Glutathione Catalysis of Interconversion of Acitretin and Its 13-*cis* Isomer Isoacitretin

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Abstract □ The ability of glutathione to catalyze the *cis–trans* isomerization of acitretin and isoacitretin was explored. Glutathione catalyzed the isomerization reaction in both directions; the reaction rate varied with glutathione concentration, atmospheric exposure condition, and identity of starting isomer. The ability of this widely distributed molecule to catalyze this interconversion nonenzymatically may contribute to the presence of both isomers in vivo after administration of either isomer.

Acitretin, an aromatic analogue of retinoic acid, is being developed for use in the treatment of psoriasis. It is a member of the retinoid family, a group of compounds related to retinol (vitamin A). The tetraene side chain in these structures exists in the *trans* configuration; however, upon exposure to light, the double bonds at the C-9, C-11, and C-13 positions can undergo *trans–cis* isomerization.[1]

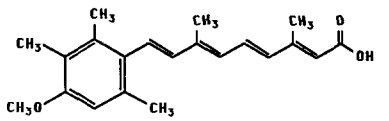
Vitamin A has several pharmacological activities, among them promoting normal epithelial differentiation and proliferation. Vitamin A deficiency leads to a keratinizing metaplasia of epithelial cells,[2] a condition which is also observed in skin disorders involving keratinization, such as psoriasis. The use of therapeutic doses of vitamin A for the treatment of these conditions results in the severe side effects associated with hypervitaminosis A syndrome. Because of this low therapeutic index, synthetic retinoids have been prepared and tested for toxicity and efficacy in these diseases.

Acitretin is one of several synthetic retinoids to show promise in the treatment of hyperkeratotic skin disease.[3] It is the major free acid metabolite of the ethyl ester etretinate (Tegison) and, like the ester, possesses pharmacological activity. The potential advantage that acitretin offers over etretinate lies in the differences between their pharmacokinetic profiles: etretinate displays an extremely prolonged terminal half-life of elimination after multiple dosing in humans,[4–6] while acitretin is cleared much more rapidly.[7]

After acitretin administration to animals or humans, both acitretin and its 13-*cis* isomer, isoacitretin, are observed in plasma. The structures of these compounds are shown below. Following multiple oral doses of acitretin or humans, isoacitretin appears in plasma at trough concentrations which are approximately five times higher than those of the parent drug.[7]

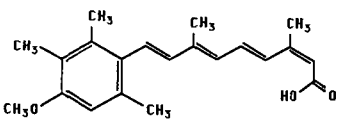
Shih et al.[8] studied the isomerization of all-*trans*- and 13-*cis*-retinoic acid. They reported that certain thiol-containing compounds, including glutathione, chemically catalyzed the isomerization reactions for these compounds. Examination of various compounds showed that only those containing a sulfhydryl group, a thiocyanate group, or a thione were active as catalysts. The ability of microsomes, boiled microsomes, apoferritin, and albumin to catalyze these reactions was attributed to their sulfhydryl group content rather than to any enzymatic activity. The authors concluded that it was unlikely that the in vivo interconversion of retinoic acid and 13-*cis*-retinoic acid was predominantly enzymatic, because small thio-containing molecules could catalyze the reaction and because boiled microsomes retained part of their catalytic activity.

a.



Acitretin

b.



Isoacitretin

Glutathione, the most prevalent intracellular thiol, is involved in many biological processes, including protein and DNA synthesis, transport, enzyme activity, metabolism, and cellular protection.[9] It is ubiquitously distributed in mammalian tissues, with intracellular concentrations of glutathione ranging from 0.5 to 10 mM;[9] depending on nutritional status, hepatic levels range from 3 to 10 mM.[10] Plasma glutathione concentrations are in the micromolar range, with rat plasma containing 26 μM.[9]

Because of the ability of glutathione to catalyze the isomerization reactions of retinoic acid and 13-*cis*-retinoic acid, and because of its wide distribution in mammalian systems, the present work focused on glutathione as a potential catalyst. The purpose was to determine if glutathione could catalyze the isomerization of acitretin and isoacitretin.

Experimental Section

Materials—Acitretin, etretinate, and isoacitretin were gifts of Hoffmann-La Roche (Basel, Switzerland). Sodium *p*-hydroxymercuribenzoate, glutathione, and Tris HCl were obtained from Sigma (St. Louis, MO). All remaining chemicals and solvents were obtained from Fisher (Springfield, NJ). All chemicals were used as received without further purification.

Prepurified nitrogen was obtained from Central Welding Supplies (Lexington, KY).

Study Design—Incubations of the two retinoids were carried out at 37 °C. The reaction mixture consisted of 50 μL of 10% (v/v) Triton X-100 and 4.90 mL of Tris HCl buffer (25 mM, pH 7.6) with or without added glutathione. The reaction was initiated with the addition of 50 μL (165 nmol) of either acitretin or isoacitretin in methyl acetate.

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Two glutathione concentrations were used, 0.10 mM (based on Shih et al.[4] and 7.0 mM (physiological concentration range); in addition, glutathione-free incubations were prepared to serve as controls.

At various times (0, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 16, 20, 24, 48, 72, and 96 h) after the initiation of the reaction, 200 μL aliquots of the incubation mixtures were removed to culture tubes containing sodium *p*-hydroxymercuribenzoate (a sulfhydryl reactive compound) at five times the amount of glutathione present in the sample. This excess is sufficient to completely inhibit the isomerization reaction.[8]

These incubations were carried out under two different atmospheric exposure conditions, ambient air or nitrogen. In the air experiments, no control of exposure to air was made, although the tubes were capped during incubation. In the nitrogen experiments, high-purity nitrogen was bubbled through the solutions after each sample was taken before the tubes were capped.

The samples were stored at −20 °C until extracted for HPLC analysis. All procedures were performed under yellow light to avoid photoisomerization of the compounds.

High-Performance Liquid Chromatography Analysis—*Extraction*—Standard solutions were prepared in methyl acetate and ranged from 100 to 8000 pmol/200 μL in concentration. In order to have similar solvent composition for extraction, 200 μL of 25 mM pH 7.6 Tris HCl buffer was added to each 200-uL standard and 200 μL of methyl acetate was added to each incubation mixture aliquot before extraction. Twenty-five microliters of an 8000 pmol/200 μL solution of etretinate (the internal standard) in methyl acetate and 0.5 mL of phosphate buffer (0.13 M pH 7.4) were added to each tube before extraction with 4.0 mL of ethyl ether; the ether layer was removed and evaporated to dryness under nitrogen. Following evaporation, the residue was rinsed from the wall of the tube with ~1 mL of ethyl ether, which was again evaporated to dryness under nitrogen. The samples were reconstituted with 800 μL of mobile phase; injections of 150 μL were made. Extraction efficiencies for acitretin, isoacitretin, and etretinate were 98, 85, and 95%, respectively.

High-Performance Liquid Chromatography System—The method was a modification of Hänni et al.[11] Chromatography was performed using a model LC-6A pump, a model SCL-6A/SIL-6A autoinjector, a model SPD-6AV UV/visible detector, and a model C-R3A integrator (Shimadzu Scientific Instruments, Columbia, MD). The column was packed with LiChrosorb Si-60 (5 μm, 4.0 × 125 mm; EM Science, Gibbstown, NJ) and was protected by a guard column packed with the same material. The system used a mobile phase of hexane:methyl benzoate:propionic acid (375:25:1, v/v/v) at a flow rate of 2.0 mL/min. Detection of all compounds was at 365 nm; at this wavelength and in this mobile phase, isoacitretin showed a lower absorptivity than acitretin, resulting in an absorbance measurement only 81% of that of acitretin. Quantitation was performed by the peak area ratio method using etretinate as the internal standard. Retention times for etretinate, isoacitretin, and acitretin were 2, 9, and 10.5 min, respectively. Standard curves for acitretin and isoacitretin were linear ($r > 0.99$) from 100 to 8000 pmol/200 μL; weighted linear regression was used in fitting the standard curve. The effective detection limit of the assay for both acitretin and isoacitretin was 100 pmol/200 μL. Table I summarizes the validation data for the analytical procedure. Figure 1 shows representative chromatograms.

Data Analysis—The concentration–time data for isoacitretin and acitretin were described with an open, two-compartment model with exit occurring from both compartments as shown in Scheme I, where k_{12}, k_{21}, k_{10}, and k_{20} are first-order rate constants with units of h^{-1}. Data from a matched pair of incubations were simultaneously fitted using PC NONLIN[12] in order to estimate these parameters. A matched pair of incubations consisted of two incubations under the same experimental conditions of glutathione concentration and atmospheric exposure, one starting with acitretin and one starting with isoacitretin.

Table I—Summary of Assay Validation Data

Concentration, pmol/200 μL	Interday Variability			
	Acitretin Mean (n = 6)	CV, %	Isoacitretin Mean (n = 6)	CV, %
100	102.5	9.7	103.6	4.9
1000	1004.5	3.6	1009.7	5.4
5000	5026.8	4.5	5287.5	7.1

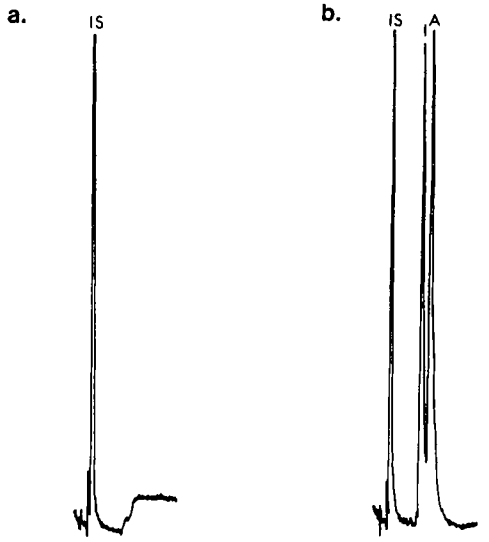
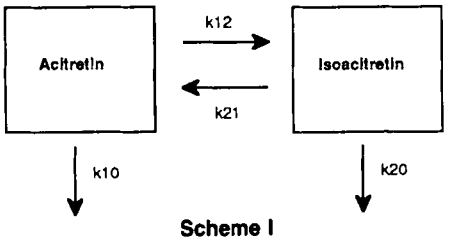


Figure 1—Chromatograms of (**a**) blank with internal standard and (**b**) acitretin and isoacitretin at 1000 pmol/200 μL. Key: (**IS**) internal standard, etretinate; (**I**) isoacitretin; (**A**) acitretin.



Scheme I

Results and Discussion

Table II summarizes the results of these studies in terms of the mean parameter estimates from the nonlinear regression analyses for the various experimental conditions. Figure 2 shows the mean concentration–time data for one of the combinations, incubation with 0.10 mM glutathione under ambient atmosphere.

In the control experiments (no glutathione present), no isomerization was observed under any combination of experimental conditions (data not shown).

There are several points to be extracted from these data. First, under every combination of experimental conditions, the conversion of isoacitretin to acitretin proceeds more rapidly than the reverse reaction. This is consistent with the expectation that, like the isomers of retinoic acid, the all-*trans* isomer is thermodynamically more stable than the 13-*cis* isomer.[13]

Under aerobic conditions, the rate for the isomerization of isoacitretin was higher at the lower glutathione concentration than it was at the higher glutathione concentration. Similarly, the rate for the isomerization of acitretin was higher at the lower glutathione concentration when exposed

Table II—Summary of Parameter Estimates for the Incubation of Acitretin and Isoacitretin with Glutathione at 37 °C

Condition	k_{12}, h^{-1}	k_{21}, h^{-1}	k_{10}, h^{-1}	k_{20}, h^{-1}
Air				
0.10 mM GSH	0.0141	0.0406	0.00572	0.0228
7.0 mM GSH	0.00241	0.00819	0.00350	0.0378
Nitrogen				
0.10 mM GSH	0.00467	0.0192	0.00290	0.0265
7.0 mM GSH	0.0349	0.0518	0.00290	0.161

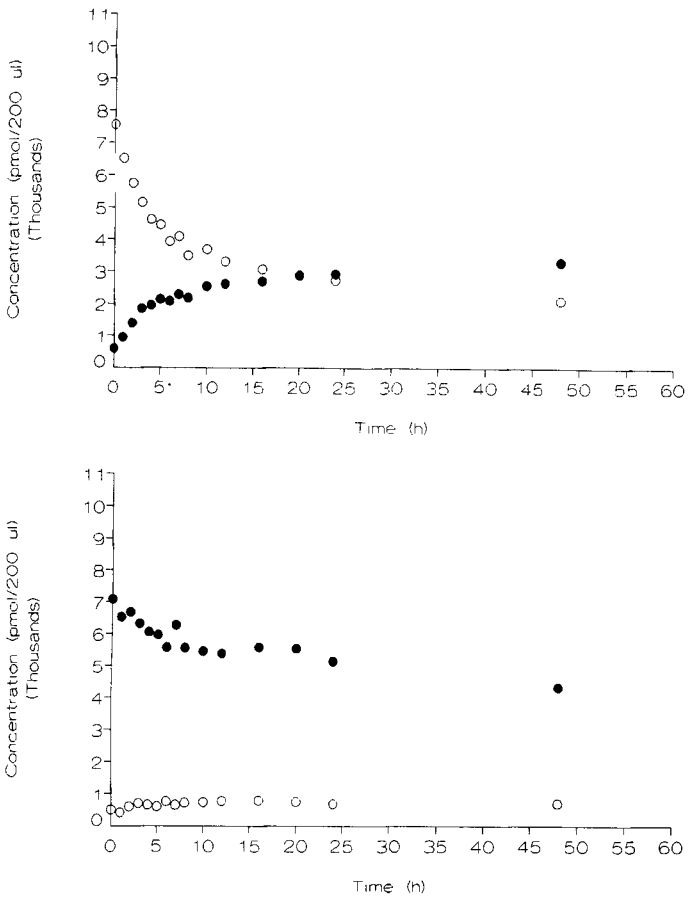


Figure 2—Mean concentration–time data for incubation of retinoids with 0.10 mM glutathione at 37 °C under ambient atmosphere. Upper panel: isoacitretin initially present. Lower panel: acitretin initially present. Key: (○) isoacitretin; (●) acitretin.

to air. Under anaerobic conditions, the opposite was true. The rates for the isomerization reactions of both isoacitretin and acitretin were lower at the lower glutathione concentration than they were at the higher glutathione concentration. The other comparisons to be made are the rates at each glutathione concentration. At 0.10 mM glutathione, the reaction rates for isomerization were higher under aerobic conditions than under anaerobic conditions. At 7.0 mM glutathione, the reaction rates were much higher under anaerobic conditions than under aerobic conditions.

In each case, the concentration–time data show a net loss of material from the system as the reaction proceeds; the formation of one isomer cannot account completely for the loss of the other. Therefore, an open, two-compartment model was proposed to describe the system, in which loss can occur from either isomer; there is no evidence that only one isomer is susceptible to this loss. All of the processes were assumed to be first-order. Table II summarizes the results of the PC NONLIN analyses. The rate constants given in the table are consistent with the relative rates as described above.

The explanations for the relative rates of the isomerization reactions under differing experimental conditions are not known. Under anaerobic conditions, the isomerization reactions proceed more rapidly at higher glutathione concentrations, which is consistent with an increased catalyst concentration causing an increased reaction rate. Under aerobic conditions, this is not the case. Shih et al.[8] observed a maximal reaction rate under aerobic conditions at glutathione concentrations of 0.08 to 0.16 mM, with decreasing reaction rates at higher concentrations; our findings are consistent with this observation. The authors suggested that glutathione and oxygen may exist as a complex at high glutathione concentrations, preventing the sulfhydryl group from serving as a catalyst and ultimately oxidizing the glutathione.

The mechanism proposed by Shih et al.[8] for the isomerization of retinoic acid and 13-*cis*-retinoic acid is nucleophilic attack by a sulfhydryl group (thiolate anion) on the unsaturated retinoid system; isomerization results from an addition–elimination mechanism. Due to the structural similarity of the retinoic acid and the acitretin and isoacitretin pairs, the same mechanism can be invoked to account for the reaction of acitretin and isoacitretin.

Our findings would suggest that after multiple dosing with acitretin, isoacitretin would be formed. However, the concentration of acitretin would exceed that of isoacitretin, based on the relative interconversion rates observed in the present study. This is not what has been observed in humans. After multiple oral dosing of acitretin, both acitretin and isoacitretin were observed in plasma, but the trough concentration of isoacitretin was approximately fivefold that of acitretin; upon cessation of therapy, plasma levels of the two compounds declined in parallel.[7] Our in vitro data cannot be readily reconciled with these in vivo observations. Available evidence suggests the presence of a relatively rapid elimination process in vivo for acitretin (k_{10}) compared with isoacitretin. In such a case, the interconversion reaction for both compounds occurs in addition to the rapid clearance of acitretin; then the rate-limiting step in the decline of the plasma concentrations of the retinoids is the rate of isomerization of isoacitretin to form acitretin. This hypothesis is supported by Paravicini et al.[7] who suggested that elimination of isoacitretin determined the terminal elimination half-life of acitretin.

Conclusions

Glutathione can catalyze the interconversion of acitretin and its 13-*cis*-isomer, isoacitretin. The ability of this widely distributed molecule to catalyze this interconversion nonenzymatically may contribute to the presence of both isomers in vivo after administration of either isomer.

References and Notes

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