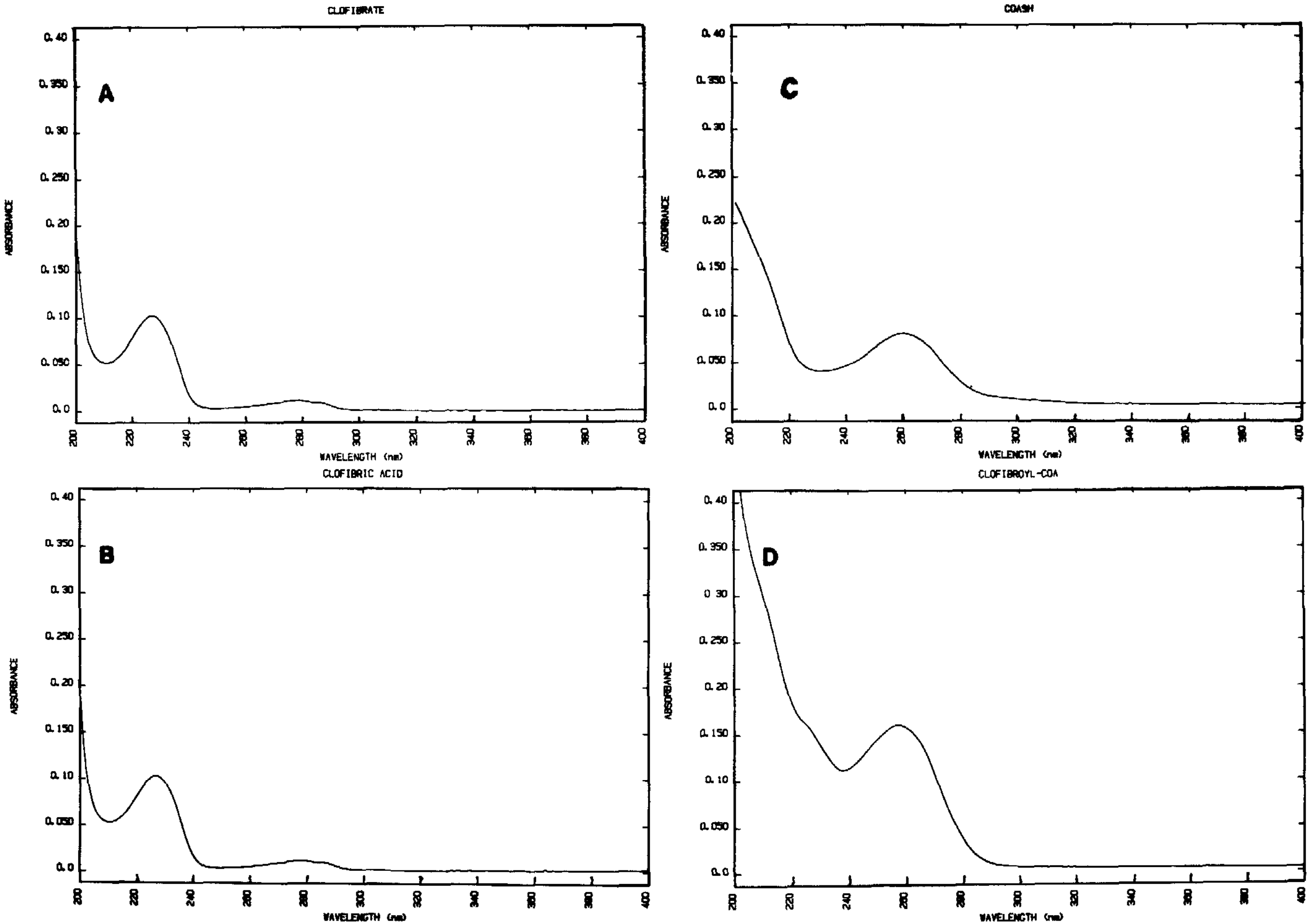


Fig. 1. The absorbance spectra of clofibrate (A), clofibric acid (B), CoASH (C) and clofibroyl-CoA (D). The corresponding standards were dissolved in 50 m*M* potassium phosphate buffer (pH 6.5). The absorbance was recorded between 200 and 400 nm using a diode array spectrophotometer, Model 8450 A from Hewlett Packard.

Other analytical methods

An enzyme able to hydrolyse clofibroyl-CoA was partially purified from the cytosolic fraction of rat liver by gel chromatography [25]. The clofibroyl-CoA hydrolase activity was assayed spectrophotometrically by monitoring the release of CoASH using a reaction mixture containing 15 m*M* Hepes buffer (pH 7.5), 2 m*M* EDTA, 0.01% Brij-58, 0.3 m*M* DTNB [5,5-dithio-bis(2-nitrobenzoic acid)], 30 μg of protein and clofibroyl-CoA obtained after HPLC elution of an acid-soluble liver extract prepared from clofibrate-treated rats (see Fig. 4B and Fig. 6). A linear relationship with respect to activity and amount of enzyme protein or substrate concentration was always maintained.

RESULTS AND DISCUSSION

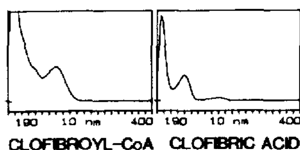
Absorbance spectra of clofibrate, clofibric acids, CoASH and clofibroyl-CoA

A Hewlett-Packard photodiode array spectrophotometer (Model 8450A) was used to record the spectra of clofibrate, clofibric acid, CoASH and clofibroyl-CoA (Fig. 1). Both clofibrate and clofibric acid showed a distinct absorbance peak at 230 nm, while clofibroyl-CoA showed an absorbance shoulder at that wavelength. However, a clear absorbance peak at 260 nm was obtained for clofibroyl-CoA (Fig. 1D), which is attributed to the absorption of the CoASH part (Fig. 1C). Thus, the spectrum of clofibroyl-CoA is different from the spectra of both clofibric acid and clofibrate.

Reversed-phase HPLC of the standards

Fig. 2 shows the elution profiles of clofibroyl-CoA and clofibric acid on reversed-phase HPLC. Absorbance of the eluate was monitored at 230 nm. In the isocratic system adopted, the retention times were 4.0 and 4.7 min for clofibroyl-CoA and clofibric acid (Fig. 2), respectively. The identity of the peaks at the corresponding retention times was confirmed by obtaining a UV spectrum of the eluate as it passed through the detector cell (insert of Fig. 2). The retention time for authentic clofibrate was 46 min in the adopted system (data not shown). These results show that clofibrate does not coelute with either clofibric acid or clofibroyl-CoA. In the subsequent experiments, conducted to optimize the conditions for alkaline hydrolysis of clofibroyl-CoA, an aliquot of standard clofibroyl-CoA was treated with base, heated to 55°C and the pH was readjusted to pH 6.5. In the chromatogram of the resulting sample, a clear absorbance peak at a retention time of 4.6 min was observed (Fig. 3). The peak was identified by the UV spectrum and, as expected, the peak represented clofibric acid (data not shown). Furthermore, Fig. 3 shows that there was no evidence of compounds coeluting with the CoASH part of clofibroyl-CoA. This method was applied in the assay of endogenous clofibroyl-CoA of rat tissue (acid-insoluble clofibroyl-CoA).

When the column was eluted with a 1:1 mixture of water and methanol, severe tailing of both clofibric acid and clofibroyl-CoA was observed. Addition of a phosphate solution instead of water, however, with the same amount of methanol, reduced the tailing of the compounds and subsequently a better separation of clofibroyl-CoA and clofibric acid was obtained. A concentration of 52% (v/v) methanol and 28 m*M* potassium phosphate buffer (pH 4.2) was



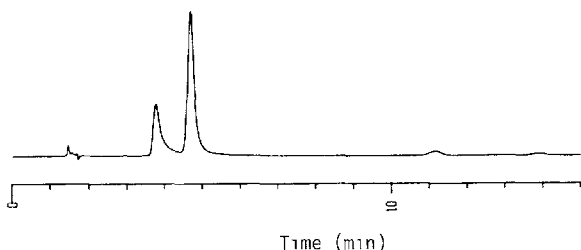


Fig. 2. Chromatographic separation of clofibroyl-CoA (first peak) and clofibric acid (second peak) standards. The absorbance spectra of the chromatographic peaks, tentatively identified as clofibroyl-CoA and clofibric acid, are shown in the upper left-hand corner of the figure.

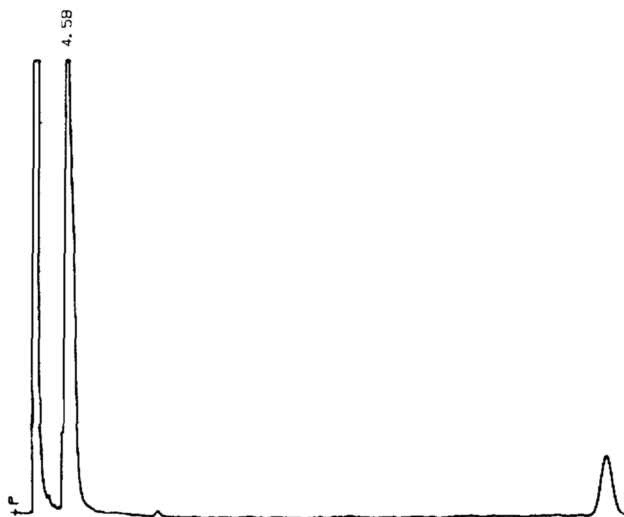


Fig. 3. HPLC elution pattern of clofibroyl-CoA after alkaline hydrolysis under standard conditions. The peak at retention time 4 58 min is clofibric acid

found to be optimal and the remaining chromatograms in this report were performed under these conditions.

Early in the development of this method, we found that a baselıne separation of clofibric acid and clofibroyl-CoA was sensitive to the pH of the solution in which they were prepared. At pH 3.5—4.0, the clofibroyl-CoA peak had severe tailing. At pH 6.5—7.0, however, a clear separation of the peaks was obtained, but at that pH the clofibroyl-CoA standard was sensitive and some degradation was found. After 12 h at 4°C, the amount of clofibroyl-CoA in tissue extracts adjusted to pH 7.0 was 60% of that found at time zero.

Standard curves

A standard curve was obtained with pure clofibric acid and clofibroyl-CoA (data not shown). Each standard curve showed a good correlation between the area of peaks measured by absorbance at 230 nm and the amount of clofibric acid and clofibroyl-CoA. The following regression lines of the corresponding compounds were: clofibric acid, $y = 3.7 \cdot 10^5 x + 2.9 \cdot 10^4$; clofibroyl-CoA, $y = 4.1 \cdot 10^4 x + 6.6 \cdot 10^3$. A linear standard curve of clofibroyl-CoA was also obtained at 260 nm (data not shown). The detection limit with electronic integration at 230 nm was ca. 5 and 2 pmol per injection for clofibroyl-CoA and clofibric acid, respectively, with a signal-to-noise ratio of 15.

Applications

The method reported here has been used to determine clofibric acid and clofibroyl-CoA in biological samples. At the high methanol concentration of the solvent system used, the large amount of UV-absorbing materials present in the liver extract (compounds of low molecular weight and of ionic nature, e.g. ATP, dithiothreitol and CoASH) were eluted in the solvent front.

After clofibrate administration to rats, six peaks with retention times of 3.6, 4.0, 4.4, 4.6, 5.8 and 7.0 min, respectively, were observed on the chromatogram of an acid-soluble liver extract (Fig. 4B). The UV-absorbing material in the tissue extract, which eluted near the front of the chromatographic run, did not interfere with determination of the six unknown peaks. In the extracts of livers from control animals, no absorbance at all within this retention-time period was observed at 230 nm (Fig. 4A).

The identity of the peaks at the different retention times was investigated by obtaining UV spectra of the material as it passed through the detector cell. The absorbance peak at a retention time of 4.4 min showed a spectrum comparable to clofibroyl-CoA, but the characteristic absorbance of the CoASH part of the compound was weak. However, the on-line UV spectrum of the substance of this peak was very similar to the UV spectrum of standard clofibroyl-CoA at low concentration (Fig. 5), where the absorbance peak of the CoASH part (at 260 nm) was not so discernible as with a higher concentration of clofibroyl-CoA.

The substance of the absorbance peak at a retention time of 4.6 min (Fig. 4B) showed a spectrum comparable to the clofibric acid standard (data not shown). Definite proof of the composition of the possible clofibroyl-CoA and clofibric peaks in the chromatogram was obtained in experiments with the enzyme clofibroyl-CoA hydrolase [16]. This enzyme catalyses the reaction:

clofibroyl-CoA + $H_2O \rightleftharpoons$ clofibric acid + CoASH

and the equilibrium appears to be to the right. Thus, this enzyme reaction can be used to quantify the clofibroyl-CoA. After addition of clofibroyl-CoA hydrolase, more than 96% of the material in the clofibroyl-CoA peak (retention time of 4.4 min) was consumed, with concomitant formation of clofibric acid at a retention time of 4.6 min (data not shown). To determine whether there were any materials coeluting with clofibroyl-CoA, an aliquot of a liver extract from clofibrate-treated animals was treated with base to hydrolyse clofibroyl-CoA. The pH of the extract solution was adjusted to 12 and the extract was

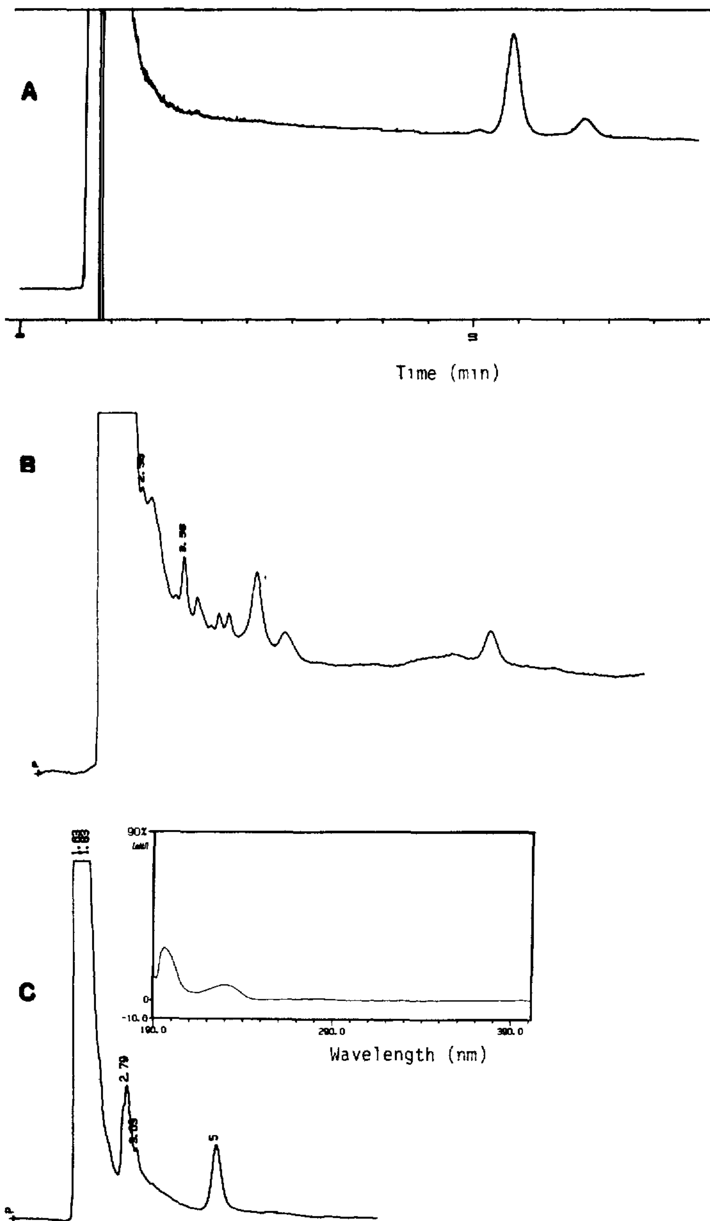


Fig. 4. HPLC elution pattern of an acid-soluble extract (A, B) and an acid-insoluble extract (C) of liver from normal rats (A) and clofibrate-treated rats (B, C). Absorbance of the effluent was recorded by a photodiode array detector at 230 nm. The absorbance spectrum of the chromatographic peak (retention time, t_R = 5.0 min) (C), tentatively identified as clofibric acid, is shown. The absorbance peaks at t_R = 4.4 min and t_R = 4.6 min (B) were identified as clofibroyl-CoA and clofibric acid, respectively.

heated at 55°C for 1 h, after which the pH was readjusted to ca. 7.0 with perchloric acid and 1.44 *M* potassium hydroxide—1.2 *M* potassium bicarbonate. In the chromatogram of the resulting sample, there was no evidence of compounds coeluting with the CoA part of the possible clofibroyl-CoA after

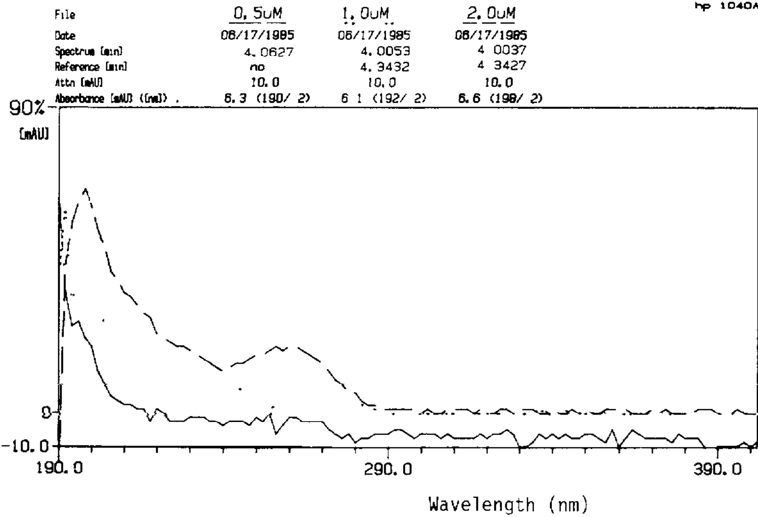


Fig. 5. Absorbance spectra of clofibroyl-CoA standards of different concentrations: 2.9 μM clofibroyl-CoA (- - -), 1.0 μM clofibroyl-CoA (· · · · ·), 0.5 μM clofibroyl-CoA (——). Absorbance of the eluates was recorded by a photodiode array detector

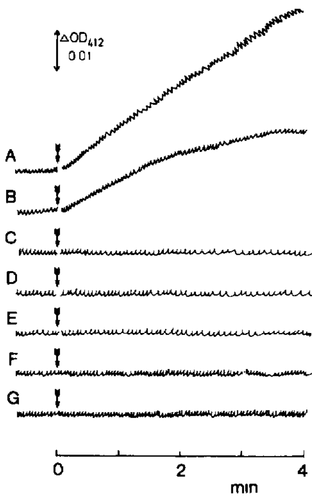


Fig. 6. Clofibroyl-CoA hydrolase activity assayed by measuring the release of thiol groups from clofibroyl-CoA detected by DTNB at 412 nm. The enzyme was partially purified from a cytosol fraction of liver from clofibrate-treated rats (see Experimental). The substances in the six unknown peaks in chromatogram B of Fig. 4. and an extract spiked with clofibroyl-CoA (trace A) were used as substrates (200 μl) for the purified clofibroyl-CoA hydrolase. Trace G: substance of peak, t_R = 3.6 min; trace F: substance of peak, t_R = 4.0 min; trace E: substance of peak, t_R = 4.6 min; trace D: substance of peak, t_R = 5.8 min; trace C: substance of peak, t_R = 7.0 min; trace B: substance of peak, t_R = 4.4 min; trace A: substance of peak (t_R = 4.4 min) after injection of a liver extract spiked with 3.0 μM clofibroyl-CoA

treatment of the sample with base (insert to Fig. 4C). However, the UV spectrum of the clear peak at a retention time of 5.0 min was similar to the clofibric acid standard spectrum (insert to Fig. 4C). In another experiment, we investigated whether there were any materials coeluting with the possible

clofibroyl-CoA and clofibric acid. An extract was spiked with a standard solution of both clofibric acid and clofibroyl-CoA immediately before injection. The standards coeluted with the peaks of absorbance from the extracts, with retention times of 4.4 and 4.6 min, respectively (data not shown).

Finally, fractions of the elution pattern of an extract from liver of clofibrate-treated rats (Fig. 4B) were collected. These fractions, containing possible clofibroyl-CoA as well as clofibric acid, were used as substrate for the clofibroyl-CoA hydrolase. Hydrolysing activity was assayed by measuring the release of thiol groups from clofibroyl-CoA detected by DTNB. Fig. 6 shows that the substance of the possible clofibroyl-CoA peak in the chromatogram after elution of a liver extract of clofibrate-treated rat was a substrate for the enzyme. Fractions other than the clofibroyl-CoA and the clofibric acid peaks contained no substrate for the enzyme.

The retention times of clofibroyl-CoA and clofibric acid are critically dependent on the methanol concentration and the pH of the solution in which they were prepared. Comparison of Figs. 2 and 3 with Fig. 4B and C shows that changes in the amount of methanol and the pH result in small changes in the retention times. However, these small differences of the two parameters had no significant effect on the separation and quantitation of clofibroyl-CoA and clofibric acid in biological samples (Fig. 4B). Altogether, these results show that clofibroyl-CoA is undoubtedly formed in rats after clofibrate administration. The formation of clofibric acid from clofibrate seemed to be catalysed by the microsomal carboxylesterase (p*I* 6.0), as this enzyme has high activity toward clofibrate [19—20]. As shown in Fig. 4B, four other peaks were found in the liver extract from clofibrate-treated rats. These clofibrate-derived unknown substances disappeared after alkaline hydrolysis (Fig. 4C), suggesting the presence of an ester bond.

The method described in this report is useful for the determination of free clofibric acid and clofibroyl-CoA under different situations. The analysis is direct and rather fast, and linearity is excellent. We have applied this method to quantitation of clofibric acid and especially clofibroyl-CoA in biological extract. To our knowledge, it is the first time that the formation of clofibroyl-CoA in a biological extract has been reported. In an acid-soluble extract of liver of clofibrate-treated rats, the content of clofibric acid and clofibroyl-CoA were 0.12 ± 0.04 nmol/mg of protein ($n = 5$) and 0.19 ± 0.05 nmol/mg of protein ($n = 5$), respectively.

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