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Isolation and Identification of a Major Urinary Canthaxanthin Metabolite in Rats

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Abstract: The urinary metabolic pattern after administration of the radiolabeled non-provitamin A carotenoid canthaxanthin was investigated in rats. In the rather complex HPLC urinary metabolic pattern a fraction was found which was conjugated. Deconjugation of the polar conjugates with glusulase, purification of the metabolite with HPLC and identification with GC-MS and NMR revealed that it was 3-hydroxy-4-oxo-7,8-dihydro-beta-ionone. This structure was confirmed by comparisons with HPLC retention times, UV/VIS- and NMR-spectroscopy and GC-MS of the synthesized compound.

Key words: Carotenoids, canthaxanthin, metabolism, urinary metabolite, rat

Introduction

Carotenoids are the most widespread group of naturally occuring pigments. The orange-red mushroom chanterelle *(Cantharellus cinnabarinus)* contains the oxo-carotenoid canthaxanthin. Whereas the conversion of β-carotene and other provitamin A carotenoids to retinal and retinoic acid is well documented in rat and man [1–5], little information is available on the metabolism of non-provitamin A carotenoids like canthaxanthin in mammals. Mathews-Roth et al [6] reported that canthaxanthin is not metabolized in rats and monkeys. We have reinvestigated that question by altering the positions which are radiolabeled and, in contrast to Mathews-Roth et al [6], have found that canthaxanthin is metabolized in rats.

Materials and Methods

Materials: ^{14}C-Canthaxanthin (specific activity 96 μCi/mg) labeled at positions 6, 7, 6' and 7' formulated in liposomes and reference substances used as HPLC and GC-MS standards were obtained from F. Hoffmann-La Roche, Basel, Switzerland, and glusulase (β-glucuronidase and sulfatase, helix pomatia juice) from IBF (France). All other chemicals used were of reagent grade or HPLC-grade and were purchased either from Sigma (Buchs, Switzerland) or Merck (Darmstadt, Germany).

Animals and experimental procedures: Male Wistar rats (Brl:RORO, about 10 weeks old), obtained from Biological Research Laboratories, Füllinsdorf, Switzerland, were provided diets (Kliba 31-331-DVOD BD without vitamins A and D; analyses of the content per kg diet of vitamin A, carotenes and the xanthophylls lutein and zeaxanthin revealed 2200 IU/kg, 4.4 mg/kg and 9.3 mg/kg, respectively) *ad libitum.* Four daily doses of about 0.2 mg of ^{14}C-canthaxanthin were given orally to 5 rats and the

urine was continuously collected on dry ice and stored at –20°C.

Urine preparation and HPLC analysis: In order to increase the ionic strength solid NaCl, corresponding to about 0.5 M final concentration, was added to the pooled urine samples which were centrifuged and the resultant supernates of approximately 185 ml filtered through a 0.45 µm filter (Nalge, Rochester, N.Y., USA). The total radioactivity in the filtrate was about 12.5 µCi. HPLC was carried out using the system 1090-series from Hewlett Packard with diode array detection. For the detection of radioactive compounds the HPLC radioactivity monitor LB 507A (Berthold) was used, equipped with the 200 µl flow cell GT-200U4 and connected to a W+W recorder 1100 and a Hewlett-Packard PC. Based on the HPLC radioactivity chromatogram obtained from a preliminary analysis of 1 ml from the urine filtrate (Fig. 1), four fractions (F1–F4) were selected for HPLC step 2. The urine filtrate of about 184 ml was then applied to semi preparative columns (Table I, HPLC steps 1–4) and fractions corresponding to F1–F4 of the preliminary run collected. In order to test whether the fractions were conjugated as glucuronides or sulfates, the four fractions were treated with glusulase; only F2 was almost completely cleaved and a main peak in HPLC analysis (Table II, HPLC step 4) found (Fig. 2). Further purification was done according to the scheme shown in Table I.

NMR and GC-MS analysis: The purified urinary metabolite and the synthesized reference substance were analysed using ^{1}H-NMR-spectrometry (Bruker, Karlsruhe, Germany) at 400 MHz and 250 MHz, respectively, with chloroform as solvent. The residues of the dried HPLC fractions were dissolved in 50 µl silylating reagent (pyridine+ bis– (trimethylsilyl)trifluoroacetamide 1:1) and analysed by GC-MS after standing at room temperature for approximately 30 minutes. The silylated products were injected into a model 5890A gas chromatograph (Hewlett-Packard) equilibrated at 270°C which was coupled to a model 5989B mass spectrometer (Hewlett-Packard). The stationary phase was phenyl (50%)-methyl (50%)-silicon (DB-17, film thickness 0.25 µm) in a fused silica column (15 m × 0.25 mm). Helium was used as carrier at a flow rate of 0.5 m/s. The heating program for the column was 150–320°C with a heating rate of 4°C/min. Ionization was performed by electron impact at 70 eV and 250°C.

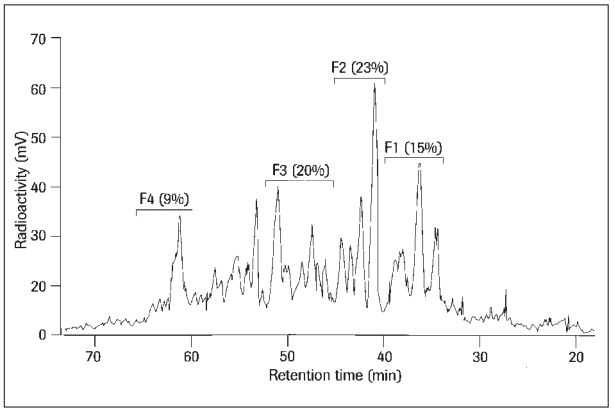

Figure 1: HPLC analysis of rat urine filtrate after oral administration of ^{14}C-canthaxanthin. HPLC was carried out by reversed phase chromatography using a RP-18 Spheri 5 µm (Brownlee LABS, dimensions of columns: 30 × 4.6 mm and 220 × 4.6 mm). Elution was performed with a flow of 1 ml/min and mobile phases A: acetic acid, pH 3.0 and B: methanol and a gradient of elution of 0% B for 6 min, 40% B in 40 min and 100% B in 30 min.

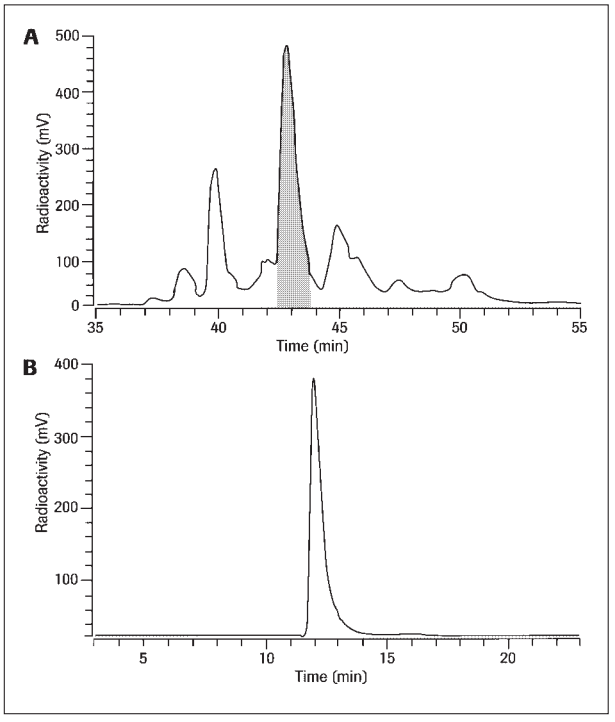

Figure 2: HPLC pattern after glusulase treatment of fraction F2 (A) and after purification of the main peak (B). Chromatogram A was obtained after purification step number 4 and chromatogram B after step number 9.

Table I: HPLC purification steps

HPLC step number	Stationary phase	Dimensions of columns (mm)	Supplyer	Mobile phase	Flow (ml/min)	Gradient of elution
1	ODS-2 Spherisorb 5 µm	40 × 8 and 2 coupled (250 × 8)	Stagroma	NaCl 0.5M / H_2O / CH_3OH (50%) / CH_3OH (100%)	2.3	one mobile phase after another
2	ODS-2 Spherisorb 5 µm	40 × 8 and 2 coupled (250 × 8)	Stagroma	A: Acetic acid adjusted to pH 3.0 B: MeOH	2.3	0% B for 3 min, 5% for 5 min, 45% in 40 min, 85% in 25 min, 100% in 5 min
3	ODS-2 Spherisorb 5 µm	40 × 8 and 250 × 8	Stagroma	A: Acetic acid adjusted to pH 3.0 B: MeOH	2.5	0% B for 3 min, 5% for 5 min, 45% in 40 min, 70% in 16 min
4	ODS-2 Spherisorb 5 µm	40 × 8 and 250 × 8	Stagroma	A: Acetic acid adjusted to pH 3.0 B: MeOH	2.5	0% B for 3 min, 5% for 2 min, 50% in 46 min, 100% in 4 min
5	RP-18 Spheri 5 µm	30 × 4.6 and 220 × 4.6	Brownlee LABS	A: Acetic acid adjusted to pH 3.0 B: CH_3CN/THF, 2:1	1.0	0% B for 3 min, 15% for 5 min, 25% in 14 min,100% in 0.1 min
6	ODS-2 Spherisorb 5 µm	40 × 4.6 and 200 × 4.6	Stagroma	A: H_2O, pH 7.0 B: CH_3CN/THF, 2:1	1.0	0% B for 3 min, 15% for 7 min, 35% in 20 min
7	ODS-2 Spherisorb 5 µm	40 × 4.6 and 200 × 4.6	Stagroma	A: Acetic acid adjusted to pH 3.0 B: CH_3CN/THF, 2:1	1.0	0% B for 3 min, 15% for 7 min, 35% in 20 min
8	ODS-2 Spherisorb 5 µm	40 × 4.6 and 200 × 4.6	Stagroma	A: Acetic acid adjusted to pH 3.0 B: CH_3CN	1.0	0% B for 3 min, 17% for 7 min, 35% in 15 min
9	ODS-2 Spherisorb 5 µm	40 × 4.6 and 200 × 4.6	Stagroma	A: H_2O, pH 7.0 B: CH_3CN	1.0	0% B for 3 min, 30% for 7.5 min, 50% for 6 min

Results and Discussion

The urine from five rats treated with radiolabeled canthaxanthin for 4 days contained about 5% of the administered radioactivity. HPLC analysis of the salt treated urine (Fig. 1) revealed a rather complex pattern which we separated into four fractions, F1–F4. Glusulase treatment of these fractions demonstrated that only fraction F2 was almost completely cleaved and in HPLC analysis gave a main peak (Fig. 2A). The deconjugated F2 fraction was further purified by the procedures shown in Table I and the purified metabolite (Fig. 2B) analysed by ^{1}H-NMR (data not shown) and GC-MS (Fig. 3A) which both identified it as 3-hydroxy-4-oxo-7,8-dihydro-betaionone. Comparison with this chemically synthesized structure revealed identical NMRs (data not shown), GC-MS data (Fig. 3A,B), HPLC retention times, and absorption spectra (Fig. 4).

The present study demonstrated that about 5% of the applied radioactivity of orally administered ^{14}C-canthaxanthin was recovered in the urine demonstrating that at least this amount was absorbed and metabolized in rats. These results clearly contrast with those of Mathews-Roth et al [6] who reported that the rat does not metabolize canthaxanthin. However, these authors used a differently labeled (at positions C15,15') ^{14}C-canthaxanthin which means that metabolites with a side chain shorter than the corresponding vitamin A compounds (like the metabolite described in the present paper) will not be identifiable because of lost label. Furthermore, the isolation procedures used by Mathews-Roth et al [6] were not suitable for the extraction of compounds with the polarity of the metabolites we found in rat urine.

A main urinary metabolite in rats was 3-hydroxy-4-oxo-7,8-dihydro-beta-ionone which was present in a conjugated form. Thus, the catabolism of the ketocarotenoid canthaxanthin included cleavage of the polyene chain at position C9, hydroxylation at position C3 of the ionone ring and hydrogenations at positions C7 and C8. It is noteworthy that in the Japanese catfish *Parasilurus asotus* there are two carotenoids, parasiloxanthin and dihydroparasiloxanthin which also are hydrogenated at positions C7,8- and C7,8,7',8' [7]. Insofar as the substrate specificity of β-carotene dioxygenase is concerned can-

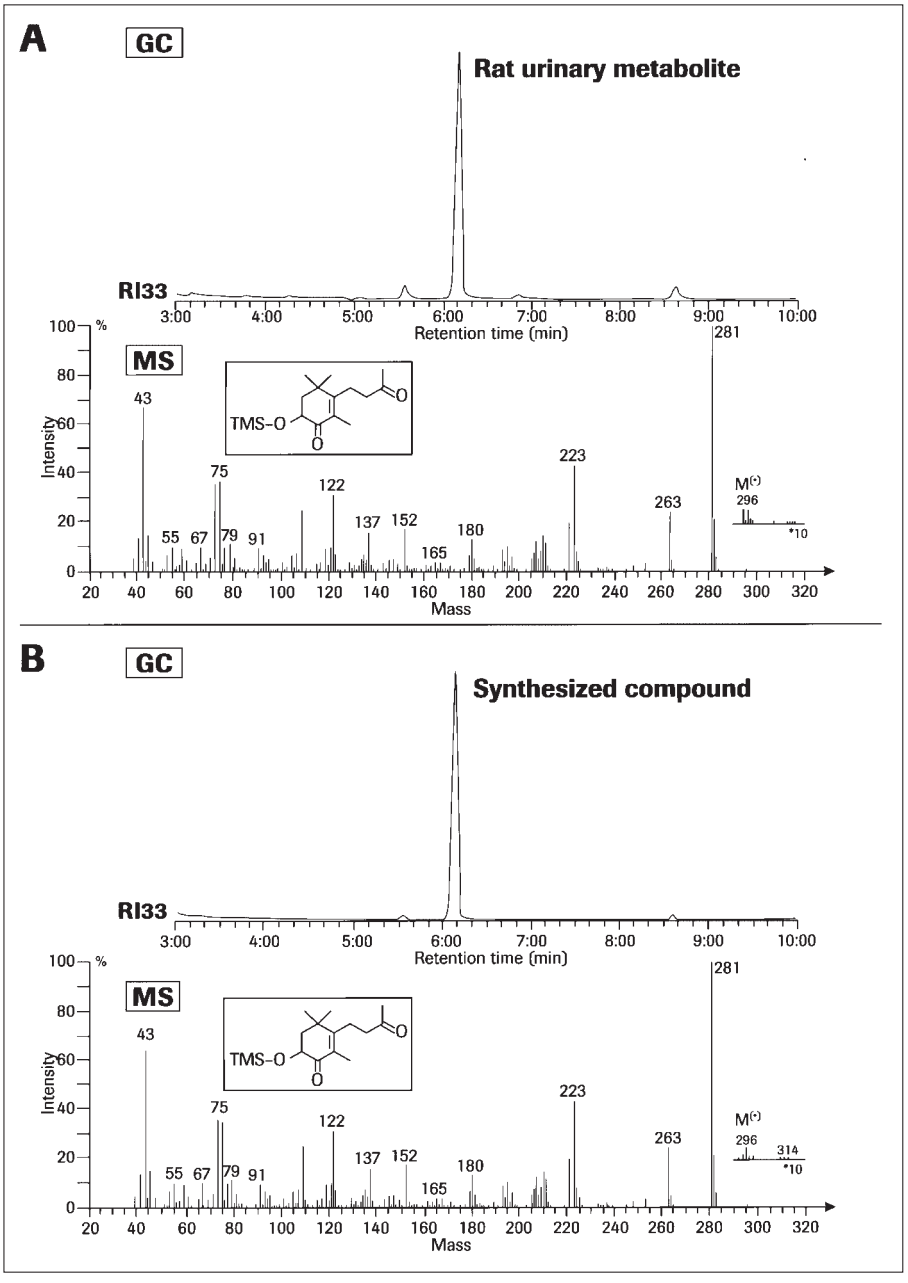

Figure 3: GC-MS of the purified urinary metabolite after derivatisation (A) and of its corresponding chemically synthesized compound (B). The structure of the urinary metabolite, 3-hydroxy-4-oxo-7,8-dihydro-beta-ionone was confirmed by comparing it with the synthesized structure.

thaxanthin was not cleaved at the central C15 = C15' double bond (Woggon WD, personal communication) indicating that no vitamin A compounds were formed. The biological significance of the metabolites derived from canthaxanthin await clarification.

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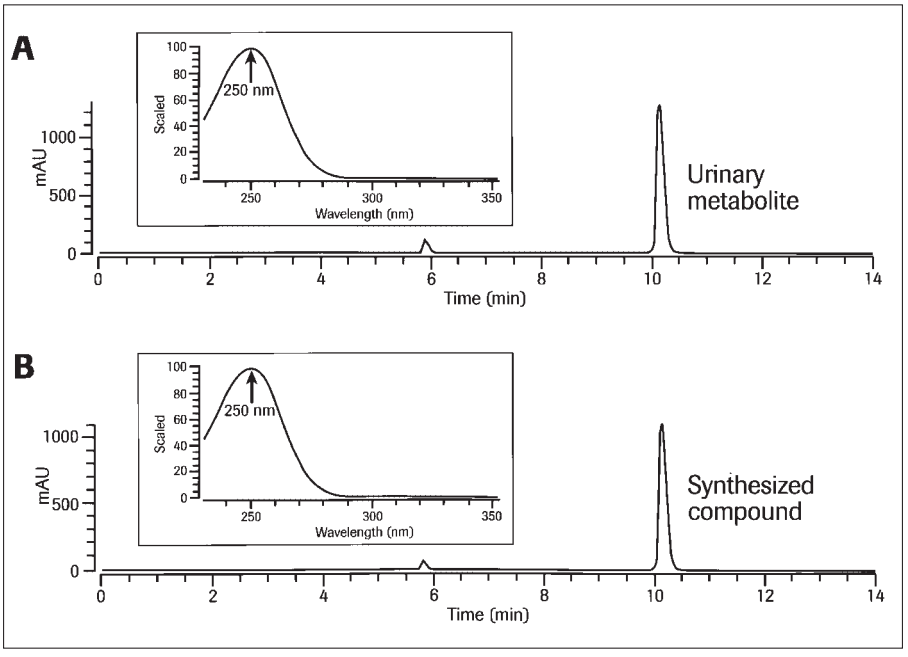

Figure 4: HPLC retention times and absorption spectra of the rat urinary metabolite (A) and its synthesized reference structure (B). The normalized absorption spectra overlapped when monitored at apex position and on both sides of the HPLC peak with absorption maxima at 250 nm.

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