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# Effect of 2-(4-aminophenylmethyl)-6-hydroxy-3, 4-dihydronaphthalen-1(2*H*)-one on all-*trans* and 13-*cis*-retinoic acid levels in plasma quantified by high perfomance liquid chromatography coupled to tandem mass spectrometry

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**Abstract**
The effect of the titled tetralone as a retinoic acid metabolism blocking agent (RAMBA) *in vivo* in comparison with ketoconazole, a well known cytochrome P450 inhibitor, was studied. Development of a HPLC/MS/MS method for the quantification of retinoic acid levels extracted from rat plasma was used to demonstrate that ketoconazole and the tetralone (100 mg/kg) enhanced the endogenous plasma concentration of retinoic acid. Levels of retinoid were raised from a control value of 0.11 to 0.15 and 0.17 ng/mL after treatment with tetralone and ketoconazole respectively showing that the tetralone and ketoconazole lead to comparable effects, indicating an inhibitory activity of the tetralone on retinoic acid metabolism.

**Keywords:** *Retinoic acid, high performance liquid chromatography, tandem mass spectrometry, liquid–liquid extraction, RA*

## Introduction

The retinoids are a large group of compounds that are structurally related to vitamin A (retinol). All-trans-retinoic acid (*at*-RA), the biologically most active metabolite of vitamin A (Figure 1), has been shown to possess selective activities in proliferation, differentiation, keratinisation, sebum production, inflammation, immune reaction and tumor prevention and therapy [1]. In addition, two natural isomers of *at*-RA, 9-*cis*-retinoic acid and 13-*cis*-retinoic acid (13-*cis*-RA) are being investigated as agents for cancer therapy. Probably because of its profound biological effects, *at*-RA is metabolized rapidly [2,3]. A major metabolic pathway of retinoic acid consists of the hydroxylation at position C-4 of its cyclohexenyl moiety to form 4-hydroxy-RA, which is then oxidized to 4-keto-RA and more polar metabolites. This rapid *in vivo* metabolism is carried out by the microsomal cytochrome P450 (CYP) enzyme system. Several CYP enzymes have been shown to be capable of metabolizing *at*-RA via the 4-hydroxylation reaction [4–8], but CYP 26 appears to be the most dedicated *at*-RA 4-hydroxylase by far [9,10].

A drug which can prolong and intensify the action of endogenous RA on epidermal cells by inhibiting P450-RA metabolising enzymes would have potential as a clinical agent in the treatment of certain skin conditions and as an anti-cancer agent. The imidazoles, ketoconazole (**3**) and liarozole (**4**) were reported as inhibitors of RA-metabolising enzymes whilst being studied as inhibitors of 17α-hydroxylase: 17,20-lyase (P450 17α) as agents for the treatment of androgen-dependent prostatic cancer by lowering testosterone levels [11]. Ketoconazole is not a suitable oral agent as an RA-mimetic for sex hormone-dependent and -independent cancers since it inhibits several other P450 enzymes on the steroidogenic pathway of androgen synthesis and, furthermore, has a poor

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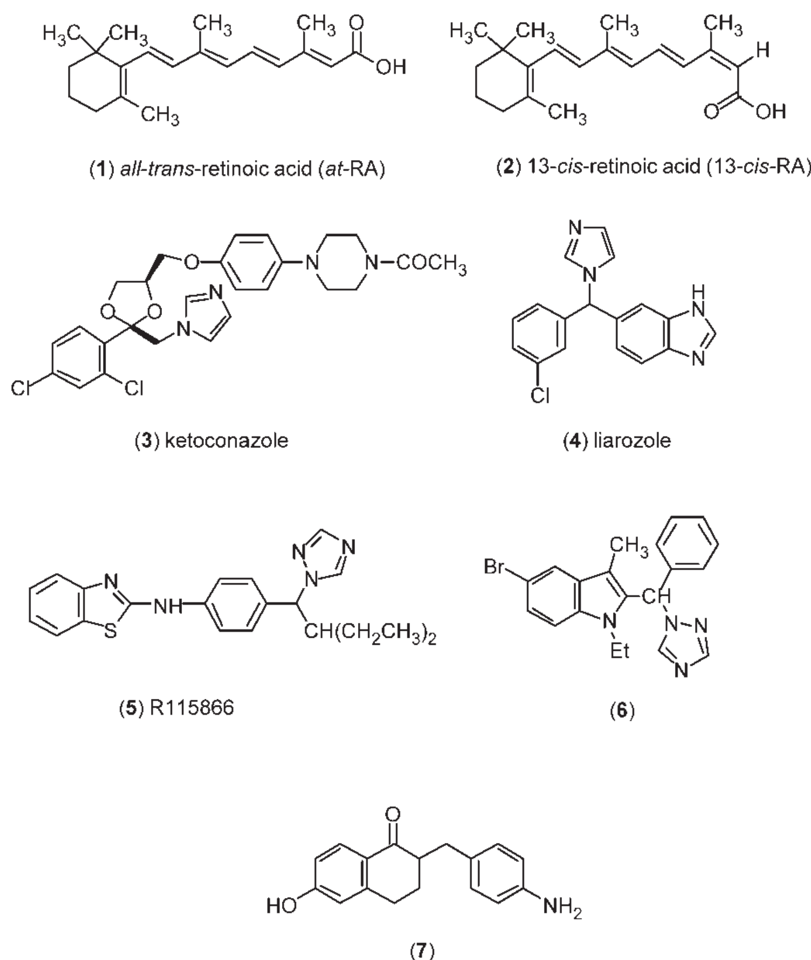


Figure 1. Structures of retinoic acid and its metabolism inhibitors.

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pharmacokinetic profile. Liarozole (Liazal®) inhibits testicular (but not adrenal) P450 17 and is a potent inhibitor of aromatase ($P450_{AROM}$), both these targets negating its potential as an oral RA-mimetic for sex hormone-independent cancers despite its effectiveness in clinical trials (oral administration) in psoriasis [12,13], as well as icthyosis and hormone resistance prostate cancer [14–18]. Fluconazole, a triazole antifungal, can reverse the decline in RA plasma levels in leukaemia patients[19].

The triazole R115866 (**5**) has been described as a novel inhibitor of CYP26 which, *in vivo* in rats after a single oral dose, increases endogenous tissue RA levels and mimics RA in several other of its biological actions [20].

Some 3-azoylmethyl-1*H*-indoles and 2, 3 or 5-(α-azolylbenzyl)-1*H*-indoles have recently been described as inhibitors of rat liver microsomal RA-metabolising enzymes; **6** was the most potent and comparable in activity with ketoconazole [21].

The titled tetralone (**7**) in cadaverous systems (pig brain, human placenta and human liver microsomes; rat and human skin homogenates) as well as *at*-RA-induced cell cultures (human male genital fibroblasts and HaCat cells) was more potent than or equipotent with ketoconazole in the cadaverous systems but less active towards RA-induced cell culture systems [22]. Examination of the data suggests that RA-induction generates metabolising enzymes not present in the cadaverous systems, which were more susceptible to inhibition by ketoconazole than (**7**). The development in the design, synthesis and evaluation of the Retinoic Acid Metabolism Blocking Agents (RAMBAs) has been comprehensively reviewed [23].

The development of a method based on high performance liquid chromatography coupled to tandem mass spectrometry (HPLC/MS/MS) for the determination of low plasma levels of *at*-RA is described here as well as its applications to a study of the *in vivo* effects of the tetralone (**7**) in comparison with ketoconazole, on *at*-RA levels after administration in male rats.

## Materials and methods

### *Chemicals*

13-*cis* Retinoic acid (13-*cis*-RA), all-trans retinoic acid (*at*-RA) and retinal (RAL) were purchased from

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Sigma-Aldrich (Sigma-Aldrich Chemie GMBH, Steinheim, Germany). Water was Millipore grade which was prepared in our laboratory. Methanol and diethyl ether (HPLC-grade) were purchased from Fischer Scientific UK Limited (Loughborough, UK), ethyl acetate from Accros Organics (New Jersey, USA), and tert-butyl methyl ether from Fluka (Sigma-Aldrich Chemie GmBH). Glacial acetic acid was obtained from Carl Roth GmBH (Karlsruhe, Germany). The preparation of 2-(4-aminophenyl-methyl)-6-hydroxy-3,4-dihydronaphthalen-1(2*H*)-one (7) has been previously described [22].

*Standard solutions*

Stock solutions of 13-*cis*-RA, *at*-RA and RAL were prepared by dissolving in methanol at 1 mg/mL, respectively. Working solutions containing 100–1.25 ng/mL for the retinoic acids and 10 ng/mL for the retinal were obtained by sequential dilutions of the respective stock solutions in methanol.

*Plasma*

Rat plasma for the calibration curve was obtained from Pharmacelsus (Saarbruecken, Germany). Plasma was preliminary irradiated with a UV lamp (Degasa UVIS, Germany) at 4°C for 24–36 h to be free of retinoid [23].

*Plasma solution for calibration curve*

Solutions to establish the calibration curve were prepared by spiking 900 μL of rat plasma with 100 μL of working solutions to obtain calibration standards. 13-*cis*-RA and *at*-RA levels used were 0.125, 0.25, 0.5 and 1 ng/mL and for the internal standards (RAL) a constant level at 1 ng/mL was used. Subsequently, plasma solutions were extracted and assayed as described below.

*Plasma solution for quantification*

Forty three male Wistar rats (180–220 g) in three groups were treated intraperitoneally to quantify the retinoic acid level in plasma: 1) Cn group: controls received vehicle (cyclodextrin/saline) ($n = 13$); 2) Tn group: received tetralone (100 mg/kg) ($n = 14$); and 3) Kn group: received ketoconazole (100 mg/kg) ($n = 16$). Blood was collected 6h later immediately post mortem. The plasma preparation was performed by Covance Laboratories (UK).

To 900 μL of these plasma sample solutions was added 100 μL of the internal standard solution at 10 ng/mL to obtain the same internal standard concentration used for the calibration curve at 1 ng/mL for the retinal. The plasma solutions were then extracted and assayed as described below.

*Plasma extraction procedure*

After mixing 1 mL of plasma sample and 100 μL of HCl (0.2N), the compounds were extracted for 0.5 min with 1 mL of diethylether–ethyl acetate mixture (50:50, v/v) by vortex-mixing. After centrifugation at 2000 rpm for 10 min at 4°C the supernatant was collected. This extraction process was repeated twice and the extracts were vortex-mixed with 500 μL of water and centrifuged under the same conditions. The aqueous phase was frozen using an acetone and dry-ice bath, and the organic phase was removed and evaporated under a nitrogen stream. The residue was dissolved in 50 μL of methanol/tert-butyl methyl ether (50/50: v/v) containing 0.5% glacial acetic acid and transferred to an injection vial for HPLC/MS/MS analysis.

*Chromatographic conditions*

A Surveyor® MS pump with quaternary gradient system and automatic injector was used (Thermo Finnigan, San Jose, California, USA). Reversed-phase liquid chromatography was performed on octadecyl-grafted silica Nucleodur C18ec (column 70 mm × 2 mm i.d.; 3 μm) stationary phase (Macherey-Nagel, Düren, Germany). The solvents were methanol/water (50/50: v/v) (A) and methanol/tert-butyl methyl ether (50/50: v/v) (B) containing 0.5% glacial acetic acid, respectively. The gradient composition was (referring only to the percentage of solvent B): I, 10% solvent B at the injection time; II, 70% solvent B from a 30 min to 32 min; (the mobile phase changed linearly between 0 and 30 min), III, 10% solvent B at 32.10 min and maintenance of 10% solvent B for 5 min until a new injection. Total time between injections was 35 min. The flow-rate was 200 μL/min and the injected volume was 10 μL.

*Mass spectrometry*

All experiments were performed using a triple quadrupole mass spectrometer TWQ Quantum® (Thermo Finnigan, San Jose, California, USA) equipped with an electrospray interface. The operating conditions were optimized for the MS and the MS/MS analysis by direct infusion of standard solutions of retinoids in methanol/tert-butyl methyl ether (50/50, v/v) containing 0.5% glacial acetic acid. The instrument was operating in positive mode applying the parameters given in Table I. $Q_1$MS full scan permitted observation of the expected $[M + H]^+$ protonated molecular ions at m/z 301.2 and 285.4 for retinoic acids (*at*-RA and 13-*cis*-RA) and retinal, respectively

Table I. Electrospray ionization parameters.

| | | | |
|---|---|---|---|
| Spray Voltage | 4100 V | Capillary Temp. | 350°C |
| Sheath Gas Pres. | 40 psi | Tube Lens Opt. | 301.2 Th: 50.00 V |
| Aux. Gas Pres. | 10 psi | Tube Lens Opt. | 285.4 Th: 60.00 V |

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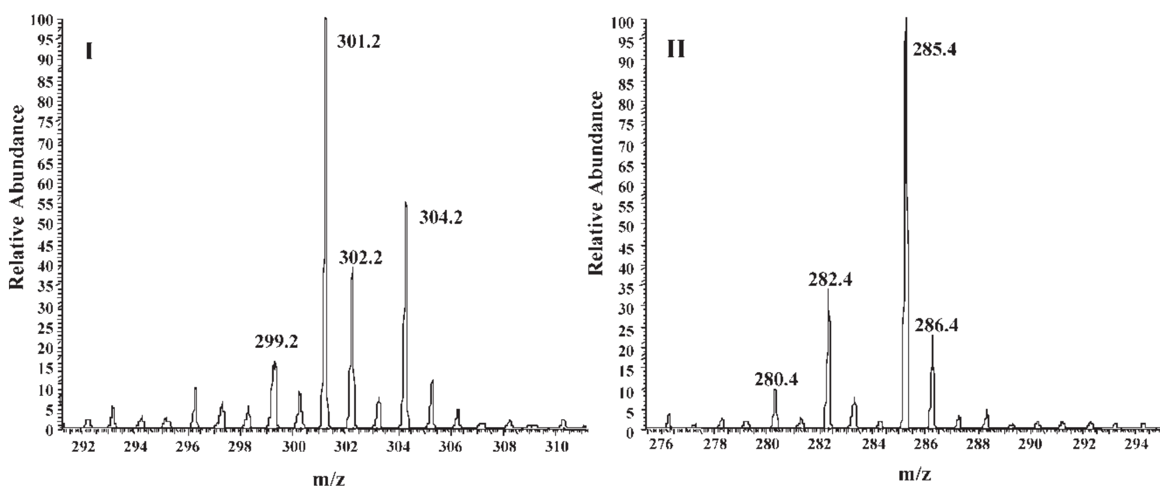


Figure 2. Mass spectra of (I) *at*-RA or 13-*cis*-RA and (II) RAL (internal standard).

(Figure 2). The transmission of these protonated molecular ions into the collision cell and the subsequent scanning of the second resolving quadrupole ($Q_3$) for fragments gave the product ion scan for each retinoid (Figure 3). The collision experiments were performed using standard instrumental conditions with a collision energy at 20 V for *at*-RA and 13-*cis*-Ra and 10 V for RAL (internal standard), and argon as collision gas at a pressure at 1.5 mTorr. Fragment ions were determined on which all the quantifications were based (Figure 3). In conclusion, product ion scan in positive ionization mode led to a large number of fragment ions, providing structural information but multiple reaction mode (MRM) acquisition mode gave more selectivity and sensitivity as it monitors only selected specific transitions. As the objective of this work was to quantify very small amounts of retinoic acid in rat plasma samples, the MRM mode was chosen despite the extensive number of transitions to be programmed for a multi-residue analysis by HPLC/MS/MS. Then retinoic acids ions at m/z 123.1 and retinal ions at m/z 161.2 (internal standard) were selected as product ions and their respective $[M+H]^+$ protonated molecular ions as parent ions for the following analysis of the extract from the rat experimental plasma in MRM acquisition mode.

### *Calculations*

The ratios of the peak area of *at*-RA and 13-*cis*-RA to the peak area of the internal standard (RAL) were determined from the chromatograms using the Quan Process and Quan Browser software (Surveyor System v 1.3, Thermo Finnigan, San Jose, California, USA). Data on plasma concentrations of analyzed compounds were obtained from least-square linear regression curves, established from four calibration points.

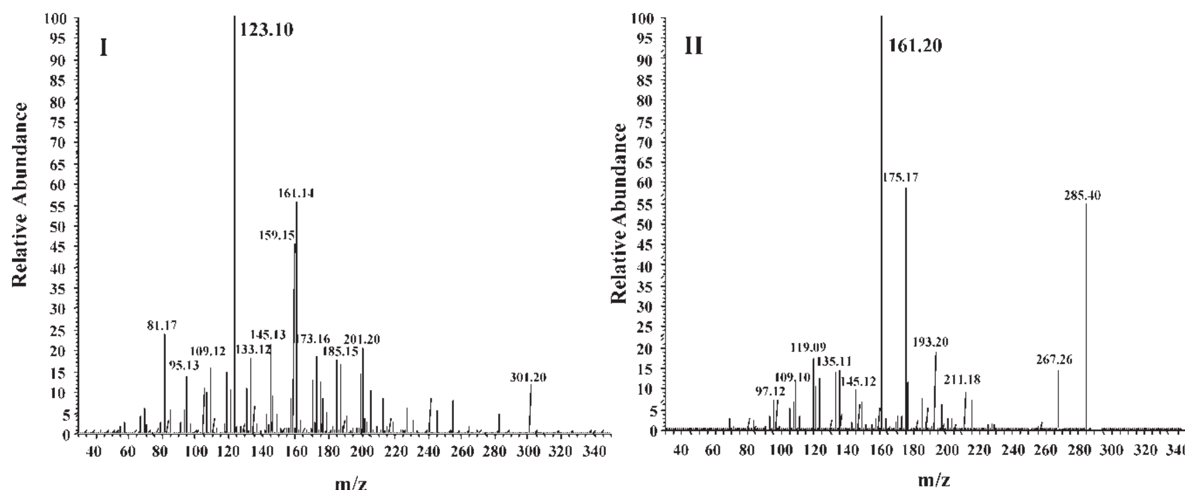


Figure 3. Product ion scan spectra of *at*-RA or 13-*cis*-RA (I — Parent ion: m/z 301.2 − $E_{CID}$ = 20 V) and RAL (internal standard) (II — Parent ion: m/z 285.4 − $E_{CID}$ = 10 V).

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Table II. *at*-RA levels (ng/mL) in controls and treated rat plasma after statistical significance test

| Rat Plasma Groups | Mean ± SD (±SEM) |
|---|---|
| Control | 0.1092 ± 0.020 (±0.006) |
| Tetralone (7) | 0.1514 ± 0.036* (±0.010) |
| Ketoconazole | 0.1719 ± 0.049** (±0.012) |

*p < 0.05; **p < 0.01; Control group is used as reference group.

Table III. 13-*cis*-RA levels (ng/mL) in controls and treated rat plasma after statistical significance test

| Rat Plasma | Mean ± SD (±SEM) |
|---|---|
| Control | 0.3292 ± 0.084 (±0.023) |
| Tetralone (7) | 0.3614 ± 0.101* (±0.027) |
| Ketoconazole | 0.3906 ± 0.091* (±0.023) |

*p > 0.05; Control group is used as reference group.

Results are expressed as mean ± SD (standard deviation). Differences between control and experimental rat plasma groups were statistically evaluated using Dunnett's Multiple Comparisons Test. Values < 0.05 were considered as significant (Tables II and III).

## Results

### *Chromatograms*

Under the described conditions, retention times for *at*-RA and 13-*cis*-RA and the internal standard RAL were 22.45, 21.20 and 20.95 minutes, respectively.

Figure 4 shows typical chromatograms obtained for the calibration solution (1 ng/mL) spiked with *at*-RA (peak I), 13-*cis*-RA (peak II) and RAL (peak III). A satisfactory HPLC separation was achieved to quantify the amount of these two isomers from endogenous plasma and treated plasma (Figure 4, upper chromatogram).

### *Linearity and limit of quantification*

The standard curves for both *at*-RA and 13-*cis*-RA showed a good linearity (Figure 5). The difference between the correlation coefficient found (0.975 for 13-*cis*-RA and 0.9643 for *at*-RA) and the optimal value, was probably due to the fact that quantifications were carried out close to the detection limit. Furthermore, extraction of such low amounts of analyte may have increased the error. The limit of quantification (LOQ), 0.125 ng/mL, was defined as the lowest level of the calibration curve.

### *Effect of tetralone and ketoconazole on plasma* at-*RA level*

To determine the ability of the tetralone (7) to affect the *in vivo* degradation of retinoic acid, rats were treated by intraperitoneal injection with vehicle (cyclodextrin), ketoconazole (100 mg/kg) and tetralone (100 mg/kg) and blood samples were collected 6 hours later, after anaesthesia, by cardiac puncture. The quantification results are shown in Table II. Mean *at*-RA levels in vehicle-treated animals (control group) was established at 0.1092 ± 0.020 ng/mL. Tetralone enhanced this value significantly (P < 0.05) to 0.1514 ± 0.036 ng/mL. The effect of ketoconazole was more so

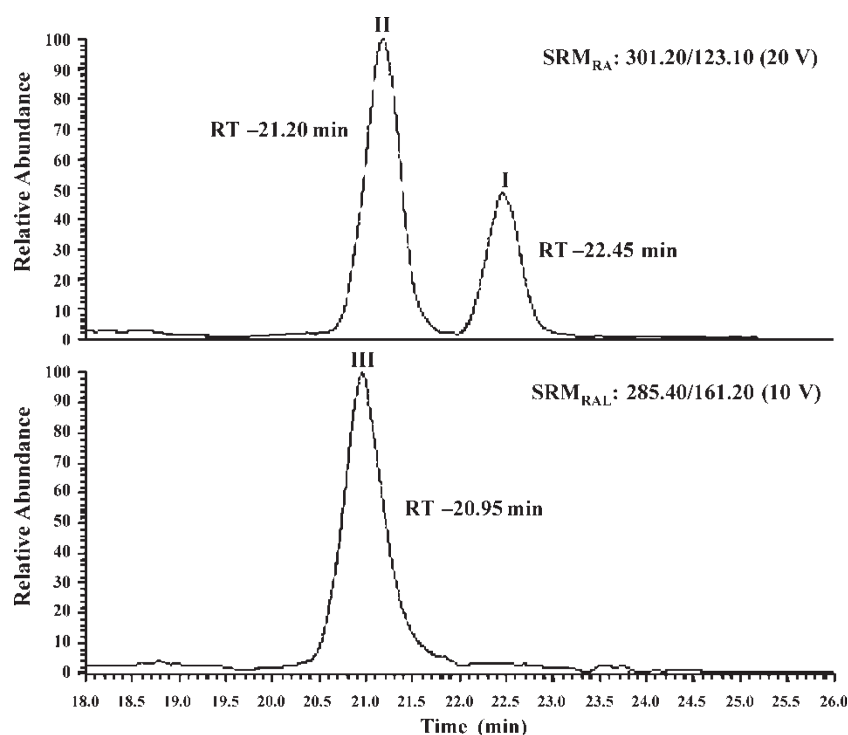


Figure 4. Typical Chromatograms obtained for calibration solution (1 ng/mL): (I) *at*-RA, (II) 13-*cis*-RA and (III) RAL (internal standard).

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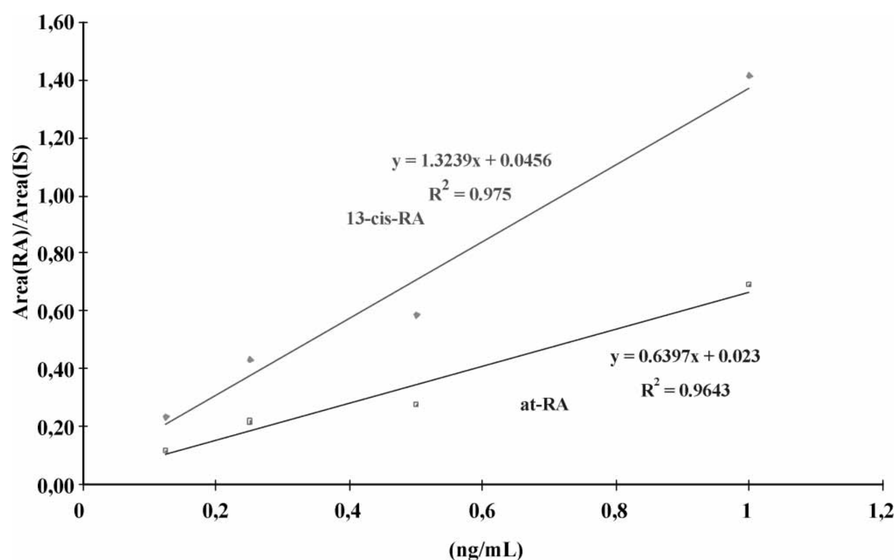


Figure 5. Standard curves for analysis of *at*-RA and 13-*cis*-RA. The ratio of peak area of analyte (RA) to the peak area of internal standard (RAL) was used to plot the standard curve and to calculate levels of *at*-RA and 13-*cis*-RA.

and we observed a significant increase ($p < 0.01$) of the *at*-RA level to $0.1719 \pm 0.049$ ng/mL.

*Effect of tetralone and ketoconazole on plasma 13-*cis*-RA level*

In parallel to the plasma *at*-RA level determination we also quantified 13-*cis*-RA levels in the same rat plasma groups (Table III) with an increase in the 13-*cis*-RA level observed after animals were treated with tetralone and ketoconazole. Indeed, the mean value (Table III) for the control rat group at $0.3292 \pm 0.084$ ng/mL increased to $0.3614 \pm 0.1010$ ng/mL after tetralone treatment. As in the case of *at*-RA, ketoconazole had a more pronounced effect and the 13-*cis*-RA level was found to be at $0.3906 \pm 0.091$ ng/mL. Treatment of animals with *at*-RA also involves a change for the 13-*cis*-RA amount in rat plasma with both the tetralone and ketoconazole but this effect was not significant ($p > 0.05$) from the control value.

## Results and discussion

An inhibitor of the metabolism of endogenous RA would be expected to have a beneficial effect on epithelial differentiation and proliferation as a RA-mimetic, with potential use as an agent for non hormone-dependent cancers and various skin conditions. The target enzymes involved in RA metabolism are a wide group of non-specific liver CYPs, among which in humans CYP2C8 is a major contributor, with additional contributions from 3A7, 3A5, 3A4, 2C9 and 1A1 [24–26]. In humans, RA administration induces another RA-metabolising enzyme CYP26 (P450RAI) which recognizes only RA as its substrate, and the expression of this isozyme can be induced by RA both *in vitro* and *in vivo* [27–29]. It seems likely that both the non-specific CYPs and CYP26 would need to be targeted since without initial RA build-up due to inhibition of non-specific enzyme action, RA would not be at a sufficiently high level to induce CYP26. Consequently these *a priori* considerations require that a prospective inhibitor as a RA-mimetic requires at least good activity against the liver microsomal CYP enzymes.

The readily detectable appearance of *at*-RA in the plasma after drug treatment can be rationalized as follows: the tissue concentration of endogenous *at*-RA is dependent on the rate of its turnover and/or its supply from or transport to other body compartments. In tissues that have the capacity to synthesize as well as degrade *at*-RA, appropriate P450 inhibition should result in increased *at*-RA levels. Such tissues could then deal with the accumulation of *at*-RA in three plausible ways: 1) biodegradation; 2) ester formation or "retinoylation", and/or 3) transport of retinoid into the blood stream, where it could be kept in storage or redistributed to other organs. In this case, *at*-RA will inevitably appear in the plasma.

Previous work [22] showed that the tetralone (7) ($IC_{50} = 12.75\,\mu M$) was two-fold more potent than ketoconazole ($22.15\,\mu M$) towards rat liver microsomal *at*-RA-metabolising enzymes, equipotent against pig brain enzymes, slightly less active against human liver (66.4% and 81.6% inhibition respectively) and human placental microsomal enzyme (67.4% and 73.9% respectively), more potent

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with human skin homogenates (71.8% and 38.8% respectively) and rat skin homogenates ($IC_{50} = 14.75\,\mu M$ and $85.95\,\mu M$ respectively. However with *at*-RA-induced cultured cellular systems, the tetralone (7) was more than one order less potent than ketoconazole; human epidermal fibroblasts ($IC_{50} = 18.5\,\mu M$ and 66% inhibition at $0.625\,\mu M$), HaCat cells ($IC_{50} = 15.5\,\mu M$ and 64% inhibition at $2.5\,\mu M$).

The constituent *at*-RA-metabolising enzymes in these cadaverous and cultured cellular systems is unclear although many P450 isoforms can metabolise *at*-RA [12–14,27–29]. A specific *at*-RA-metabolising enzyme, CYP 26, is induced by *at*-RA in many tissues [27–29] but the mix of existing *at*-RA-metabolising enzymes in the cadaverous tissue and the *at*-RA-induced enzymes (existing or new) in the living culture systems is not known or their relative contribution to *at*-RA-metabolism. However, CYP26 can be induced in rat liver, [20] MCF 7 cells [30] and HaCat cells. [31]

The ability of the tetralone (7) to increase *at*-RA plasma levels on systemic administration in rats (proof of concept) required an analytical system for measuring ng/mL levels of *at*-RA and 13-*cis*-RA in rat plasma.

HPLC coupled with UV detector or mass spectrometer is the preferred method for determining retinoids in biological samples. [32] HPLC using UV–visible detection provides a limit of quantification of 1.7 pmol [33]. However, this method suffers from poor selectivity, which makes sample preparation critical and requires careful chromatographic separation (up to 55 min/injection). In 2001, van Breemen *et al*. reported a sensitive HPLC/MS assay for simultaneous determination of retinoids in rat prostate with a limit of quantification determined at 702 fmol of *at*-RA injected on-column [34]. Here, this method was further developed for HPLC/MS/MS where the product ions from *at*-RA and 13-*cis*-RA and the internal standard (RAL) were used to quantify the small amounts of retinoic acid present in rat plasma (Figures 2 and 3).

The results show that the rats ($n = 13–16$) treated intraperitoneally at 100 mg/kg with the tetralone (7), ketoconazole or control vehicle (cyclodextrin/saline) with blood collection 6 hours later had mean *at*-RA plasma levels of $0.1514 \pm 0.036$, $0.1719 \pm 0.049$ and $0.1092 \pm 0.020$ ng/ml respectively (Table II). Statistical analysis showed that the differences between the tetralone (7) ($p < 0.05$) and ketoconazole ($p < 0.01$) levels were significant with respect to the control level but not significant between the tetralone and ketoconazole. The mean 13-*cis*-RA plasma levels were $0.3614 \pm 0.101$, $0.3906 \pm 0.091$ and $0.3292 \pm 0.084$ for tetralone, ketoconazole and control, respectively but were not significantly different ($p > 0.5$) (Table III).

In conclusion a HPLC/MS/MS method was developed for the quantification of *at*-RA levels in rat plasma after intraperitoneal administration of tetralone and ketoconazole, respectively. Treatment with the tetralone (7) led to a significant increase from 0.11 to 0.15 ng/mL, whereas ketoconazole raised *at*-Ra to 0.17 ng/mL. These findings indicate that tetralone can be considered as an inhibitor of *at*-RA metabolism *in vivo* in the rat.

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