

## **A COMPARITIVE STUDY ON THE IN VITRO HEPATIC METABOLISM OF RETINOIC ACID USING DIFFERENT SPECIES.**

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### **ABSTRACT**

All trans retinoic acid (RA), derived from the oxidative metabolism of dietary retinol (vitamin A) and  $\beta$  carotene, contributes to the growth and differentiation of mammalian epithelial tissues. RA is rapidly cleared from the plasma and suddenly metabolized in tissues. The increase of its biological potency through inhibition of its oxidative metabolism is consistent with this. This research is part of a study to develop novel compounds as inhibitors of retinoic acid metabolism that could have potential value as anticancer agent. The investigation was done to compare the in vitro metabolism of [ $^3$ H]RA by hepatic microsomes from several common laboratory animal species. Also, the ability of ketoconazole to inhibit RA metabolism was examined. The species studied were male rat, male New Zealand white rabbit, male albino mouse, male Syrian hamster, male Dunkin hartley guinea pig and male nude mouse.

The results revealed that  $k_m$  and  $V_{max}$  were species dependent. Among the animals, rat liver appeared to be the most active in metabolizing RA. Inhibition of RA metabolism by ketoconazole (100  $\mu$ M) was very similar in the hepatic microsomes of all the species examine. Overall the results indicate that male rat hepatic microsomes represent a useful enzyme source for screening novel compounds as inhibitors of RA metabolism.

**KEY WORDS:** Retinoic acid metabolism, Different species.

## INTRODUCTION

Retinoids play a crucial role in cellular differentiation and proliferation of epithelial tissue and their utility in oncology and dermatology is well documented. All- trans retinoic acid (RA) is the principal endogenous retinoid and has been used successfully in differentiating therapy of acute promyelocytic leukemia (1,2). It is currently being investigated for efficacy in the treatment and prevention of various types of cancer. One of the factors limiting its use is the observed increase in RA clearance and elimination, which occurs shortly after treatment. This increased clearance leads to reduced levels of drug in the body and loss of RA effectiveness (3-5). RA efficacy may be enhanced if clearance can be overcome, for example, through the inhibition of activity of the induced specific metabolizing enzyme(s). This requires the identification of these enzyme(s) and development of approaches to selectively inhibit their activity.

A major metabolic pathway of RA consists of the hydroxylation at position C<sub>4</sub> of its cyclohexenyl moiety, to form the inactive 4-hydroxy-RA, which is then oxidized further to keto- RA and more polar metabolites (6,7). This hydroxylation of RA is mediated by a cytochrome P- 450- dependent enzyme system (8-12). In previous work the investigation was done to elucidate the rate of RA metabolism at different tissue sites of male rat (liver, kidney, intestinal mucosa, lung, skin and brain) and its inhibition by P-450 3A4 ligand ketoconazole (13). The results of those experiments revealed that RA metabolism occurred in all organs of the male rat selected for study, with liver being the most active. Metabolism of RA was inhibited by ketoconazole at all the sites evaluated.

Presently the investigators would like to answer the following questions. Do other species metabolize RA and is this also P-450 mediated? The liver was chosen as the enzyme source for comparison because in the rat this had been shown to be the most active site of RA metabolism (13). The species studied were male and female rat, male New Zealand white rabbit, male albino mouse, male Syrian hamster, male Dunkin hartley guinea pig and male nude mouse.

## **MATERIALS AND METHODS**

### *REAGENTS:*

[11,12 <sup>3</sup>H] All – trans retinoic acid was purchased from Dupont (U.K) LTD, Stevenage, Herts. All trans retinoic acid (cold), NADPH, butylated hydroxy anisole, ketoconazole and protein standards were obtained from Sigma Chemical Company Poole, Dorset. Formic acid, ammonium acetate and Hisafe III scintillation fluid (optiphase-III) were obtained from Fisons LTD Leicestershire. All solvents used for chromatography were of HPLC grade and were obtained from Ruthburn Chemicals LTD, Walkburn, U.K. All other laboratory reagents were ANAL-R grade and obtained from British Drug House (BDH) Poole, Dorset.

### *ANIMALS:*

Healthy male New Zealand white rabbits, male albino mice, male Syrian hamsters, male dukin hartley guinea pig, male wistar albino rats were fasted overnight and killed by stunning. Nude mice fasted overnight was killed by exposure to carbon dioxide.

### *PREPARATION OF MICROSOMES:*

The livers were removed separately, washed with cold normal saline solution, then blotted, dried and weighed. The livers were cut into several pieces using scissors and then homogenized in three times their weight of phosphate buffer (50mM, pH 7.4) containing 0.25M sucrose. The homogenates were centrifuged for 20 min at 12000 rpm (10,000g) at 3<sup>0</sup>C. The pellet was discarded and the supernatant spun at 36000 rpm (100,000g) at 3<sup>0</sup>C for one hour. The pellet (microsomal fraction) was suspended in phosphate buffer using a Polter Elvehjem homogeniser. Aliquots (0.5 ml) were transferred into capped 1.5-ml plastic tubes and snap frozen in liquid nitrogen and stored at – 80<sup>0</sup>C until required for use.

### *RA METABOLISM ASSAY*

The hepatic microsomal fraction from the male albino mouse, male nude mouse male albino rat, male Syrian hamster, male New Zealand rabbit and male duakin Hartley guinea pig (0.030, 0.100, 0.120, 0.250, 0.350, 0.400 mg protein /ml final concentration respectively) were incubated with RA (0.1- 0.8 μM.), NADPH

(2mM,) and phosphate buffer (50mM, pH 7.4) in a volume of 400  $\mu$ l at 37°C for optimal periods (male rat and hamster 15 min; mouse 20 min; guinea pig and nude mouse liver 30 min; and rabbit 40 min). This optimal protein concentrations and the optimum time of incubations for different species were based on protein dependency and time dependency preliminary experiments as described in our previous report (13). The reaction was terminated by adding 1% (v/v) formic acid (0.1 ml).  $^3$ H.RA and its metabolites were extracted into ethyl acetate containing 0.05% (v/v) butylated hydroxy anisole (2x2 ml). The extract was dried out in – vacuo at room temperature and the residue dissolved in the mobile phase used for the reverse phase.

#### *HPLC SEPARATION OF LABELED MATERIAL*

The column was a 10 $\mu$ m C18  $\mu$  Bondapak (3.9 x 300 mm, Millipore) and the mobile phase was acetonitrile: water: formic acid (75:25:0.05 v/v) containing 10mM ammonium acetate. Flow rate was 1.2ml.Min<sup>-1</sup>. Eluted  $^3$ H compounds were detected on line by a model 970 detector (Reeve) using Hisafe III scintillation fluid. The retention time of  $^3$ H.RA was 10 min and oxidative metabolites of  $^3$ H.RA were detected eluting samples over 3 to 7 min. Metabolism was determined from the % conversion of RA into its metabolites based on AUC values (Figure-1).

## **RESULTS:**

#### *IN VITRO HEPATIC METABOLISM OF RA BY DIFFERENT SPECIES:*

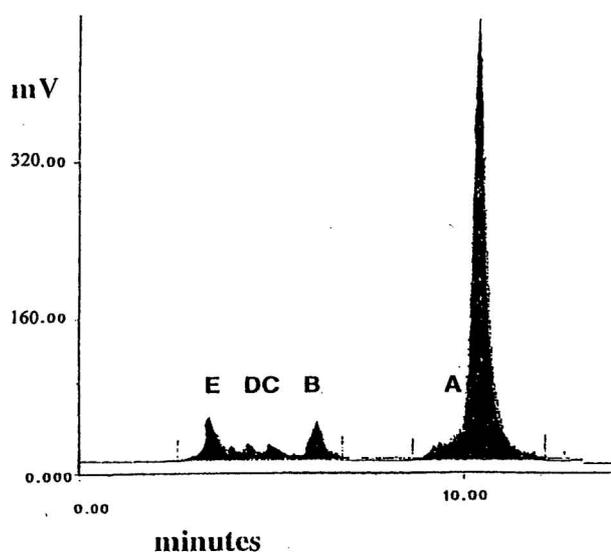
Results from the RA metabolism assays were analyzed by Lineweaver Burk plots (Figure:2). These results showed that Km and Vmax were species dependent.

Extrapolating this work to determine the efficiency of metabolism (expressed as Vmax/Km) revealed that the male rat liver was the most active in metabolizing RA (Table-1). The ranking order for metabolism of RA by the hepatic enzyme is rat (male) > mouse (male) > nude mice (male) > hamster (male) > guinea pig (male) > rabbit male.

#### *EFFECT OF KETOCONAZOLE ON THE METABOLISM OF RA*

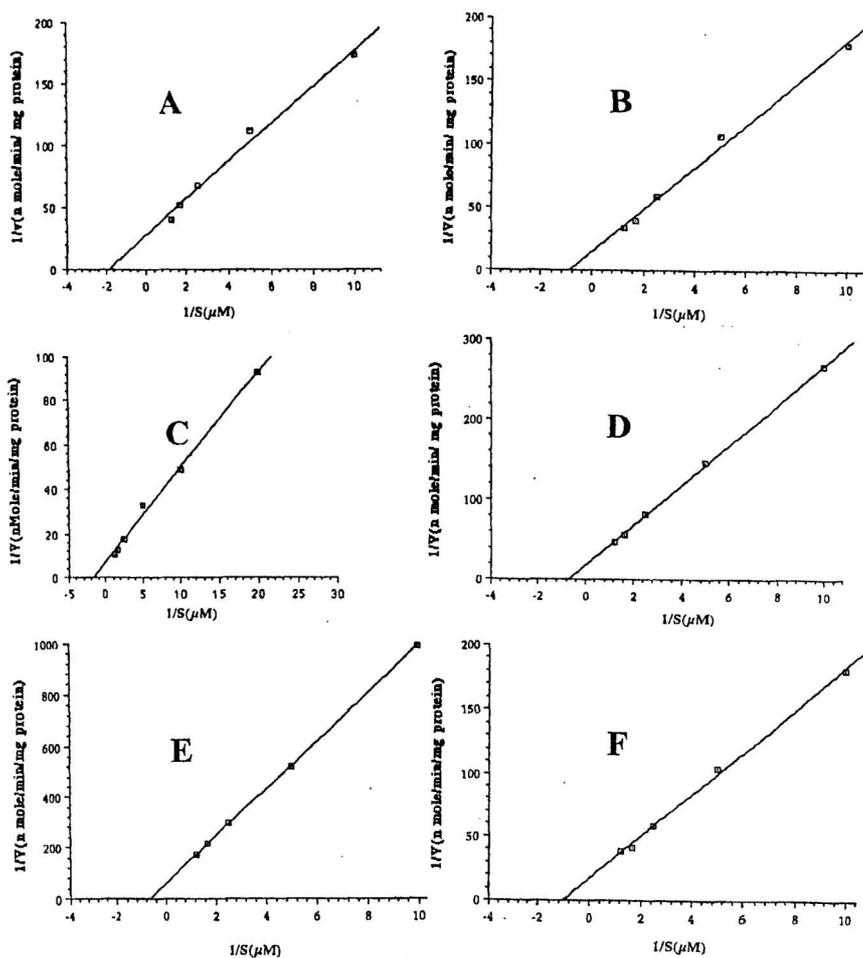
Ketoconazole (KC), a broad spectrum inhibitor of Cyt P-450 (14) was

employed to assess the extent of Cyt P- 450 dependent metabolism of RA in the species examined. A concentration of  $100\mu\text{M}$  was chosen for screening as used by Fiorella & Napoli (4) for similar type of study. The incubation system contained RA ( $3\mu\text{M}$ ), NADPH ( $2\mu\text{M}$ ), ketoconazole ( $100\mu\text{M}$ ) / solvent DMSO ( $10\mu\text{M}$ ), phosphate buffer ( $50\text{mM}$  p.H 7.4) in a volume of  $400\ \mu\text{l}$ . The reaction was started by the addition of hepatic microsomes at  $37^{\circ}\text{C}$  from different species as described above. Processing and analysis of the mixture were according to the procedure described earlier. The results showed that ketoconazole inhibited the metabolism of RA in all the microsomes samples examined (table-2). The degree of inhibition was very similar in all the species examined, and all the metabolite peaks were decreased in height /area by this ligand. This supports the view that the noted RA metabolism in these species of animals is P-450 mediated.



**FIGURE: 1** Typical HPLC separation of  $^3\text{H}$ -RA from its metabolites.

A= RA (Retinoic acid), B= 4OH-RA, C&D= unidentifie Metabolites, E= 4 keto-RA..



**FIGURE- 2 :** Representative Lineweaver-Burk plots for metabolism of retinoic acid by A: Nude mice liver microsomes, B: Mouse liver microsomes, C: Rat liver microsomes, D: Guinea pig liver microsomes, E: Rabbit liver microsomes, F: Hamster liver microsomes. Each point was the mean from three determinations, each with triplicate tubes.

Table 1: In vitro hepatic metabolism of RA by microsomes from several animals species.

Species	Km* ( $\mu\text{M}$ )	Vmax*(p mole mg protein <sup>-1</sup> .min <sup>-1</sup> )	Efficiency of metabolism Vmax/Km
Rat (male)	0.5 $\pm$ 0.1	102 $\pm$ 4	200.0
Mouse (male)	1.0 $\pm$ 0.13	67 $\pm$ 21	70.0
Nude mice (male)	0.5 $\pm$ 0.01	35 $\pm$ 1	66.0
Hamster (male)	0.9 $\pm$ 0.08	56 $\pm$ 11	62.0
Guinea Pig (male)	1.3 $\pm$ 0.18	43 $\pm$ 19	33.0
Rabbit (male)	1.5 $\pm$ 0.94	17 $\pm$ 1	11.0

Mean of three determinations (each with triplicate tubes) S.D.

Table-2: Effect of ketoconazole (100 $\mu\text{M}$ ) on the in vitro hepatic metabolism of RA by microsomes from several animal species.

Species	% Inhibition*
Rat ( male)	87.4 $\pm$ 0.4
Mouse ( male)	70.5 $\pm$ 2.4
Hamster(male)	89.7 $\pm$ 3.2
Rabbit ( male)	89.0 $\pm$ 2.5
Guinea pig (male)	90.0 $\pm$ 1.0
Nude mice (male)	82.0 $\pm$ 2.0

\*Mean of three determinations  $\pm$  s.d

#### COMPARISON OF THE RETINOIC ACID METABOLISING PATTERNS OF THE DIFFERENT SPECIES.

A quantitative comparison of the amounts of the retinoic acid metabolites 4-hydroxyretinoic acid, 4-ketoretinoic acid, and two unidentified metabolites, as determined by HPLC analysis, is presented in Table 3.

Table – 3 : Retinoic acid metabolites produced by hepatic microsomes from different species.

Species	Peak area as a percentage of the total		
	B (4-OH RA)	C&D (unidentified metabolite)	E (4-keto RA)
Rat ( male)	38.0	24.5	37.5
Mouse (male)	43.0	20.0	37.0
Nude mice (male)	40.4	25.0	34.6
Hamster (male)	38.0	29.0	33.0
Guinea pig (male)	40.0	20.0	40.0
Rabbit ( male)	37.8	26.0	36.2

Results are means, n =3

## DISCUSSION

Roberts et al (6), Van Wauwe et al (12) and Frolik (15) have suggested that retinoic acid is initially hydroxylated at the C-4 position of the cyclohexenyl ring by an enzyme with properties similar to the P450- mediated monooxygenase system.

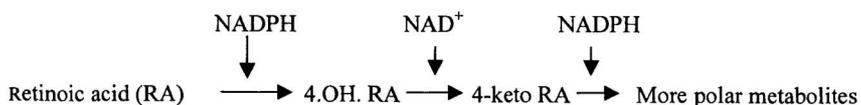


FIGURE: 3 The metabolism of retinoic acid.

Previous work of the author has also confirmed these findings (13,16). According to Roberts (6) the second step of the sequence, which is conversion of the 4-hydroxyl group to a 4-keto group, can be characterized as a dehydrogenase reaction in that  $\text{NAD}^+$  is the required cofactor. In the final reaction of the sequence, metabolism of the 4-keto derivative to metabolites that are believed to be more polar, seem also to have the characteristics of a P450-type enzyme system (12).

Quantitative analysis of the areas under HPLC peaks B (4-hydroxy retinoic acid), C, D (unidentified metabolite) and E (4-keto retinoic acid) (Figure-1) showed

the same pattern of biodegradation. For all samples examined no individual difference in the activity of dehydrogenase was found. Previous experiments showed a variation in dehydrogenase activity in different tissues of the male rat (13). This may indicate that the difference in the efficiency of hepatic metabolism among different species depends on the altered activity of 4- hydroxylase solely (table-3).

This work indicates that cellular endogenous RA could be elevated or maintained by specific inhibitors of RA metabolism. The inhibition of RA metabolism by P-450 inhibitor ketoconazole could elevate or maintain the cellular RA levels. Overall the results indicate that the male rat hepatic microsomes represent a useful enzyme source for screening novel compounds as inhibitors of RA metabolism.

All – trans retinoic acid derived from the oxidative metabolism of dietary retinol (vitamin A) and  $\beta$  carotene contributes to the growth and differentiation of mammalian epithelial tissue (1,17,18). In contrast to retinol, which is stored in tissues and which displays stable plasma levels under regulation from the tissue stores, retinoic acid is rapidly metabolized and cleared from the plasma (3,19,20). Our data indicate the feasibility to develop specific drugs that are capable of delaying the biodegradation and prolonging the biological half life of RA. Treatment with such compounds could result in higher tissue levels of RA and a more sustained response leading to improved control of epithelial differentiation.

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