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PLASMA PHARMACOKINETICS AND METABOLISM OF 13-*CIS*- AND ALL-*TRANS*-RETINOIC ACID IN THE CYNOMOLGUS MONKEY AND THE IDENTIFICATION OF 13-*CIS*- AND ALL-*TRANS*-RETINOYL-β-GLUCURONIDES

A Comparison to One Human Case Study with Isotretinoin

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ABSTRACT:

In order to compare the disposition and metabolism of 13-*cis*-retinoic acid (13-*cis*-RA) and all-*trans*-retinoic acid (all-*trans*-RA) in the nonpregnant female cynomolgus monkey, the plasma concentrations of the parent compound, the oxidized metabolites 4-oxo-13-*cis*-retinoic acid and 4-oxo-all-*trans*-retinoic acid, and the conjugate metabolites 13-*cis*-retinoyl-β-glucuronide (13-*cis*-RAG) and all *trans*-retinoyl-β-glucuronide (all-*trans*-RAG), were determined on day 1 and day 10 after oral dosing of 2 and 10 mg 13-cis- and all-trans-RA/kg/day. Both 13-*cis*-RAG and all-*trans*-RAG have been identified as major plasma metabolites in these studies using thermospray/HPLC/mass-spectrometry of the intact conjugates. AUC comparisons from 0–24 hr after administration indicated that 13-*cis*-RA treatment resulted in primarily *cis* metabolites and all-*trans*-RA treatment resulted in primarily *trans* metabolites, although low levels of isomerization products were observed. Comparison of the two doses (2 and 10 mg/kg, po) revealed that the AUCs were proportional to the dose administered. Although qualitatively similar, elimination of 13-*cis*-RA in the monkey was more rapid than in the human, and approximately a 10-fold greater dose of 13-*cis*-RA was required in the monkey to produce the AUC values comparable to the human. The elimination of all-*trans*-RA in monkey was faster than that of 13-*cis*-RA and tended to increase with repeated dosing. The main metabolite of all-*trans*-RA was found to be all-*trans*-RAG, while the major metabolites of 13-*cis*-RA were 4-oxo-13-*cis*-RA and 13-*cis*-RAG. Concentrations of both glucuronides tended to increase during the treatment period. In the human, the 4-oxo-13-*cis*-RA is by far the most predominant retinoid present during 13-*cis*-RA therapy; also, the 13-*cis*-RAG was identified as a new metabolite in humans. Both all-*trans*-RA and particularly 4-oxo-all-*trans*-RA, possibly proximate teratogenic compounds, were found in human serum. A preliminary interspecies comparison is made suggesting that the mouse may be an appropriate species for the study of all-*trans*-RA teratogenesis, while the monkey may be of some advantage for the study of 13-*cis*-RA teratogenesis.

Isotretinoin (13-*cis*-RA[2]) is a human teratogen that causes a characteristic pattern of congenital defects involving craniofacial, cardiac, thymic, and central nervous system structures in human infants exposed to this compound *in utero* during the first trimester (1–4). This compound is only marginally teratogenic in the mouse after a single high dose (5–7), but recently has been shown to be teratogenic after multiple high dose administration (8, 9). All-*trans*-RA is teratogenic in all laboratory animals at doses as much as 10 times lower than those used in the studies with 13-*cis*-RA (10). Transplacental pharmacokinetics in the mouse and rat show that the *trans* forms readily reach the embryo compared to the *cis* forms and that this correlates directly to their teratogenicity (9, 11, 12). It has been suggested that the metabolic conversion to the *trans* isomer may be playing a major role not only in the teratogenicity of 13-*cis*-RA after multiple-dose administration in the mouse (9) but also in rat whole embryo cultures (13) and in humans (14). Since all-*trans*-RA has been reported to be an endogenous morphogen controlling local spatial relationships in early embryonic development (15, 16), it is of interest to investigate the metabolic conversion of 13-*cis*-RA to this form and, in addition, to the 4-oxo-all-*trans*-RA metabolite, which has likewise been shown to readily reach the mouse embryo and is teratogenic (9, 12).

We have selected the nonhuman primate for our studies because all-*trans*-RA induces craniofacial dysmorphia (17–19) and the associated abnormalities, including malformed ears, hypertelorism, retrognathia, exophthalmos, and hypoplastic viscerocranium, are suggestive of defective neural crest cell migration (19). The dose level and duration of dose administration necessary to produce developmental toxicity remain controversial (17–22) and, to date, metabolic and pharmacokinetic studies of retinoids are lacking in the nonhuman primate.

In order to select an appropriate dose and dosing regimen for developmental toxicity studies of retinoids in the nonhuman primate, disposition and metabolic studies of 13-*cis* and all-*trans*-RA are needed. The present studies were designed to determine the metabolic profile, dose dependency, and the possibility of accumulation in plasma of 13-*cis* and all-*trans*-RA after their administration in the cynomolgus monkey. The reti-

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[2] Abbreviations used are: RA, retinoic acid; RAG, retinoyl-β-glucuronide; TSP-HPLC-MS, thermospray-HPLC-mass spectrometry.

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noid serum concentration of one woman during treatment with 13-*cis*-RA has been presented for a direct comparison with our comprehensive studies in the nonhuman primate.

Materials and Methods

Laboratory Precautions. All animal experiments and laboratory procedures were carried out in a darkened room with yellow light to prevent photoisomerization.

Chemicals and Reagents. 13-*cis*-RA, 4-oxo-13-*cis*-RA, and 4-oxo-all-*trans*-RA were gifts from Hoffmann-La Roche (Basel, Switzerland). All-*trans*-RAG was a gift from Dr. A. B. Barua. All-*trans*-RA and retinol were purchased from Sigma (Munich, FRG). β-Glucuronidase solution from *Escherichia coli* was obtained from Boehringer (Mannheim, FRG). Methanol (HPLC grade), ethanol, isopropanol, acetic acid, ammonia, and ammonium acetate (all of analytical grade) were obtained from Merck (Darmstadt, FRG). Water for the HPLC was purified using a Milli Q water purification system. Stock solutions of retinoids were prepared by dissolving 10 mg of the compound in 100 ml ethanol. These solutions were kept in glassware at −20°C, tested regularly for stability, and freshly prepared at least every 6 weeks.

Subjects: Cynomolgus Monkeys (*Macaca fasicularis*). Four adult female monkeys (3.5–4.2 kg) were housed and maintained individually in accordance with the American Association for Accreditation of Laboratory Animal Care and National Center of Toxicological Research animal care guidelines. Access to high-protein monkey chow (Ralston Purina, St. Louis, MO) was provided twice per day and water was provided *ad libitum*. Temperature and relative humidity were maintained at 25 ± 2°C and 50 ± 4%, respectively, under a 12-hr light/dark cycle with yellow lights coming on at 6:00 a.m.

Dosing Procedure. The chair-conditioned monkeys were placed in chairs each morning between 8:00–9:30 for dosing and blood collection. The monkeys were dosed for 10 consecutive days by gastric intubation with a dose volume of 1 ml/kg followed by a 6 ml soybean oil flush of the intragastric tube. Four studies were conducted with the same four monkeys. The studies were conducted in the following order: 1) 2 mg/kg all-*trans*-RA; 2) 2 mg/kg 13-*cis*-RA; 3) 10 mg/kg all-*trans*-RA; and 4) 10 mg/kg 13-*cis*-RA. At least 3 weeks separated each study. This low to high order of dosing was used to assure that no carryover occurred between experiments. This was then confirmed by HPLC measurements. The dosing solutions were prepared at a concentration of 17 mg/ml *cis*-RA or all-*trans*-RA in a solution of 1:11 ethanol/soy bean oil. The dosing solutions were prepared fresh each week, stored at 5°C in the dark, and sonicated for 30 sec before use. Aliquots of the dosing solutions (1 ml) were stored and analyzed at the same time and in the same manner as the plasma samples for later verification of dose purity. HPLC analysis revealed that both the all-*trans*-RA and 13-*cis*-RA dosing solutions were greater than 98% pure; less than 1% of the 13-*cis*-RA was present in the all-*trans*-RA dosing solution, and less than 0.5% of the all-*trans*-RA as found in the 13-*cis*-RA dosing solution.

Collection of Samples. Blood samples (2.0 ml) were collected by venous puncture in heparinized 3-ml syringes with 23-gauge needles. Collection times were 0, 0.5, 1, 2, 4, 8, 24, 48, 72, and 96 hr after dose 1 on day 1 and after dose 10 on day 10. Each sample was centrifuged at 2000*g* for 2 min and the plasma transferred to a screw-cap tube, frozen on dry ice, and stored at −80°C until HPLC analysis.

Human Case Study. Venous blood samples (5 ml) were drawn from a female who was being treated for acne with 0.7 mg/kg 13-*cis*-RA (Accutane). Samples were collected 23 hr after dosage on day 26 of treatment and 1, 2, 4, and 5 hr after dosage on day 27. Samples were allowed to clot and were then centrifuged to obtain serum. The samples were protected from light and stored at −20°C.

HPLC Assay. A fully automated reversed-phase HPLC procedure with a precolumn switching technique was employed (11). The samples were extracted with an equal volume of isopropanol, frozen in liquid nitrogen overnight, and then centrifuged at 4°C for 20 min at 6000*g* (Haraeus Christ Minifuge 2). The supernatant was then injected directly into the HPLC system (23). A binary gradient system allowed baseline separation of the retinoids within 15 min. In brief, two precolumns were used (20 × 4 mm cartridges prepacked with Lichrosorb RP 18, 10 μm, VDS-Säulentechnik, Berlin, FRG) and an analytical column (125 × 4.6 mm), which was slurry-packed with Spherisorb ODS II 3 μm (Phase Sep, Norwalk, CT) in our laboratory.

The mobile phase was a mixture of 40 mM ammonium acetate (adjusted to pH 7.3 with 25% NH_3) and methanol. The gradient was formed by mixing these two solvents so that the initial methanol concentration was 55%, reaching 100% at the end of the run. The flow rate was 2.0 ml/min and the eluate was monitored at 354 nm. Calibration was performed by an external standard method (23).

Treatment of Plasma Samples With β-Glucuronidase. A 100-μl aliquot of monkey plasma from animals that had been administered either 10 mg/kg/day of 13-*cis*-RA or all-*trans*-RA was treated with 30 μl β-glucuronidase (5 units/ml) for 2 hr at 37°C. The untreated plasma sample from the same monkey was used as reference.

Collection of HPLC Peaks for TSP-HPLC-MS Analysis. Approximately 1 ml of corresponding fractions containing the retinoyl-glucuronides from each of 20 HPLC runs was collected from plasma from monkeys treated with 10 mg/kg/day of either 13-*cis*-RA or all-*trans*-RA in order to make up about 150 ng/ml of each compound. They were prepared on a solid-phase Varian AASP system (Varian Ass., Darmstadt, FRG) with a C-8 cartridge (ICT, Frankfurt, FRG) that had been preconditioned with 1 ml methanol and 1.2 ml 40 mM ammonium acetate buffer, collected in 0.5 ml methanol, and evaporated with N_2 down to 20 μl methanol. This procedure was likewise carried out with human serum and urine. These aliquots were used for the HPLC-MS analysis.

The stock solution of all-*trans*-RAG, as well as a UV-treated stock solution and the fractions collected from HPLC experiments containing the retinoyl-glucuronides, were prepared as above.

TSP-HPLC-MS Analysis. Chromatography of all samples was performed with a Spherisorb ODS II (3 μm, 125 × 4.6 mm) column. The mobile phase was 80% methanol in 50 mM ammonium acetate buffer with a flow rate of 1.5 ml/min. The HPLC eluent was introduced directly into a Finnigan MAT 4610 quadruple mass spectrometer equipped with a Finnigan MAT Thermospray LC/MS interface and an INCOS 2100D data system. The vaporizer temperature was 115°C, the jet temperature 220°C. The mass spectrometer recorded positive mass spectra. A mass range of *m/z* 250 to *m/z* 520 was scanned every 4 sec.

Data Evaluation. Comparison of the day-1 *vs.* the day-10 AUC values was accomplished by calculating the differences in the group means and testing for significant effects ($p < 0.05$) by Student's *t* test. The Satterthwaite's method was used to adjust the degrees of freedom when variances were unequal.

Results

Identification of Intact 13-*cis* and all-*trans*-RAG by TSP-HPLC-MS. The RAGs were analyzed with a directly coupled TSP-HPLC-MS to gain further structural proof. Injection of the reference compound all-*trans*-RAG resulted in the elution of an HPLC peak that yielded a thermospray-ionization mass spectrum compatible with the structure of this synthetic compound: *m/z* 499 corresponds to the sodium adduct ion $[M\ Na]^+$; *m/z* 301 to the protonated molecule, with loss of the sugar moiety $[MH\text{-}176]^+$; *m/z* 343 to a characteristic fragment ion that has not yet been identified. *M/z* 267, 313, and 322 all belong to the background (24).

Treatment of a stock solution of all-*trans*-RAG with UV light resulted in the appearance of an additional HPLC peak with a slightly earlier retention time, but exhibiting an identical mass spectrum upon TSP-HPLC-MS analysis (fig. 1*A*). This fraction very likely corresponds to 13-*cis*-RAG.

Analysis of the biological samples by TSP-HPLC-MS was done in the mass fragmentography mode by recording the mass chromatograms of the two characteristic ions *m/z* 301 and 343. The various fractions obtained by HPLC analysis (pooled from several HPLC runs as necessary to increase the amount of sample

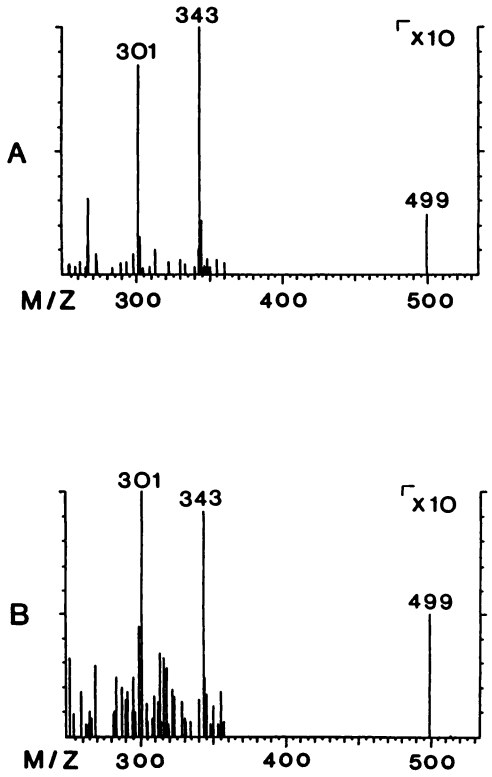

FIG. 1. *A: Mass spectrum of 13-cis-RAG collected standard (produced by UV irradiation of all-trans-RAG). B: Mass spectrum of 13-cis-RAG collected from human serum.*

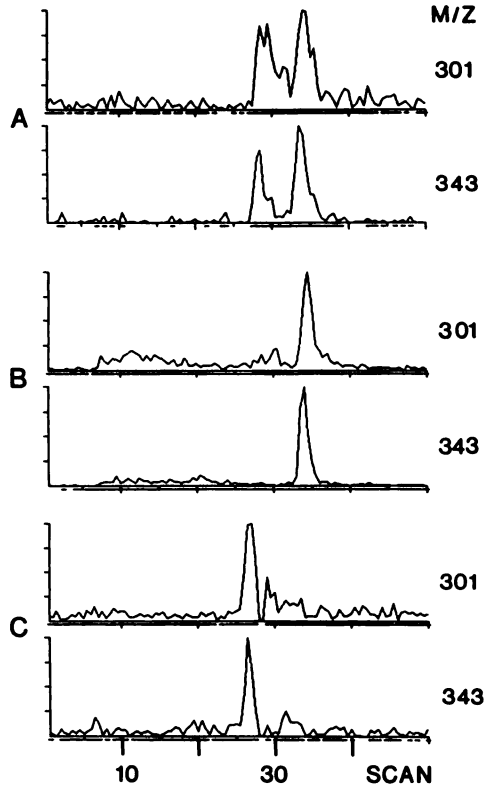

FIG. 2. *Mass chromatograms obtained by TSP-HPLC-MS analysis of UV light-treated synthetic all-trans-RAG. B: Mass chromatograms indicating all-trans-RAG in plasma of monkeys treated with 10 mg all-trans-RA/kg (TSP-HPLC-MS analysis). C: Mass chromatograms indicating 13-cis-RAG in plasma of monkeys treated with 10 mg 13-cis-RA/kg (TSP-HPLC-MS analysis).*

available) of the plasma samples were reinjected into the TSP-HPLC-MS system. Fig. 2*A* shows the mass chromatograms obtained by TSP-HPLC-MS analysis of UV-light-treated all-*trans*-RAG, fig. 2*B* the corresponding analysis of monkey plasma from all-*trans*-RA-treated monkey, and fig. 2*C* the analysis of plasma from 13-*cis*-RA-treated monkey. The earlier HPLC peak eluting corresponded to the putative 13-*cis*-RAG, which was closely followed by the fraction corresponding to the all-*trans*-RAG.

In Fig. 3, the mass chromatograms from TSP-HPLC-MS analysis of human serum and urine from a woman treated with 0.7 mg 13-*cis*-RA/kg are shown. In both urine (*A*) and plasma (*B*), 13-*cis*-RAG could be detected.

Further information on the presence of the glucuronides was obtained by treatment of the plasma samples with β-glucuronidase. Such enzymatic treatment resulted in complete disappearance of the HPLC peaks corresponding to the putative 13-*cis* and all-*trans*-RAGs, with a concomitant increase of the 13-*cis* and all-*trans*-RA peaks, respectively.

Administration of all-*trans*-RA in the Monkey. Following administration of 2 mg/kg daily, maximal concentrations of all-*trans*-RA on the first day of treatment (420 ng/ml) were twice as high as on the 10th day (210 ng/ml) (table 1). Also, the AUC value of all-*trans*-RA was halved during the 10-day treatment period. About 2% of the 13-*cis* isomer was present on day 1, which increased to 10% on day 10 (*cf.* C_{max} values in table 1). The main metabolite was identified as the all-*trans*-RAG; the maximal concentrations of this conjugate were lower than those of the parent drug; however, due to slower elimination, the AUC values of the glucuronide exceeded corresponding values of the parent drug (table 1). Only traces of 4-oxo-all-*trans*-RA (20 ng/ml) were found.

All-*trans*-RAG was also the major metabolite following administration of 10 mg/kg all-*trans*-RA (table 2). On day 1, both C_{max} and AUC values of this metabolite were lower than corresponding values of the parent drug, while the reverse was found on day 10. This again demonstrates the slow elimination of this conjugate, which accumulated during the treatment period by a

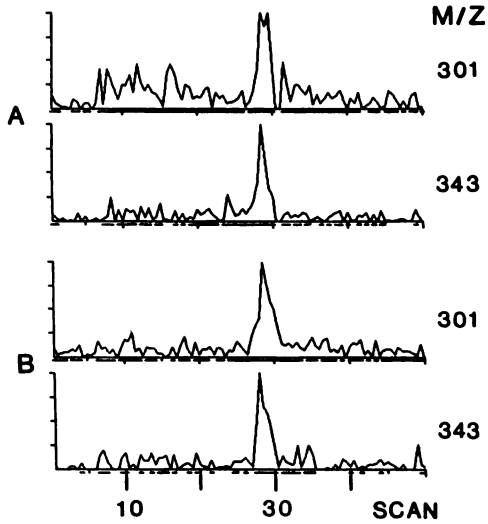

FIG. 3. *Mass chromatograms indicating 13-cis-RAG in urine from a woman treated with 0.7 mg 13-cis-RA/kg/day (TSP-HPLC-MS analysis). B: Mass chromatograms indicating 13-cis-RAG in serum from a woman treated with 0.7 mg 13-cis-RA/kg/day (TSP-HPLC-MS analysis).*

TABLE 1

Plasma C_{max} and AUC (24-hr) values in the cynomolgus monkey on the first and 10th day after daily dosing with 2 mg/kg/day all-trans- and 13-cis-RA

Mean values of four nonpregnant female cynomolgus monkeys ± SE.

	all-*trans*-RA				13-*cis*-RA			
	Day 1		Day 10		Day 1		Day 10	
	C_{max}	AUC	C_{max}	AUC	C_{max}	AUC	C_{max}	AUC
	ng/ml	*ng/ml × hr*	*ng/ml*	*ng/ml × hr*	*ng/ml*	*ng/ml × hr*	*ng/ml*	*ng/ml × hr*
all-*trans*-RA	420 ± 100	928 ± 233	210 ± 100	432 ± 196	7 ± 2	NC[a]	7 ± 2	NC
13-*cis*-RA	10 ± 5	NC	20 ± 10	NC	280 ± 75	2247 ± 638	210 ± 60	1862 ± 301
4-oxo-13-*cis*-RA	NC	NC	NC	NC	70 ± 30	907 ± 406	80 ± 25	909 ± 313
13-*cis*-RAG	NC	NC	NC	NC	6 ± 0.5		20 ± 45	140 ± 43
all-*trans*-RAG	150 ± 40	1244 ± 205	110 ± 5	705 ± 139	NC	NC	NC	NC

[a] NC = not calculated.

TABLE 2

Plasma C_{max} and AUC (24-hr) values in the cynomolgus monkey on the first and 10th day after daily dosing with 10 mg/kg/day all-trans and 13-cis-RA

Mean values of four nonpregnant female cynomolgus monkeys ± SE.

	all-*trans*-RA				13-*cis*-RA			
	Day 1		Day 10		Day 1		Day 10	
	C_{max}	AUC	C_{max}	AUC	C_{max}	AUC	C_{max}	AUC
	ng/ml	*ng/ml × hr*	*ng/ml*	*ng/ml × hr*	*ng/ml*	*ng/ml × hr*	*ng/ml*	*ng/ml × hr*
all-*trans*-RA	1200 ± 345	4607 ± 1194	460 ± 125	1557 ± 484	30 ± 5	152 ± 46	30 ± 5	181 ± 62
13-*cis*-RA	40 ± 15	315 ± 103	30 ± 10	281 ± 80	1020 ± 230	7247 ± 1014	910 ± 160	5687 ± 757
4-oxo-all-*trans*-RA	NC[a]	140 ± 40	NC	275 ± 133	NC	NC	NC	NC
4-oxo-13-*cis*-RA	NC	NC	NC	NC	260 ± 100	4321 ± 914	390 ± 50	4862 ± 1423
13-*cis*-RAG	NC	NC	NC	559 ± 289	40 ± 15	789 ± 139	120 ± 25	1415 ± 196[b]
all-*trans*-RAG	360 ± 135	3201 ± 1149	760 ± 145	7892 ± 1569	20 ± 5	314 ± 147	40 ± 15	541 ± 274

[a] NC = not calculated.
[b] Different from day 1, $p < 0.05$.

factor of about 2, in regard to both C_{max} and AUC values. On the other hand, the C_{max} and AUC values of the administered all-*trans*-RA decreased during the 10-day treatment period (table 2, fig. 4). The AUC of the 13-*cis* isomer was about 6% of the AUC of the administered drug on day 1, increasing to about 17% on day 10. Even lower values were found for the AUC values of the 4-oxo-all-*trans*-RA (3% of the AUC value of all-*trans*-RA on day 1; 7% on day 10). Maximal concentrations of the administered drug and its metabolites were reached between 3 and 6 hr after administration.

Administration of 13-*cis*-RA in the Monkey. Following repeated administration of 2 mg/kg 13-*cis*-RA, both the C_{max} and AUC values of this drug tended to decrease slightly from day 1 to day 10 (table 1). The elimination of 13-*cis*-RA tended to be slower than that of the all-*trans*-RA, so that the AUC values of 13-*cis*-RA after administration of this isomer greatly exceeded corresponding AUC values of the all-*trans*-RA after administration of the *trans* isomer (table 1). The main metabolite of 13-*cis*-RA was the 4-oxo-13-*cis*-RA; its concentrations and AUC values were about 40% of those of the parent drug. Only very small amounts of the all-*trans*-RA and of the 13-*cis*-RAG were found.

The relatively slow elimination of 13-*cis*-RA was particularly apparent following administration of 10 mg/kg (table 2, fig. 5). Maximal concentrations of this drug were reached between 1–3 hr after administration (about 1 μg/ml), which decreased to about 100 ng/ml at 24 hr; 13-*cis*-RA was detectable at 48 hr (30 ng/ml), 72 hr (10 ng/ml), and 96 hr (3 ng/ml) after the last treatment.

13-*cis*-RA was eliminated more slowly and, therefore, reached higher AUC values as compared to the all-*trans*-RA (table 2). The major metabolite was found to be 4-oxo-13-*cis*-RA, the AUC values of this metabolite reached 59 and 85% of corresponding AUC values of the administered drug on day 1 and 10 of treatment, respectively. Although the C_{max} values of the 4-oxo metabolite were lower than those of the parent drug, both compounds reached similar levels 24 hr after drug application (fig. 5). The concentrations and AUC values of 13-*cis*-RAG were usually 5–10% of the corresponding values of the administered drug. This metabolite accumulated significantly during the treatment period; its AUC value following day-10 administration reached 25% of the AUC value of the administered drug. The concentrations and AUC values of the further metabolites all-*trans*-RA (2–3% of the administered 13-*cis*-RA) and all-*trans*-RAG were typically low (fig. 5, table 2). Maximal concentrations were reached between 1–4 hr except for the glucuronides, which reached their C_{max} values somewhat later (3–6 hr).

Human Serum Concentrations During 13-*cis*-RA Therapy. 13-*cis*-RA and all of its metabolites appear to have reached a steady state by day 27 of treatment because no significant changes in plasma concentrations were seen during the 23 hr of sample collection (fig. 6). The major metabolite was 4-oxo-13-*cis*-RA with C_{max} and AUC values about 4-fold higher than those of the parent compound (table 3). There was about a 6% isomerization of 13-*cis*-RA to all-*trans*-RA, and about a 4% isomerization of 4-oxo-13-*cis*-RA to 4-oxo-all-*trans*-RA. 13-*cis*-RAG (TSP-HPLC-MS mass spectrum given in fig. 1*B*) was found at concentrations equivalent to 4-oxo-all-*trans*-RA. Small amounts of all-

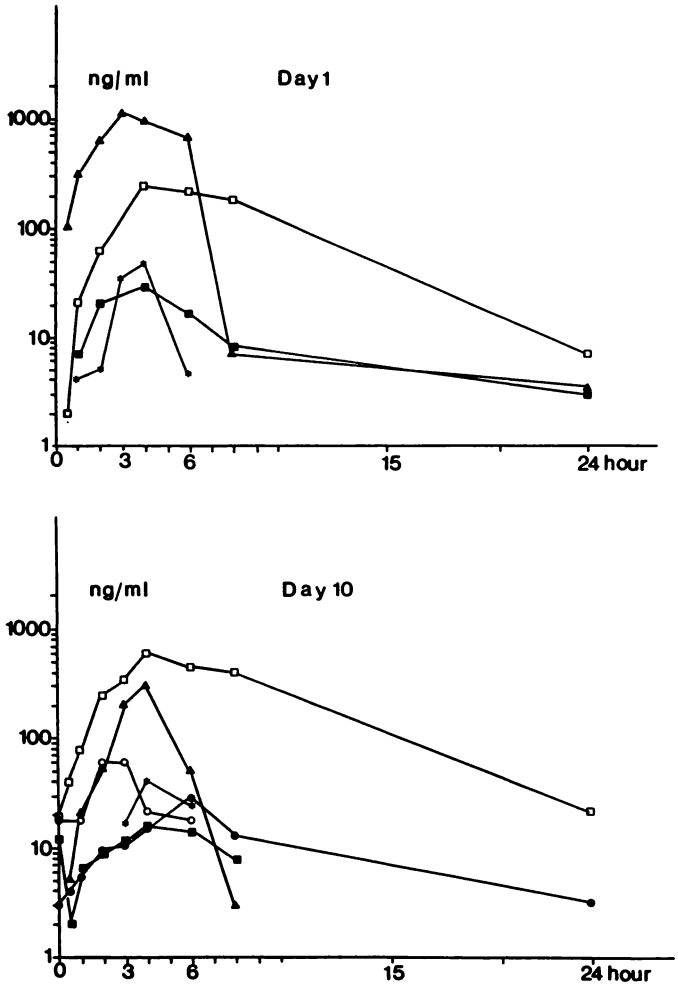

FIG. 4. *The time-course of the median concentrations of all-trans-RA and its metabolites on day 1 (top) and day 10 (bottom) in four monkeys that had been administered 10 mg all-trans-RA/kg/day.*

■ = 13-cis-RA; O = 4-oxo-13-cis-RA; ● = 13-cis-RAG; ▲ = all-trans-RA; * = 4-oxo-all-trans-RAG; □ = all-trans-RAG.

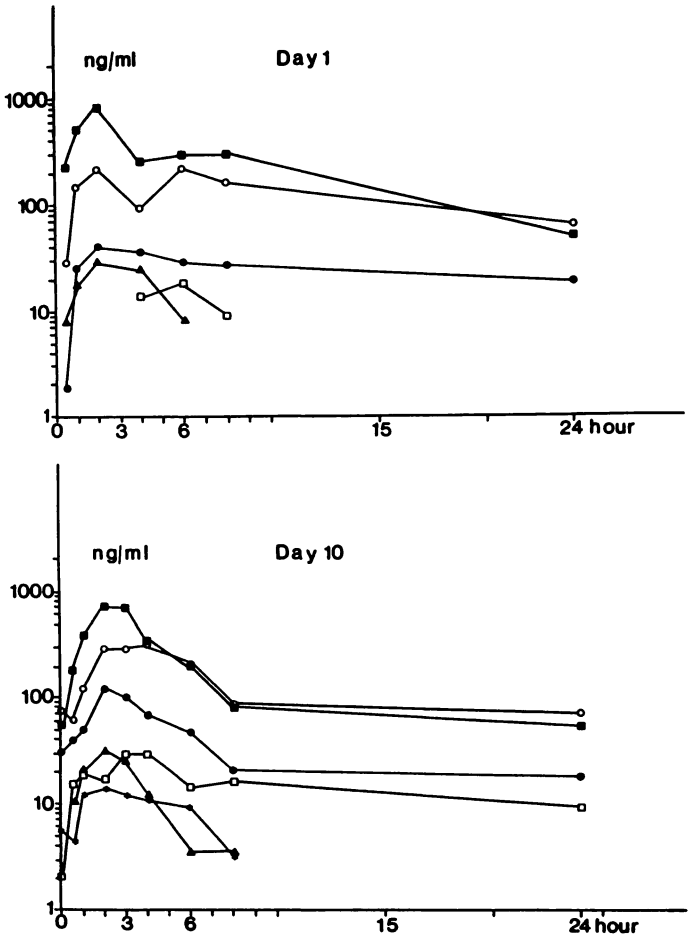

FIG. 5. *The time-course of the median concentrations of 13-cis-RA and its metabolites on day 1 (top) and day 10 (bottom) in four monkeys that had been administered 10 mg 13-cis-RA/kg/day.*

■ = 13-cis-RA; O = 4-oxo-13-cis-RA; ● = 13-cis-RAG; ▲ = all-trans-RA; * = 4-oxo-all-trans-RAG; □ = all-trans-RAG.

trans-RAG also were detected. 13-*cis*-RAG also was detected in the urine at 2 hr at 100 ng/ml (fig. 3).

Discussion

This study was designed to obtain basic pharmacokinetic and metabolic data of 13-*cis* and all-*trans*-RA in the monkey and to compare the results to corresponding data obtained in other species in order to evaluate the suitability of the monkey for investigations designed to evaluate retinoid teratogenesis (25, 26). In spite of considerable inter-individual variation of pharmacokinetic data in the monkey, the following general conclusions can be reached.

Both the plasma pharmacokinetics and the metabolic patterns of the two retinoic acid isomers differ greatly in the monkey. The plasma elimination of all-*trans*-RA was faster than that of the 13-*cis*-RA at both dose levels studied. Interestingly, repeated daily administration of all-*trans*-RA appeared to induce its own metabolism, so that the AUC value of this drug on day 10 was only one-third of the AUC value on day 1. The concentrations of 13-*cis*-RA decreased only slightly during the 10-day treatment period after repeated administration of the 13-*cis* isomer. These results agree with those obtained by Kalin et al. (27) in the

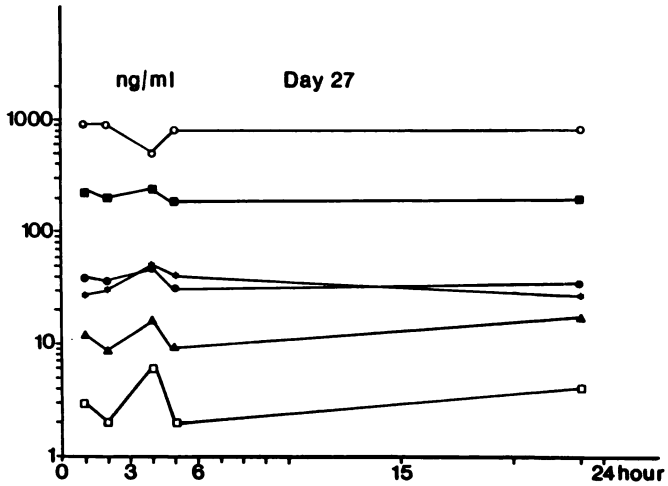

FIG. 6. *Concentrations of 13-cis-RA and its metabolites on day 27 in serum from a woman who had been treated with 0.7 mg 13-cis-RA (Isotretinoin, Accutane)/kg/day.*

■ = 13-cis-RA; O = 4-oxo-13-cis-RA; ● = 13-cis-RAG; ▲ = all-trans-RA; * = 4-oxo-all-trans-RAG; □ = all-trans-RAG.

TABLE 3

Serum C_{max} and AUC values in one female human

Values shown are from day 27 of treatment with 0.7 mg/kg/day 13-*cis*-RA.

Compound	C_{max}	AUC (24-hr)
	ng/ml	*ng/ml × hr*
13-*cis*-RA	270	4850
all-*trans*-RA	16	280
4-oxo-13-*cis*-RA	910	19450
4-oxo-all-*trans*-RA	40	809
13-*cis*-RAG	50	850
all-*trans*-RAG	6	70

mouse; also in that species, metabolism was induced by all-*trans*-, but not 13-*cis*-RA.

The main metabolite of all-*trans*-RA was found to be all-*trans*-RAG, with smaller amounts of 13-*cis*-RA, 4-oxo-all-*trans*-RA, and 13-*cis*-RAG also present. In contrast, the main metabolite of 13-*cis*-RA was found to be 4-oxo-13-*cis*-RA, followed by significant amounts of 13-*cis*-RAG and minor amounts of all-*trans*-RAG and all-*trans*-RA. Importantly, the concentrations of both glucuronides tended to increase during the 10-day treatment period; since conversion of all-*trans*-RA to all-*trans*-RAG was the predominant metabolic pathway, induction of glucuronidation may largely explain the decreasing all-*trans*-RA levels during the 10-day treatment period.

The two glucuronides of all-*trans* and 13-*cis*-RA have been identified by an HPLC-MS system directly coupled with a thermospray interface, both by their mass spectra and HPLC retention times. To our knowledge, this is the first published report on these conjugates as major plasma metabolites following pharmacological or toxicological dosing with all-*trans*- and 13-*cis*-RA. The all-*trans*-RAG has previously been identified as an endogenous component of human and rat blood, and was shown to be present in rat liver and bile (28–31). The present studies now show that all-*trans*-RAG is the predominant metabolite in monkey plasma after all-*trans*-RA administration, and 13-*cis*-RAG is an important metabolite of 13-*cis*-RA in monkey plasma and human serum after 13-*cis*-RA administration.

Drug glucuronides are usually less active or inactive in comparison to the parent drug, since glucuronidation increases water solubility, facilitating urinary and biliary excretion. There are exceptions to this rule, including some estrogens (32, 33) and morphine (34) where glucuronides may play an important role in the pharmacological and toxicological action. This also may be true for retinoic acid: the glucuronide of retinoic acid shows interesting biological actions, such as support of growth of vitamin A-deficient rats, inhibition of the induction of neoplastic changes in the mammary gland cultures, and induction of cellular differentiation in several systems (*cf.* 31). In contrast, its teratogenic action has so far not been demonstrable, even at high doses (35). The explanation for this apparent dissociation between the pharmacological activity and teratogenicity of RAG comes from our observation that RAG is not transferred to the mouse embryo in any significant amount; the same holds true for 13-*cis*-RAG (9).

The human data demonstrated that by day 27 (fig. 5), the parent compound and metabolites in the serum had reached a steady state. As in the literature (36–39), 4-oxo-13-*cis*-RA was found at concentrations higher than for the parent compound. Our data correspond to that of Brazzel *et al.* (36), who showed that minimum steady-state concentrations for 4-oxo-13-*cis*-RA were about 900 ng/ml and for 13-*cis*-RA about 200 ng/ml. These investigators did not report the presence of the *trans* isomer and further metabolites as in the present report. However, the more recent work of Wyss and Bucheli (39) showed that 24 hr after 4-month treatment with 0.5 mg/kg 13-*cis*-RA, there were concentrations of all-*trans*-RA (8 ng/ml) and 4-oxo-all-*trans*-RA (37 ng/ml) in the plasma, which is in close agreement with our findings (fig. 6). A discrepancy can be seen in the levels for 4-oxo-13-*cis*-RA and 13-*cis*-RA, ours being far higher at 24 hr after treatment than those of Wyss and Bucheli (39). Our higher concentrations of these metabolites would be expected based on the half-lives described by Brazzel *et al.* (36): 36.5 hr for 13-*cis*-RA and 49.5 hr for 4-oxo-13-*cis*-RA, respectively. The novelty of our studies is that we have detected both glucuronides in human serum after treatment with 13-*cis*-RA, the concentrations of 13-*cis*-RAG being relatively high (40 ng/ml). This compound was likewise found in the 2-hr urine sample at similar concentrations to those in the serum and has been identified with TSP-HPLC-MS (fig. 3).

A preliminary inter-species comparison of the pharmacokinetics and metabolic profiles of all-*trans*-RA in mouse (9, 12) and monkey, and of 13-*cis*-RA in mouse (9, 12), monkey, and human, can now be made. The predominant metabolite of all-*trans*-RA in the mouse is 4-oxo-all-*trans*-RA; in the monkey it is all-*trans*-RAG. The predominant metabolite of 13-*cis*-RA in the mouse is 13-*cis*-RAG; in monkey and human it is 4-oxo-13-*cis*-RA, although this metabolite is much more predominant in the human than in the monkey. All-*trans*-RA is more rapidly cleared than 13-*cis*-RA in the monkey, while the reverse was observed in the mouse.

AUC for 13-*cis*-RA: human $\gg$ monkey > mouse; for all-*trans*-RA: mouse > monkey. Thus, in some aspects of retinoic acid metabolism, the monkey resembles the human more closely than the mouse. However, the clearance rates of the two retinoic acids are high in the monkey. Thus, the AUC value for all-*trans*-RA was even lower in monkey plasma than mouse plasma, possibly because of the efficient glucuronidation of all-*trans*-RA in the monkey. From the pharmacokinetic viewpoint, therefore, the mouse appears to be an appropriate species for the study of all-*trans*-RA-induced teratogenicity.

The AUC of 13-*cis*-RA in monkey plasma was about 2.5 times higher in the monkey than the corresponding value in the mouse. Both in regard to the metabolic pattern (predominance of the 4-oxo metabolite) and exposure level (AUC), the monkey appears to be somewhat better suited as a model for the human for the study of 13-*cis*-RA teratogenesis. However, the clearance of 13-*cis*-RA in the monkey is much higher than in the human. Approximately 5 mg/kg/day would have to be administered in the monkey (equivalent to 10× the human therapeutic dose) in order to achieve plasma exposure levels comparable to those observed in human therapy (when adding the AUCs of 13-*cis*-RA and its 4-oxo metabolite). (While the galley proofs of this paper were reviewed, the study of Hummler *et al.* (40) appeared in the literature. These authors found significant teratogenic effects in the cynomolgus monkey following a dosing regimen of 2.5 mg/kg/day from day 10 to 25 of gestation followed by 2 × 2.5 mg/kg/day on days 26 and 27 of gestation. Thus, our prediction made above (5 mg/kg/day of isotretinoin should be tested in the monkey as a possible teratogenic dosing regimen) was indeed a good choice, again pointing out the great value of pharmacokinetics for risk assessment using experimental

animals.)

Because of the high teratogenic potency and efficient placental transfer of all-*trans*-RA and 4-oxo-all-*trans*-RA as compared to the corresponding 13-*cis* isomers in the mouse, we have previously hypothesized that the all-*trans* isomers are the proximate teratogens after 13-*cis*-RA treatment (6, 9, 11, 12, 14, 41, 42); all-*trans*-RA can interact with both the newly discovered nuclear receptor (43, 44) and the cytosolic binding protein, cellular retinoic acid binding protein. All-*trans*-RA also appears to be an endogenous morphogen playing a major role in embryonic development (15, 16). Indeed, in our human case, both all-*trans*-RA and 4-oxo-all-*trans*-RA were found in considerable amounts (AUCs pooled = over 1000 ng/ml × hr for a 24-hr period). In both monkey and mouse, the levels of both isomers were much lower, making both of these species unsuitable for the study of this hypothesis unless two or more doses administered each day would produce higher relative AUC values for all-*trans*- and 4-oxo-all-*trans*-RA. Thus, the human appears to be unique so far in regard to low clearance of administered 13-*cis*-RA, high AUC values of 4-oxo-13-*cis*-RA, and significant amounts of 4-oxo-all-*trans*-RA. Placental transfer studies must be performed in the monkey before it can be decided whether this species is of major advantage for the study of 13-*cis*-RA teratogenesis. The present study shows that pharmacokinetics can play a major role in the selection of the appropriate animal species and dosing regimen to save time, expense, and, above all, animals, and improve the extrapolation of findings in animals to humans.

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