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In Vitro Metabolism of Acitretin by Human Liver Microsomes: Evidence of an Acitretinoyl-coenzyme A Thioester Conjugate in the Transesterification to Etretinate

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ABSTRACT. The aromatic retinoid acitretin is the primary active metabolite of etretinate, and in this study we investigated the ethyl esterification of acitretin to etretinate using [^{14}C]acitretin and human liver microsomes. Samples were analysed by TLC, HPLC, and LC–MS. Essential requirements for the transesterification reaction were identified and included viable microsomal protein, ATP, CoASH, and ethanol. Human liver microsomes catalysed formation of acitretinoyl-CoA at the rate of 0.08 ± 0.02 nmol/min/mg (mean ± SD, $N = 10$). Acitretinoyl-CoA was pivotal for the transesterification to etretinate and in the presence of methanol, ethanol, *n*-propanol, *n*-butanol, and hexanol, the corresponding esters, namely methyl-, ethyl (etretinate)-, propyl-, butyl-, and hexyl-acitretinate, were formed. On average, 1.7% of the acitretin present in the incubation was converted to etretinate in the presence of ethanol. In the absence of ethanol, transesterification did not proceed. Inhibition of the ester hydrolysis of etretinate by *bis-p*-nitrophenylphosphate (BNPP, 1 mM) prevented futile cycling of etretinate via acitretinoyl-CoA. An additional finding was that acitretin (15–30 μM) activated significantly human liver microsomal long-chain fatty acid-CoA ligase (E.C.6.2.1.3, LCL), resulting in enhanced formation of palmitoyl-CoA. This study demonstrated that in the presence of ethanol the ethyl esterification of acitretin to etretinate proceeds via formation of acitretinoyl-CoA. Predicting clearance of acitretin *in vivo* via this unique metabolic pathway will be a challenge, as the intracellular concentration of ethanol could never be predicted with any degree of accuracy in humans. BIOCHEM PHARMACOL **60**;4:507–516, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. acitretin; etretinate; human liver microsomes; conjugation; acitretinoyl-CoA; transesterification

Acitretin (Ro 10-1670, all-trans-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraenoic acid), an aromatic retinoid used in the treatment of severe psoriasis and keratinoid disorders, is the primary active metabolite of the ethyl ester, etretinate. Both are therapeutically valuable retinoids, though with a teratogenic liability. The clinical usefulness of etretinate is limited in humans because of evidence of extensive accumulation in adipose tissue during chronic dosing. Acitretin was subsequently developed, as it was considered that the presence of the more polar carboxylate moiety would enhance elimination. Studies conducted in humans established that the primary metabolite of acitretin was 13-*cis* acitretin, but low concentrations of etretinate were also detected in plasma [1]. Ethyl esterification was not considered a normal route of metabolism for carboxylic acids, but studies conducted by Chou *et al.* [2, 3] and Laugier *et al.* [4] confirmed that ethyl esterification of acitretin to etretinate occurred in the presence of ethanol. Metabolism of acitretin to etretinate was demonstrated using human liver microsomes, and the process had a primary requirement for CoASH, ATP, and ethanol [5]. The authors proposed that the metabolic pathway involved initial formation of acitretinoyl-CoA, which was then transesterified by ethanol, forming etretinate.

The role of hepatic microsomal long-chain fatty acid-CoA ligase (E.C.6.2.1.3.) in metabolism via acyl-CoA formation is well established for many carboxylic acid xenobiotics, [6] but there are limited reports of CoA conjugation of retinoids. Formation in rats of a taurine conjugate of retinoic acid is indicative of prior formation of retinoyl-CoA [7] and similarly, the production of ethyl retinoate from all-*trans*-retinoic acid is again suggestive of retinoyl-CoA formation [8]. To date, a role for human liver

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microsomal CoA ligases in the metabolism of acitretin has not been established.

Although ethyl esterification is an uncommon route of metabolism for xenobiotic carboxyic acids, free fatty acids are known to esterify with short-chain alcohols to form fatty acid ethyl esters. This reaction can be catalysed by both a cytosolic enzyme in the absence of ATP and CoA [9] and a hepatic microsomal enzyme that is both ATP- and CoA-dependent [10]. The latter would appear to fulfil the criteria for the putative ethanol acyltransferase involved in the ethyl esterification of acitretin.

The objectives of this *in vitro* study were threefold: firstly, to investigate the role of human hepatic microsomal LCL* in the metabolism of acitretin; secondly, to determine whether substitution of ethanol with methanol, butanol, propanol, and hexanol results in formation of the corresponding esters; and thirdly, to determine if ethanol precursors such as acetaldehyde and ethylacetate could be utilised for the formation of etretinate.

MATERIALS AND METHODS

Materials

F. Hoffman-La Roche supplied [^{14}C]acitretin (sp. act. 57.3 mCi/mmol), acitretin (Ro 10-1670), and etretinate (Ro 10-9359). Coenzyme A, ATP, NADP, and palmitic acid were purchased from Sigma Chemical Co. and [^{14}C]palmitic acid (sp. act. 55 mCi/mmol, purity 98.8%) was from Amersham. Glucose-6-phosphate dehydrogenase was supplied by Boehringer Mannheim. All other reagents used were of the highest analytical grade available.

Human Liver Microsomes

Human liver specimens (H6, H24, H25, H27, and H30) were obtained with the consent of relatives from organ transplant donors; details of the tissue used have been published previously [11]. Approval for use of the tissue for the drug metabolism studies was obtained from the Flinders Medical Centre Committee on Clinical Investigation (Adelaide, Australia). *In vitro* studies investigating esterification of acitretin were conducted in Basel, Switzerland using pooled human liver microsomes from 9 human livers obtained in accordance with the guidelines of the institution's Ethical Committee from hepatic surgical resections at the Hospital Hautepierre (Strasbourg, France). Hepatic microsomes prepared using a standard differential ultracentrifugation technique were stored at −80° until used, and all preparations contained <1% mitochondria or peroxisomes.

* *Abbreviations:* BNPP, *bis-p*-nitrophenylphosphate; and LCL, long-chain fatty acid-CoA ligase.

Human Liver Long-Chain Fatty Acid-CoA Ligase Assay

All assays were conducted under dimmed light conditions using either amber or foil-covered glassware. Preliminary studies established linearity of the reaction with respect to incubation duration and protein concentration. The standard incubation medium (0.2 mL) contained Tris–HCl (150 mM, pH 7.7), $MgCl_2$ (6.2 mM), Triton X-100 (0.05%), EDTA (2 mM), ATP (2 mM), CoASH (0.6 mM), dithiothreitol (1 mM), microsomal protein (10 μg/mL), 2 μM [^{14}C]palmitic acid (sp. act. 5.8 mCi/mmol), and acitretin (1.5–30 μM) in ethanol (4 μL, 2% final solvent concentration). Samples were prewarmed (1 min, 37°) and the reaction initiated by addition of ATP (20 μL). The reaction was terminated after 10 min and [^{14}C]palmitoyl-CoA extracted and quantified as described previously [12]. Data were analysed using a two-tailed paired *t*-test and a level of significance of $P < 0.05$.

Identification and Quantification of Acitretinoyl-CoA

The standard incubation medium (0.2 mL) was used with the following modifications, microsomal protein (500 μg/mL) and 30 μM [^{14}C]acitretin (sp. act. 24.3 mCi/mmol). Samples were prewarmed (1 min, 37°) and the reaction initiated by addition of CoASH (20 μL). At 30 min, the reaction was quenched by placing tubes in an ice slurry and samples were analysed immediately by TLC. For LC–MS analyses, the reaction volume was 0.5 mL and contained 40 μM [^{14}C]acitretin. After incubation, 1 volume of acetonitrile was added to precipitate protein and 0.5 mL of water was added to the supernatant prior to LC–MS analysis (see below).

TLC

Samples were spotted (30 μL) using the CAMAG, Nanomat III apparatus onto silica plates (acid-washed silica gel, 254-nm fluorescent indicator on glass, particle size 5–17 μm) and chromatographed using *n*-butanol/acetic acid/water (5:2:3, v/v) [13]. Authentic palmitoyl-CoA was used as a positive control standard for alkaline hydrolysis. Plates were air-dried, visualised at 254 nm, and used either for subsequent determination of −SH group presence or for quantification of acitretinoyl-CoA. Presence of -SH group was determined by spraying plates with 3% sodium hydroxide in 95% methanol followed by a nitroprusside-based reagent [14]. The presence of −SH was revealed by the appearance of a brilliant pink colour.

Quantification of [^{14}C] Acitretinoyl-CoA

Following resolution of samples by TLC, [^{14}C]acitretin standards (0.015–0.12 nmol, sp. act. 20 mCi/mmol) were applied in duplicate to the plates prior to placing in a phosphorimager cassette (Molecular Dynamics). The screen was allowed to develop for 24 hr before scanning

with a Molecular Dynamics PhosphorImager. Images were down loaded to files and volume quantification of [^{14}C]acitretinoyl-CoA and the [^{14}C]acitretin standard curves undertaken using the associated ImageQuant software. [^{14}C]Acitretin standard curves (N = 8) were linear with correlation coefficients of $r > 0.99$. Reproducibility of the technique was evident from coefficients of variation of 6.6% for the lowest [^{14}C]acitretin concentration (0.015 nmol) and 1.4% for the highest concentration (0.12 nmol). The lower limit of detection of 0.015 nmol [^{14}C]acitretin was sufficient to enable detection of 0.05% of the total concentration of acitretin added to the incubation.

Hydrolysis of Etretinate

Human liver microsomes (500 μg/mL) were incubated (30 min) (as above) in the presence of etretinate (30 μM). Metabolite formation was assessed by TLC as described. Subsequent studies using microsomal esterase inhibitors were conducted with etretinate in the presence of either 0.1 mM mercuric chloride, 0.01 mM *p*-chloromercuriphenylsulphonic acid, or 0.1 or 1 mM BNPP. Samples containing acitretin (30 μM) in the presence or absence of the inhibitors were included as positive controls to confirm catalytic integrity of LCL.

Ethyl Esterification of Acitretin

[^{14}C]Acitretin (sp. act. 57.3 mCi/mmol) was dissolved in ethanol to yield a stock solution containing 1 mM (55 μCi/mL) acitretin. An aliquot (10 μL) of this stock was mixed with 0.5 mg/mL of microsomal protein diluted with sodium phosphate buffer (0.1 M, pH 7.4) and kept chilled on ice. The final concentration in the incubation was 20 μM acitretin (1.1 μCi/mL) and 2% solvent in a final volume of 500 μL. For reactions involving esterification with other solvents (acetaldehyde, ethyl acetate, methanol, *n*-butanol, *n*-propanol, and *n*-hexanol), an appropriate aliquot of [^{14}C]acitretin was dispensed from the ethanolic stock solution and dried by evaporation under a stream of nitrogen. Acitretin was reconstituted by the addition of sodium phosphate buffer (0.1 M, pH 7.4), sonication (15 min at 40°, ultrasonic water bath, Bransonic), followed by the addition of the test solvent (final concentration 2%). An NADPH-regenerating system containing NADP (sodium salt, 10 mM), glucose 6-phosphate (di-sodium salt, 100 mM), $MgCl_2$ (100 mM) in 0.1 M phosphate buffer (pH 7.4) was constituted. Glucose-6-phosphate dehydrogenase (2U/100 μL) was added and the media incubated for 2 min at room temperature to generate NADPH. The mix was then placed on ice, and ATP (1 mM), CoA (0.6 mM), and the NADPH-regenerating system were added to the enzyme fraction with the substrate, acitretin, and the co-substrate, the test solvent. After 60-min incubation at 37°, the esterification reaction was stopped by placing the tubes on ice and by the addition of 1 volume of methanol. The precipitated proteins were removed by centrifugation (13,000 *g*, 10 min) and the supernatants (800 μL) were analysed by HPLC as described below.

Analysis of Acitretin Esters by HPLC

The HPLC system comprised a gradient pump (model L-6200A, Merck-Hitachi), an auto-injector (model AS-400, Merck-Hitachi), UV monitor, wavelength 360 nm (SPD-10A Shimadzu), and radioactivity monitor equipped with an yttrium glass cell of 150 μL (LB-506 C-1, Berthold). The chromatograms were recorded and peak detection and integration performed using integration software (Winflow, Berthold AG). The stationary phase was Inertsil ODS-3, (150 × 3 mm, 5-μm column, Ergatech) with a fibreglass prefilter with a restrain capacity of 1–2 μm (Knauer Column Inlet Filter, Knauer). The mobile phases 1.5% ammonium acetate and 0.1% acetic acid in water pH 3.5 (phase A) and acetonitrile (phase B) were mixed in a linear gradient from 20 to 60% B (0 to 20 min), from 60 to 95% B (20 to 35 min), and kept at 95% B (35 to 80 min), followed by an equilibration at 20% B for 10 min. Using a flow rate of 0.5 mL/min under these conditions, retention times were 30.8 min for acitretin, 42.1 min for ethyl-acitretinate (etretinate), 40.4 min for methyl-acitretinate, 43.7 min for propyl-acitretinate, 45.1 min for butyl-acitretinate, and 49.3 min for hexyl-acitretinate.

Analysis of Acitretinoyl-CoA and Acitretin Esters by LC–MS

An Inertsil ODS-80A (2.1 × 150 mm) column (Ergatech) was used to separate reaction products for MS analysis. The column was developed with 5 mM ammonium acetate and acetonitrile with a linear gradient of 0 to 60% solvent over 60 min with a flow rate of 0.2 mL/min. For LC–MS of acitretin esters, HPLC fractions (see above) containing the esterification product were combined and solvent was removed in a SpeedVac concentrator. The samples (0.1–0.27 μg) were dissolved in 200 μL acetonitrile and 50 μL was analysed. Negative ion electrospray spectra of acitretinoyl-CoA were obtained using a Perkin Elmer Sciex API 150 Ex spectrometer equipped with a turbo ion spray source. Authentic acetyl-CoA (Sigma) was used as a reference control. Positive ion spectra were recorded for the acitretin esters with the use of an atmospheric pressure chemical ionisation interface. Authentic ethyl-acitretinate (etretinate) was used as a positive control.

RESULTS

Effect of Acitretin on Microsomal LCL Activity

The results indicate that acitretin at concentrations of 15 and 30 μM significantly activated human liver microsomal LCL (Table 1). Although there was evidence of the trend for acitretin to induce formation of palmitoyl-CoA at concentrations of 1.5 and 3 μM, this did not attain statistical significance ($P < 0.1$)

TABLE 1. Effect of acitretin on human liver microsomal long-chain fatty acid-CoA ligase activity

	Acitretin (μM)				
Human liver	Control	1.5	3	15	30
H6	2.32	1.52	1.65	2.4	2.49
H24	2.92	3.38	3.07	4.02	3.91
H25	2.63	3.67	2.69	3.19	3.42
H27	1.82	2.04	2.19	2.67	2.36
H30	4.09	4.32	4.93	4.19	5.57
Mean	2.76	2.99	2.91	3.29*	3.55†
±SD	±0.76	±1.04	±1.11	±0.71	±1.06

Human liver microsomes were incubated with acitretin (1.5–30 μM) and 2 μM [^{14}C]palmitic acid as described in Materials and Methods. Values for individual livers are the means of duplicate incubations and expressed as nmol palmitoyl-CoA formed/min/mg.

* and † Indicates a significant difference from control incubations at $P < 0.05$ and $P < 0.025$, respectively.

Identification and Quantification of Acitretinoyl-CoA

In preliminary studies, TLC of microsomes from liver H27 incubated with [^{14}C]acitretin indicated an apparent increase in the formation of the putative acitretinoyl-CoA from 0.06 nmol at 10 min to 0.18 nmol at 30 min. Metabolite production was not observed in the absence of either protein or CoASH. Following hydrolysis with methanolic NaOH, the metabolite reacted positively for a -SH group. The R_f values (migration of solute relative to mobile phase front) were acitretinoyl-CoA 0.58–0.61, palmitoyl-CoA 0.57–0.6, and unreacted acitretin was located at the solvent front. In addition, similar to palmitoyl-CoA, the acitretinoyl-CoA conjugate was cleaved by alkaline hydrolysis (50 μL 10 M NaOH at 55° for 1 hr) as indicated by disappearance of the metabolite on planar chromatography.

Negative ion electrospray mass spectra were obtained for the product generated by incubation of human liver microsomes with [^{14}C]acitretin in the presence of CoA and ATP. The molecular mass of [^{14}C]acitretinoyl-CoA corresponds to 1077 Da at the neutral state. Figure 1 shows the negative molecular ion (M-1) for the *in vitro* product at *m/z* 1076.5. Peaks at *m/z* 996.5 and 1018.5 were assigned to [M − HPO_3 − 1] and its sodium salt (M − HPO_3 − 1 + Na^+), resulting from the loss of a phosphate group from the CoA-moiety. Two other peaks (*m/z* 1098.8 and *m/z* 1120.5) were assigned to the sodium salt of the negative molecular

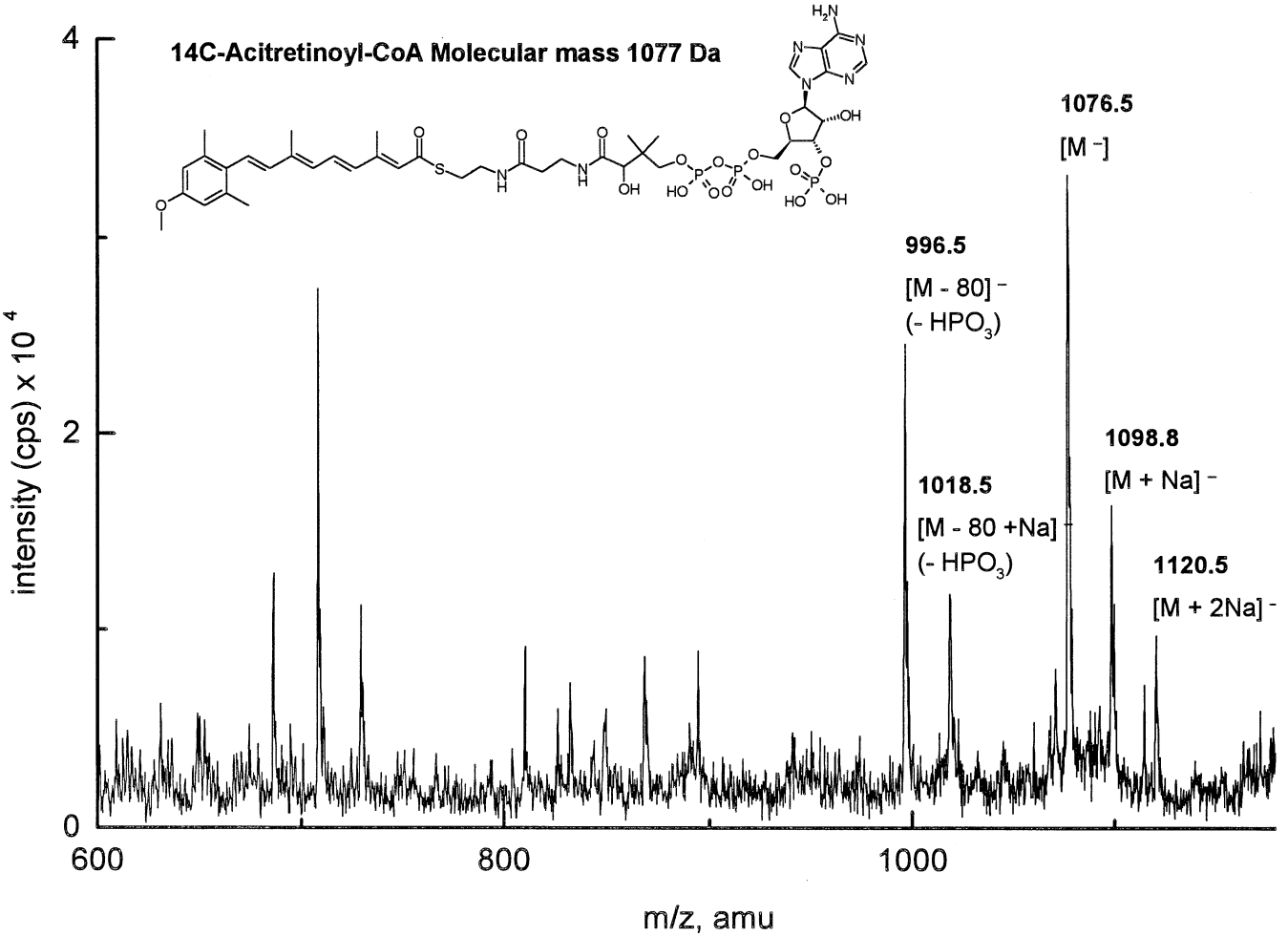

FIG. 1. Negative ion electrospray mass spectra of the product of acitretin and CoA. The inset shows the structure of acitretinoyl-CoA; the negative molecular ion was identified at *m/z* 1076. amu, atomic mass units.

ion ($M - 1 + Na^+$) and the di-sodium salt of the negative molecular ion ($M - 1 + 2Na^+$), respectively. These mass data confirmed that acitretinoyl-CoA was the product of the microsomal CoA ligase reaction.

Quantification using phosphorimager analyses indicated that acitretin was metabolised to acitretinoyl-CoA at a rate of 0.04 ± 0.03, 0.06 ± 0.02, 0.07 ± 0.02, 0.09 ± 0.02, and 0.08 ± 0.02 nmol/min/mg (mean ± SD, N = 4) for the five livers. Similar studies conducted using etretinate established that initial hydrolysis of etretinate to acitretin was essential for the subsequent formation of acitretinoyl-CoA, as inclusion of the covalently reacting esterase inhibitor BNPP completely prevented formation of the CoA thioester. Under identical incubation conditions, metabolism of acitretin to acitretinoyl-CoA was not inhibited by BNPP, indicating that microsomal LCL activity remained intact. Interestingly, metabolite formation and, by inference, hydrolysis of etretinate was not inhibited by the sulphydryl-blocking agents mercuric chloride and *p*-chloromercuriphenylsulphonic acid.

Formation of Etretinate by Esterification of Acitretin with Ethanol

In the presence of ethanol, etretinate formation from acitretin was dependent on viable microsomal protein and the co-factors ATP and CoASH (Fig. 2A). Using these conditions, an average of 1.7% (11 pmol/min/mg) of acitretin in the incubation was converted into etretinate. In the absence of either ethanol or co-factors, no etretinate formation was detected. Additional evidence for the participation of enzymatic processes in the transesterification reaction was also apparent from the lack of etretinate formation by heat-inactivated (5 min at 50°) human liver microsomes. Our studies also established that NADPH was not a necessary factor for transesterification because in its absence, etretinate formation was detected with human liver microsomes in the presence of ATP, CoA, and ethanol. Ethanol, NADPH, and liver microsomes by themselves did not generate etretinate, but instead substantial amounts of polar metabolites were detected (Fig. 2B). These metabolites represented oxidative products of acitretin and were not characterised further in this study.

Esterification of Acitretin with Other Alcohols

Incubation of pooled human liver microsomes with acitretin, ATP, CoASH, NADPH, and various hydroxyl-containing compounds resulted in formation of the corresponding acitretin esters with methanol, ethanol (control), *n*-propanol, *n*-butanol, and hexanol. The identity of the ester product was inferred initially from the appearance of a chromatographic peak on HPLC corresponding to a radiolabelled product not observed with incubations with ethanol. The substituent on acitretin was reflected by its retention time on HPLC. Thus, methyl-acitretinate was the most polar ester, followed by ethyl-acitretinate (etretinate), propyl-acitretinate, butyl-acitretinate, and hexyl-acitretinate. The quantity of acitretin ester formed was dependent on the chain length of the alcohol, such that the larger the substituent, the less product formed (Table 2).

Positive chemical ionisation mass spectra of these products show the expected molecular masses of the protonated positive ion species (Fig. 3, A–F). *In vitro* transesterification of acitretinoyl-CoA with ethanol yielded a similar mass spectrum (3B) as authentic synthetic etretinate (3A). Due to the use of [^{14}C]acitretin, the mass of the corresponding *in vitro* ethyl ester of acitretin was larger by 2 Da. All acitretin esters exhibited a characteristic fragment with a positive ion molecular mass of *m/z* 311.3 Da corresponding to the carbocation fragment of acitretin ($O = C^+ - R$), incremented by methyolate (31 Da, 3C), ethyolate (45 Da, 3, A and B), propyolate (59 Da, 3D), butyolate (73 Da, 3E), and hexyolate (101 Da, 3F) to generate the protonated positive ion masses ($M + H^+$) depicted.

Incubation of Acitretin with Acetaldehyde and Ethyl Acetate

When acitretin was incubated with liver microsomes in the presence of ATP, CoASH, NADPH, and 2% acetaldehyde, traces of etretinate were observed. The amount of etretinate formed accounted for 0.025% conversion of acitretin into etretinate, corresponding to 0.17 pmol/min/mg protein compared to 11 pmol etretinate/min/mg protein in the presence of 2% ethanol. Traces of etretinate were also observed when incubating liver microsomes with acitretin in the presence of ATP, CoASH, NADPH, and 2% ethyl acetate (Table 2). The amounts of etretinate formed accounted for 0.1% of the acitretin present in the incubation, corresponding to a formation rate of 0.7 pmol etretinate/min/mg protein or 6% of the rate when 2% ethanol was present in the incubation.

DISCUSSION

Previous studies established that a trace amount of etretinate was formed following administration of acitretin to humans [2]. Ethyl esterification, however, was not considered a normal metabolic pathway, but subsequent studies reported the possible involvement of a CoA thioester intermediate in this process [5]. Hepatic microsomal long-chain fatty acid-CoA ligase (LCL) is principally associated with the activation of long-chain fatty acids (C_{10}–C_{22}) to their respective acyl-CoA esters prior to the utilisation in a wide variety of metabolic pathways. In addition to the metabolism of fatty acids, LCL also catalyses CoA conjugation of a diverse array of xenobiotics including the 2-arylpropionate nonsteroidal anti-inflammatory drugs [6] and the fibric acid derivatives nafenopin [15], clofibrate, and ciprofibrate [16]. Formation of a CoA conjugate may not be unusual for retinoids, as Miller and DeLuca [8] reported activation of retinoic acid to ethyl retinoate, and

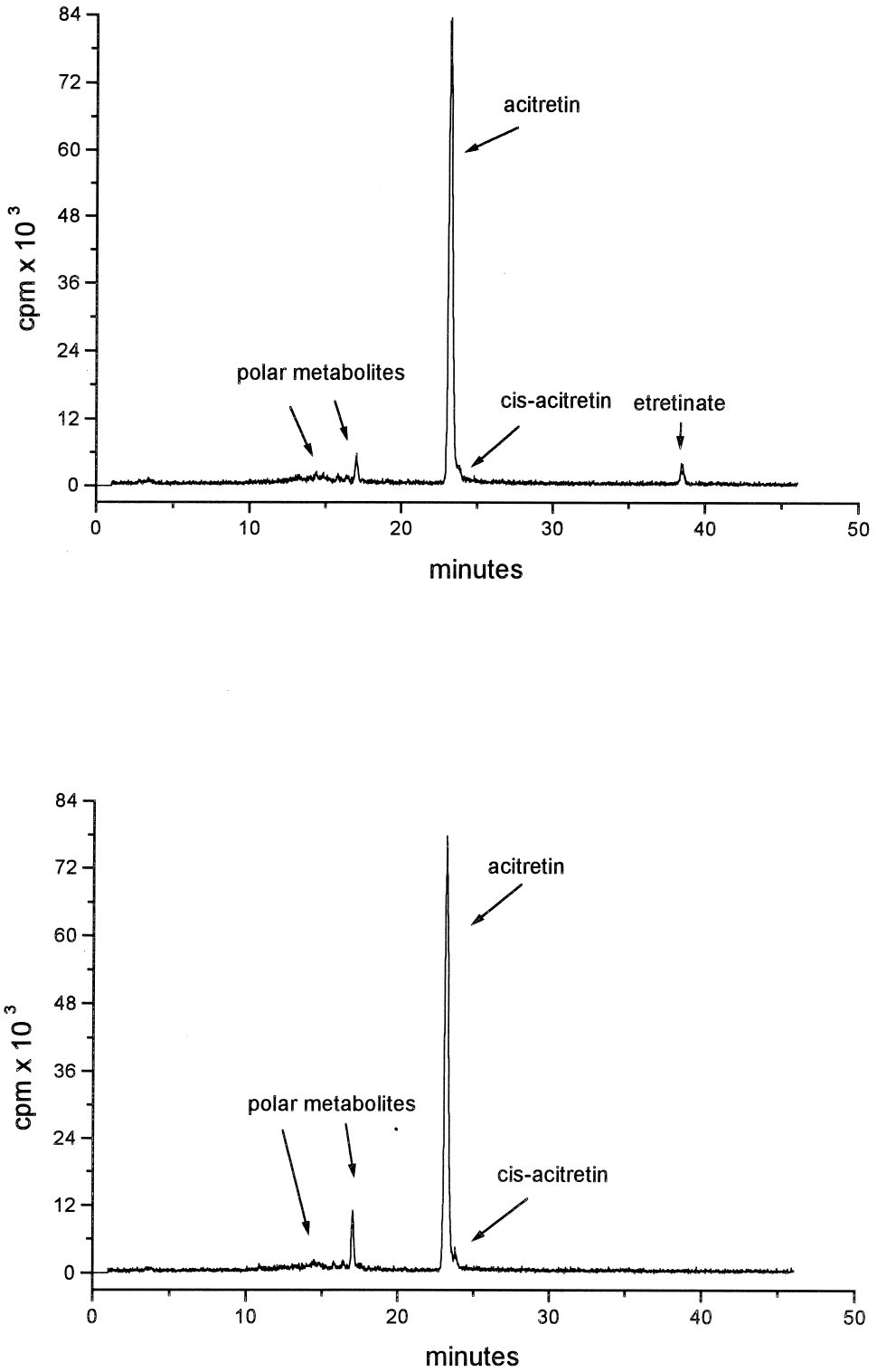

FIG. 2. Reverse-phase HPLC chromatograms of acitretin, *cis*-acitretin, and etretinate. Human liver microsomes were incubated (60 min, 37°) in the presence of [^{14}C]acitretin (20 μM, 1.1 μCi/mL), ethanol (2% v/v), and either ATP and CoASH (1A) or an NADPH-generating system (1B). Protein was precipitated and the compounds analysed by HPLC as described in Materials and Methods.

formation of a taurine conjugate of 13,14-dihydro-9-*cis*-retinoic acid has been described in rat [17].

This study is the first to report that human liver microsomes catalyse formation of acitretinoyl-CoA. After positive identification of the metabolite by LC–MS, we demonstrated that acitretin was metabolised to acitretinoyl-CoA by human liver microsomes at approximately one-fortieth of the rate of formation of palmitoyl-CoA. Another finding from the present study was that acitretin also significantly activated human liver microsomal LCL, result-

TABLE 2. Effect of various alcohols, acetaldehyde, and ethyl acetate on the ethyl esterification of acitretin

Co-substrate	Esterified acitretin*
2% ethanol	1.7
2% ethanol, minus CoA or ATP	0
2% ethanol, inactivated microsomes	0
2% methanol	2.3
2% propanol	1.7
2% butanol	0.6
2% hexanol	0.2
2% acetaldehyde	0.02
2% ethylacetate	0.1

Human liver microsomes were incubated (60 min) in the presence of acitretin (20 μM) and either various alcohols, acetaldehyde, or ethylacetate (2%, v/v). The corresponding esters were analysed by HPLC as described in Materials and Methods.
*Percent acitretin converted into the corresponding ester.

ing in enhanced formation of palmitoyl-CoA. This observation of enhanced fatty acid activation *in vitro* may be relevant to the clinical situation, as increases in serum triglycerides and cholesterol have been described repeatedly after treatment with retinoids [18]. Complex interrelationships between contributing pathways make elucidation of a mechanism of drug-induced hyperlipidaemia difficult, but it is noteworthy that acitretin activated *in vitro* a key enzyme that provides fatty acyl-CoAs for the synthesis of complex lipids including di- and triacylglycerols and cholesterol esters. Following administration, etretinate is hydrolysed to acitretin *in vivo* and although not the focus of this study, it is conceivable that activation of human liver LCL by acitretin may be the common link between both etretinate and acitretin and the clinical observation of hyperlipidaemia.

Multiplicity of rat liver microsomal LCLs has been reported [19] and to date, two LCLs have been cloned from human liver [20, 21]. Clearly, acitretin functions both as a substrate for CoA conjugation and an activator of fatty acid conjugation. At present, the form(s) responsible for the metabolism of acitretin has not been identified, but these data clearly indicate an interaction between acitretin and the catalysis of palmitic acid by a human liver microsomal long-chain fatty acid-CoA ligase.

Many esters used therapeutically are rapidly hydrolysed *in vivo*, and the presence of non-specific carboxylesterases in hepatic microsomes of many species has been known for a number of years. Recently, chemically distinct classes showing differing patterns of induction and inhibition have been identified [22–25]. Of the inhibitors used in this study, BNPP prevented initial ester hydrolysis of etretinate to acitretin and hence formation of acitretinoyl-CoA. This observation clearly established that in the case of etretinate, formation of a CoA conjugate occurred subsequent to hydrolysis to acitretin. Ethyl esterification of the metabolite acitretinoyl-CoA would then result in futile cycling to etretinate, thus potentially contributing further to tissue deposition of etretinate (Fig. 4).

Ethyl esterification has been described for a number of compounds, with the esterification of fatty acids in particular having received considerable attention. In contrast to acitretin, ethanol esterification of fatty acids has been described as a process that does not involve the formation of an acyl-CoA thioester intermediate. The formation of fatty acid ethyl esters in the presence of ethanol in a number of tissues has been attributed to an ATP/CoA-independent fatty acid ethyl ester synthase [26–30]. In addition to the cytosolic enzymes, a microsomal ATP/CoA-dependent ethanol acyltransferase has been identified in rat liver [10, 31]. Similar enzymatic processes have also been described for the transesterification of cocaine to cocaethylene [32] and incorporation of 7-hydroxy-tetrahydrocannabinol into lipids [33–35], although the enzyme systems involved have not been characterised in detail. Acitretin is not unique in its esterification, but the enzyme(s) involved differ from those catalysing the ethyl esterification of fatty acids, because the intracellular localisation of the enzyme(s) is microsomal and not cytosolic and there is an obligatory requirement for ATP and CoASH.

Using human liver microsomes supplemented with ethanol, we confirmed that ATP and CoA were obligatory co-factors for the formation of etretinate from acitretin. In the absence of any of these components, etretinate was not detected. The requirement of these factors for esterification of acitretin had been reported earlier by Schmitt-Hoffmann *et al.* [5]. Further evidence for the participation of enzymatic processes in the transesterification reaction was evident from the absence of etretinate formation by heat-inactivated human liver microsomes. In contrast to Schmitt-Hoffmann *et al.* [5], NADPH was not a necessary factor for transesterification. In fact, ethanol, NADPH, and liver microsomes by themselves did not generate etretinate, but instead substantial amounts of polar metabolites were detected.

Acetaldehyde and ethyl acetate do not contain free hydroxyl groups capable of forming esters with a carboxylic acid. In the case of acetaldehyde, an energy source-dependent reaction is needed to reduce the aldehyde to an alcoholic group. This occurs *in vivo* as a mechanism of detoxification of acetaldehyde by alcohol dehydrogenase. This reaction is energetically less favoured compared to the reverse reaction, the oxidation of ethanol to acetaldehyde. Ethyl acetate is an ester resulting from condensation of ethanol with acetic acid. In the presence of acetaldehyde and ethyl acetate, trace amounts of etretinate corresponding to 1.5% and 6%, respectively, of the amount when ethanol was present in the reaction were formed. This may be accounted for by either traces of ethanol pre-existing in the solvents or generated during the assay, by the reduction of acetaldehyde in the presence of NADPH, or by the generation of ethanol following ester cleavage of ethyl acetate by microsomal esterases.

In conclusion, enzymes present in human liver microsomes are able to catalyse the esterification of acitretin following formation of the pivotal intermediate, acitreti-

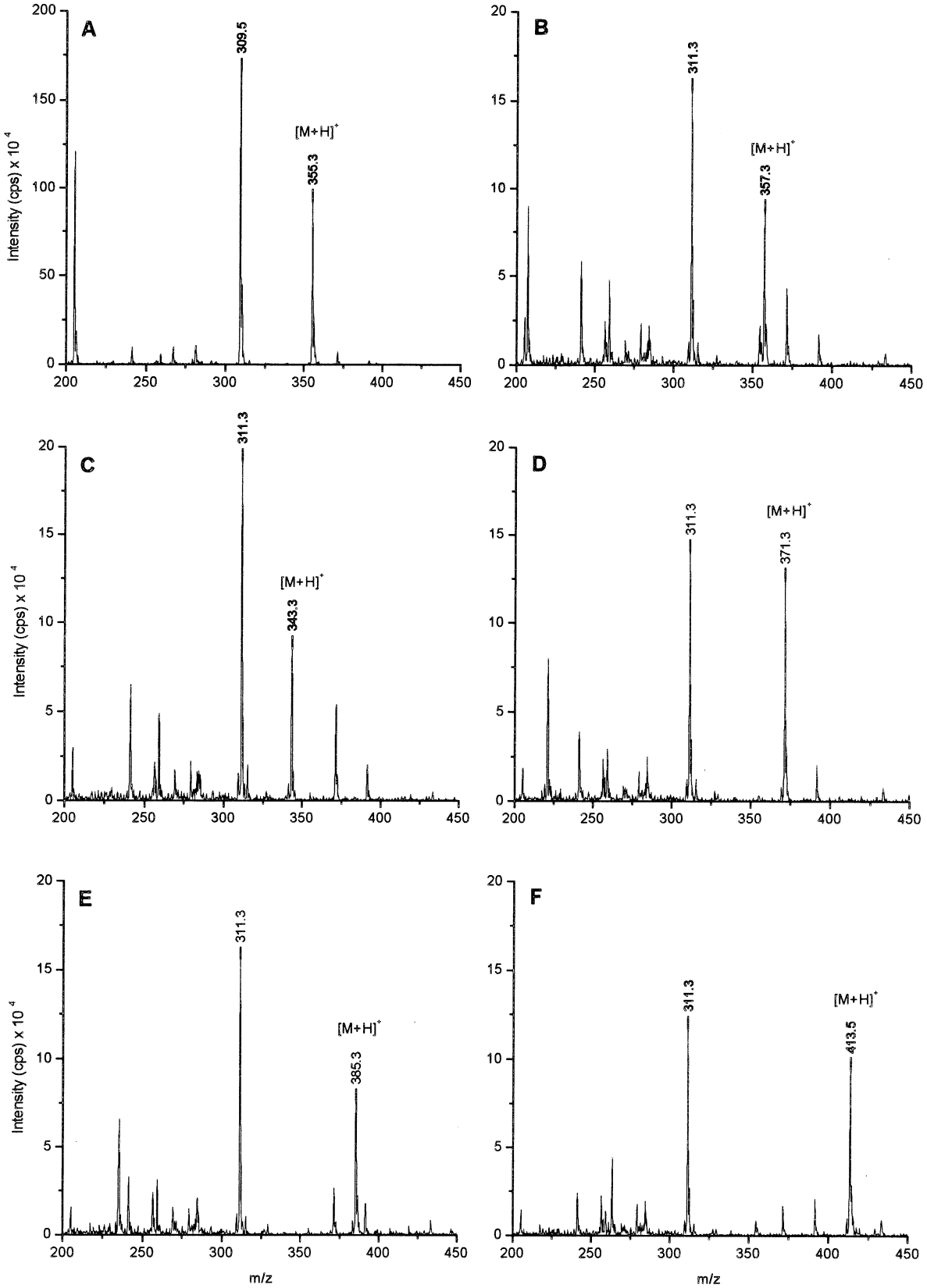

FIG. 3. Positive ion chemical ionisation mass spectra of the transesterification products of acitretinoyl-CoA and various alcohols. (A) The protonated positive molecular ion of authentic ethyl-acitretinate (etretinate) was identified at *m/z* 355.3. Loss of ethanoloate results in the carbocation of acitretin at *m/z* 309.5. (B) The product of *in vitro* transesterification of [^{14}C]acitretin in the presence of ethanol yields a protonated positive ion with *m/z* 357.3. The protonated positive ions of the further transesterification products of [^{14}C]acitretinoyl-CoA exhibit *m/z* 343.3 for methanol (C), *m/z* 371.3 for propanol (D), *m/z* 385.3 for butanol (E), and *m/z* 413.5 for hexanol (F).

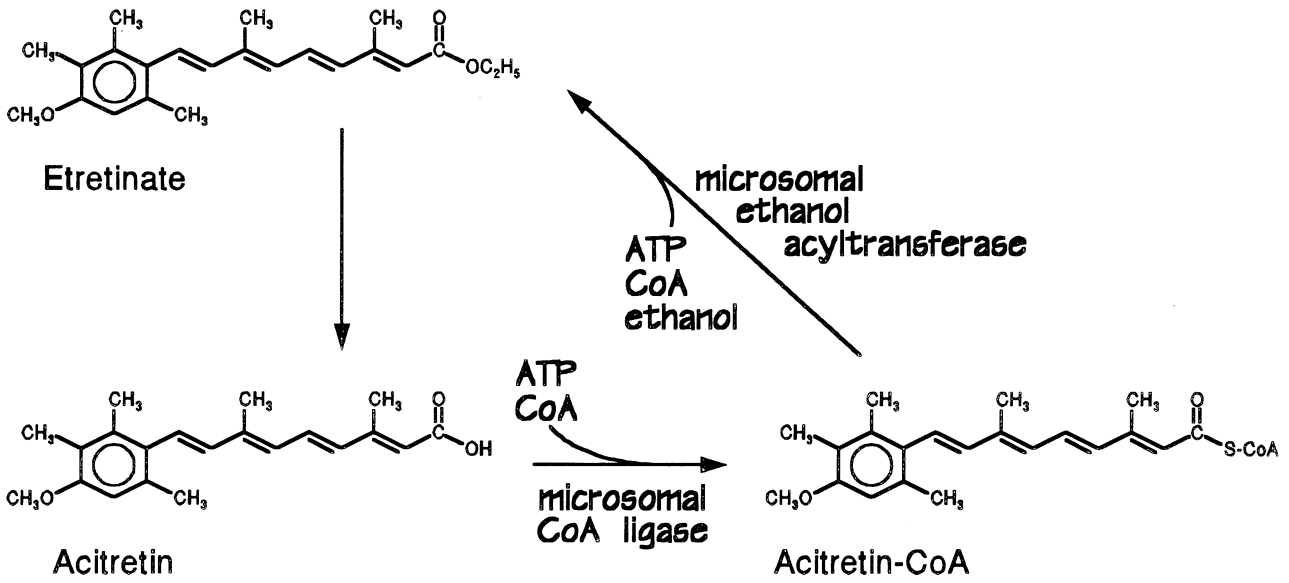

FIG. 4. Proposed metabolic pathway for the ethyl esterification of acitretin in humans.

noyl-CoA. This activated intermediate can then react with suitable alcohols to form the corresponding esters (Fig. 3). This was found to be the case for ethanol, methanol, butanol, propanol, and hexanol. Ethanol formation is not a physiological process occurring in humans and therefore, ethyl esters such as etretinate are not formed under normal physiological conditions. In humans, exogenous ester-containing compounds, present in food or drugs, are cleaved by esterases, giving rise to small amounts of ethanol, which are then metabolised efficiently by alcohol dehydrogenase. The amounts would be significantly less than from ingestion of any ethanolic beverages.

In this study, we have clearly established the mechanism of the ethyl esterification of acitretin in humans. This unique metabolic pathway is dependent on two factors: formation of acitretinoyl-CoA and the intracellular concentration of ethanol. The former may be predicted *ex vivo*, but the latter will be influenced in humans by societal, physiological, and genetic variables. Predicting *in vivo* clearance via this metabolic route will be a challenge, as the intracellular concentration of ethanol could never be predicted with any degree of accuracy in humans.

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