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Quantification of retinoyl-β-glucuronides in rat urine by reversed-phase high-performance liquid chromatography with ultraviolet detection

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Abstract

A method is presented for the quantitation of the glucuronide conjugates of 4-oxo-all-*trans*-, 4-oxo-13-*cis*-, 13-*cis*-, 9-*cis*- and all-*trans*-retinoic acids in rat urine utilizing solid-phase extraction and gradient reversed-phase HPLC. The range of the R.S.D. (relative standard deviation) for both the inter- and intra-assay precision was 1.45–11.60%. The recovery of all retinoyl-β-glucuronides from rat urine ranged between 89 and 99%. The limit of detection was 0.01 μg/ml using 5 ml of rat urine. This method was applied to quantitate the amount of retinoyl-β-glucuronides produced in urine after the single and multiple oral administrations of 13-*cis*-, 9-*cis*- and all-*trans*-retinoic acids to rats.

Keywords: Retinoyl-β-glucuronides; Retinoic acid

1. Introduction

Retinoids have a well-established value as dermatological agents. Both Accutane (13-*cis*-retinoic acid) and Retin-A (all-*trans*-retinoic acid) have been used for the treatment of mild to severe cystic acne [1]. Tegison (etretinate) and Soritane (acitretin), both synthetic retinoids, are effective anti-psoriatic agents [2,3].

More recently, retinoids have been shown to have chemotherapeutic potential [4]. All-*trans*-retinoic acid (Vesanoid) is currently the first modality in the treatment of patients diagnosed with acute promylocytic leukemia (APL) [5]. The oral administration of Vesanoid induces complete remission for the vast majority of APL patients. However, this remission is short-lived and must be supplemented by conventional chemotherapy in order for patients not to relapse. Experimental clinical trials have shown that 13-*cis*-retinoic acid may not only prolong but also improve the quality of life in infants diagnosed with juvenile chronic myelogenous leukemia [6]. In combination with α-interferon, 13-*cis*-retinoic acid has also been shown to be beneficial for the treatment of cervical cancer [7]. The retinoid, 9-*cis*-retinoic acid, has been shown to be a potent inhibitor of mammary carcinogenesis induced by *N*-nitroso-*N*-methylurea in Sprague–Dawley rats [8].

As previously mentioned, the chemotherapeutic value of all-*trans*-retinoic acid is short-lived in APL patients. This loss of potency has been associated, in vivo, with a decrease in the area under the plasma

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concentration–time curve (AUC) [9]. However, this phenomenon is not unique to APL patients. Both mice and monkeys demonstrate reduced AUC's after multiple oral administrations of all-*trans*-retinoic acid [10,11]. This reduction in AUC can be attributed to one or more of the following factors: (1) decreased absorption; (2) increased metabolism (induction of P450 and/or conjugation enzymes) and (3) induction of plasma and/or cellular binding proteins.

Glucuronidation is an important mechanism by which nonpolar, water insoluble compounds are converted, in vivo, to more water soluble compounds for enhanced elimination. Glucuronidation of 13-*cis*-retinoic acid predominates in the mouse after oral administration of 13-*cis*-retinoic acid [12]. This event is most likely responsible for the decreased teratogenicity of 13-*cis*-retinoic acid in the mouse because the glucuronide conjugate is rapidly and easily eliminated by the kidney and also does not cross the placenta. However, in both monkeys and humans, glucuronidation plays a minor role in the metabolism of 13-*cis*-retinoic acid after oral administration of the same [12].

This study is part of a larger study to characterize the oral pharmacokinetics of all-*trans*-, 13-*cis*- and 9-*cis*-retinoic acids in the rat. The purpose of this specific study is to investigate whether glucuronidation of all-*trans*-retinoic acid significantly contributes to the reduced AUC's produced upon multiple oral dosing with all-*trans*-retinoic acid and to compare the amount of retinoyl-β-glucuronides produced in urine after single and multiple oral administrations of all-*trans*-, 13-*cis*- and 9-*cis*-retinoic acids in the rat.

Conventional methods quantitate retinoyl-β-glucuronide conjugates in urine indirectly [13,14]. The concentration of the conjugate is based on the concentration of parent drug and metabolite observed after hydrolysis (chemical or enzymatic). A method [15] has been recently described which does quantitate the intact conjugate in rat plasma, but not in urine. To quantitate the intact retinoyl-β-glucuronides, a method employing solid-phase extraction (SPE) followed by reversed-phase HPLC was developed. Extraction of retinoids from biological matrices utilizing solid-phase extraction [16] and analysis by reversed-phase HPLC [17] have been previously reported.

2. Experimental

2.1. Materials and reagents

All-*trans*-, 9-*cis*-, 13-*cis*-, 4-oxo-all-*trans*- and 4-oxo-13-*cis*-retinoyl-β-glucuronides and acitretin-β-glucuronide (Fig. 1) were synthesized by modifications to a previously published method [18]. HPLC grade methanol, acetonitrile and ethyl acetate were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Certified ammonium acetate was also obtained from Fisher Scientific. Distilled water was purified using a Mill-Q UF Plus water purification unit, Millipore (Bedford, MA, USA). Bond-Elut C_{18} disposable extraction columns (3 ml, 500 mg) were purchased from J.T. Baker Chemical Co. (Phillipsburg, NJ, USA). Drug free, control rat urine was collected from adult male CD rats (Charles River, Wilmington, MA, USA) and pooled.

2.2. Laboratory precautions

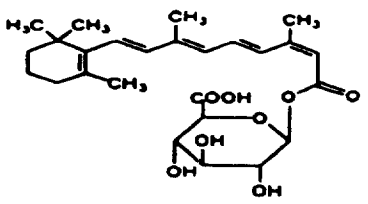
All-retinoyl-β-glucuronides are light sensitive and unstable at ambient temperatures. Therefore, all procedures were carried out under yellow lights, using amberized glassware and sample preparation and extraction were performed over ice.

2.3. Animal study

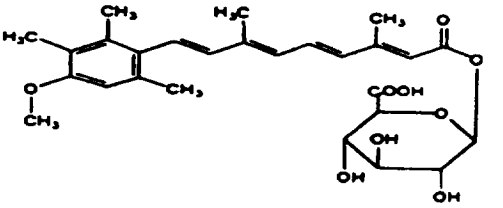
Adult male CD rats weighing 275–370 g were administered single, daily oral doses of 20 mg/kg of all-*trans*-, 13-*cis*- and 9-*cis*-retinoic acids, suspended in corn oil, for four consecutive days. Control animals received only corn oil. The animals were housed in metabolic cages with free access to food and water for the entire study. Urine samples, 24 h, were collected into plastic conical centrifuge tubes over dry ice and stored at −70°C until analysis.

2.4. Solutions and standards

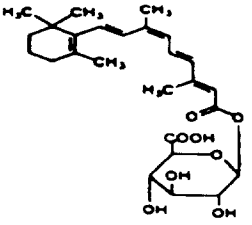
Stock solutions of the five retinoyl-β-glucuronides and the internal standard, acitretin-β-glucuronide, were prepared by dissolving approximately 3–5 mg of each compound into separate 10-ml amberized volumetric flasks and diluted to the mark with methanol. A sufficient amount of each stock solution was then placed into amberized 10-ml volumetric



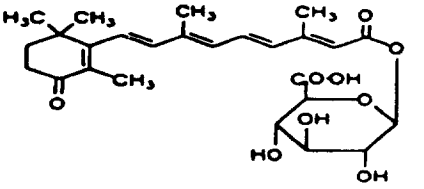
13-cis retinoyl-β-glucuronide



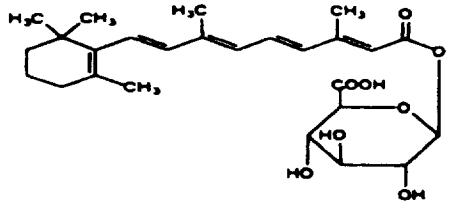
Acitretin-β-glucuronide (I.S.)



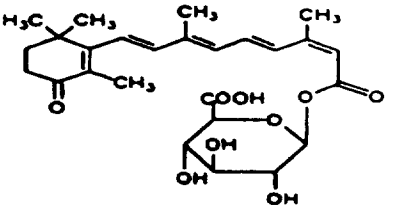
9-cis retinoyl-β-glucuronide



4-oxo-All-trans retinoyl-β-glucuronide



All-trans retinoyl-β-glucuronide



4-oxo-13-cis retinoyl-β-glucuronide

Fig. 1. Structures of the compounds.

flasks and diluted again with methanol to yield final concentrations of 0.5, 1.0, 2.5, 5.0, 12.5 and 25.0 μg/ml of the retinoyl-β-glucuronide standards and 40 μg/ml of the internal standard. These solutions served as the working solutions. All stock and working solutions were stored at −20°C under argon.

2.5. Preparation of samples

The calibration curve was prepared daily by mixing 100 μl of the retinoyl-β-glucuronide standard and 25 μl of the internal standard working solutions to 5 ml of control rat urine. This yielded final concentrations of 0.01, 0.02, 0.05, 0.10, 0.25 and 0.50 μg/ml of the five retinoyl-β-glucuronides and 0.20 μg/ml of the internal standard. For analysis, both experimental and quality control samples were fortified with 100 μl of methanol and the same volume of the internal standard as was used to prepare the calibration standards. Quality control (QC) samples were prepared at two different concentrations by fortifying 50 ml of control rat urine with an intermediate solution containing 25 μg/ml of the five retinoyl-β-glucuronides to yield final concentrations of 0.40 and 0.03 μg/ml. Samples (5 ml) of the quality control pools were then placed into 15-ml disposable conical plastic centrifuge tubes and stored at −70°C, immediately.

2.6. Sample extraction

Retinoyl-β-glucuronides were extracted from rat urine utilizing solid-phase extraction (SPE). A VAC ELUT SPS 24 vacuum manifold (Varian, Harbor

City, CA, USA) was employed to provide vacuum to the SPE columns. The SPE columns were preconditioned with 6 ml of methanol followed by 9 ml of water, applying vacuum after the addition of each solvent. The sample (5 ml) was then applied to the preconditioned SPE columns and pulled into the column with vacuum. The columns were then washed with 9 ml of water, 8 ml of 25% methanol in water and 5 ml of acetonitrile, successively, applying vacuum after each addition. The retinoyl-β-glucuronides and internal standard were eluted from the columns with 3.5 ml of methanol, applying the vacuum to dryness at this step only. The methanol fractions were then evaporated to dryness using a TurboVap evaporator set to 25°C (Zymark, Hopkinton, MA, USA) and the residues reconstituted into 120 μl of mobile phase A prior to reversed-phase HPLC analysis.

2.7. Instrumentation

The HPLC instrument consisted of: a Hewlett-Packard Model 1050 quaternary pump, a Hewlett-Packard Model 1050 multi-wavelength detector and a Hewlett-Packard Model 1050 autosampler equipped with the 100-vial tray extension, all from Hewlett-Packard Analytical Instruments (Valley Forge, PA, USA). A Model BF004 column heater (Applied Separations, Allentown, PA, USA) was employed to maintain constant column temperature. The 100-vial tray extension was cooled using a refrigerated re-circulator, Model 9105 (Fisher Scientific). Zorbax ODS C_{18} columns (5 μm, 25 cm× 4.6 mm) were purchased from MAC-MOD Analytical (Chadds Ford, PA, USA).

2.8. Chromatography

Reversed-phase HPLC utilizing step gradient elution was employed to resolve the five retinoyl-β-glucuronides and internal standard. The stationary phase used was mentioned in the previous section. Mobile phase A consisted of 1 *M* ammonium acetate–water–methanol in a ratio of 4:496:600 (v/v/v) whereas mobile phase B consisted of methanol–ethyl acetate in a ratio of 400:100 (v/v). Initial conditions were 100% mobile phase A which were held for 5 min. During the next 5 min the percentage of mobile phase B increased linearly to 20%. At 10 min the mobile phase composition was 80% A and 20% B. This composition was held for 10 min. Over the next 3 min, the percentage of mobile phase B increased linearly to 40%. During the next 7 min the percentage of mobile phase B increased linearly to 80%. This composition (20% A and 80% B) was held for 2 min before returning to initial conditions. The total run time, including re-equilibration, was 40 min. The column temperature was maintained at 45°C and the flow-rate was 1 ml/min. The samples were maintained at 8°C while in the autosampler. The retinoyl-β-glucuronides were detected by their UV absorbance at 363 nm. Sample volume injected onto the column was 50 μl. Typical retention times were 6.3, 7.0, 19.2, 20.5, 21.3 and 15.0 min for 4-oxo-all-*trans*-, 4-oxo-13-*cis*-, 13-*cis*-, 9-*cis*- and all-*trans*-β-glucuronides and acitretin-β-glucuronide, respectively. Fig. 2A is the elution profile of a standard solution containing all five retinoyl-β-glucuronides and the internal standard, acitretin-β-glucuronide. Fig. 2B is the chromatogram of an extract from drug-free rat urine which was fortified with internal standard only. No interfering peaks were observed in the regions of the chromatogram where the retinoyl-β-glucuronides elute.

2.9. Treatment of data

The signal from the UV detector was captured by a Nelson Chromatography 2600 System, version 5.2.0 (Cuperino, CA, USA) and a linear calibration curve was constructed using a weighted (1/y) least squares regression of concentration versus the peak-height ratio (analyte/internal standard) utilizing an RS/1 based program (DMLIMS+, PennCorp, PA, USA). This was used to calculate concentrations for the individual QC and experimental samples using their respective peak-height ratios.

The stability of analytes in control rat urine under different storage conditions and periods was evaluated by calculation of mean (geometric) differences with 90% confidence intervals. Descriptive statistics were used (arithmetic mean, %R.S.D.) to summarize other results.

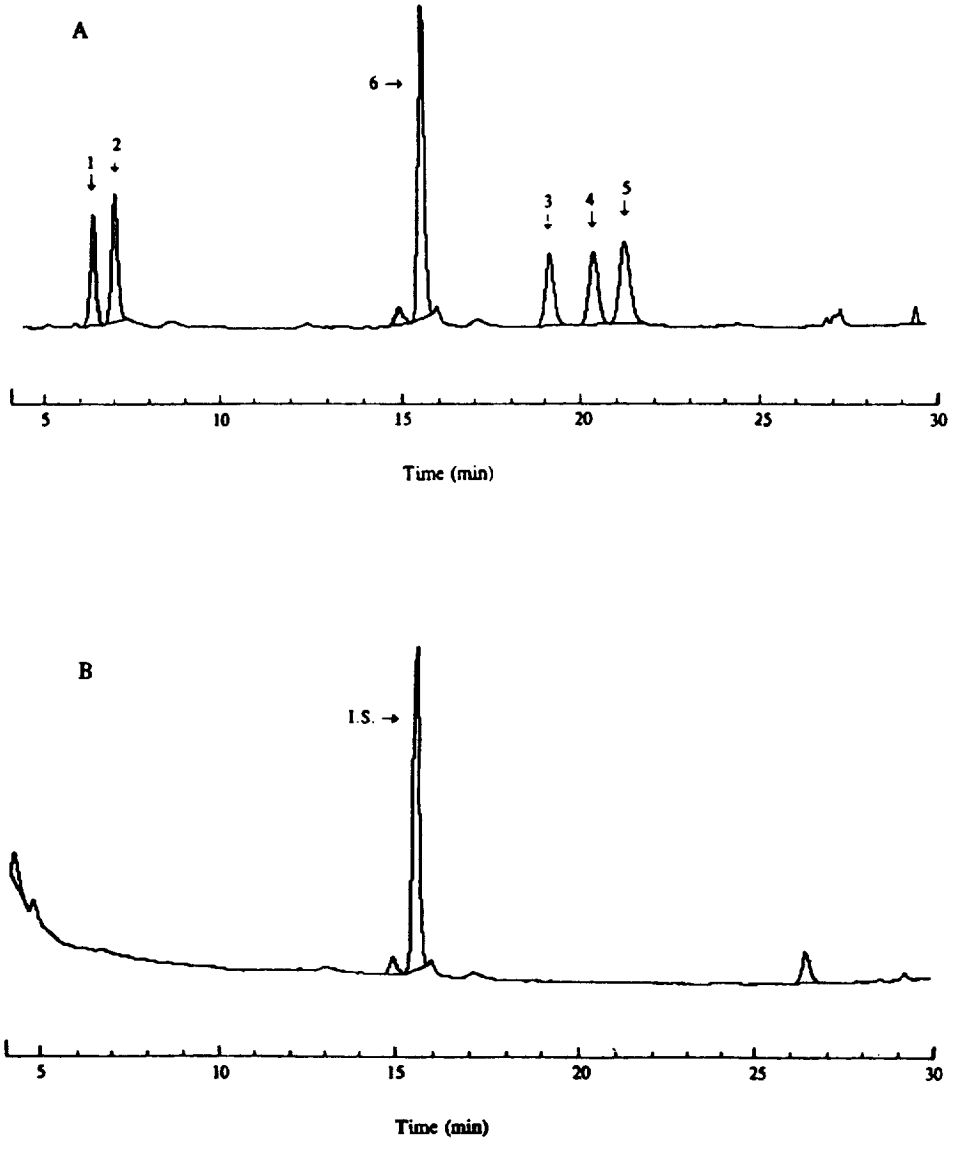


Fig. 2. (A) Chromatogram of a standard solution containing: 1=4-oxo-all-*trans*-, 2=4-oxo-13-*cis*-, 3=13-*cis*-, 4=9-*cis*-, 5=all-*trans*-retinoyl-β-glucuronides (104 ng each) and 6=internal standard (417 ng); (B) Chromatogram of drug-free rat urine fortified with 1000 ng of internal standard, acitretin-β-glucuronide.

3. Results and discussion

3.1. Precision and accuracy

The inter-assay precision for the retinoyl-β-glucuronides was estimated by calculating the mean analytical value obtained for each concentration of duplicate QC samples on a tray. This was done on several separate days ($n=3$), and the %R.S.D. for each QC concentration level was calculated. These calculations are summarized in Table 1. The intra-assay precision was assessed by calculating the mean value obtained from each QC concentration. The calculated %R.S.D. was based on one standard curve with $n=5$ for all QC concentrations. These calculations are presented in Table 1. The precision at the

Table 1
Inter-day and intra-day reproducibility expressed as %R.S.D.

Retinoyl-β-glucuronide	Inter-day R.S.D. (%) (n=3)		Intra-day R.S.D. (%) (n=5)	
	0.03 μg/ml[a]	0.40 μg/ml[a]	0.03 μg/ml[a]	0.40 μg/ml[a]
4-Oxo-all-*trans*-	11.60	1.24	9.75	3.34
4-Oxo-13-*cis*-	3.79	2.05	8.39	3.27
13-*cis*-	1.83	1.94	3.22	2.27
9-*cis*-	6.86	3.70	9.30	5.48
All-*trans*-	1.72	3.72	4.31	1.45

[a] QC concentrations.

limit of detection (0.01 μg/ml) was also assessed by calculation of the %R.S.D. for each analyte. The %R.S.D. values at the limit of detection for 4-oxo-all-*trans*-, 4-oxo-13-*cis*-, 13-*cis*-, 9-*cis*- and all-*trans*-retinoyl-β-glucuronides were 5.61, 0, 6.22, 5.61 and 6.22%, respectively.

The accuracy of the method was assessed by determining the percent error observed in the analysis of QC samples. The mean concentration for each QC was subtracted from the theoretical concentration over three calibration curves. The residue was divided by the theoretical value and converted to percent. These calculations are summarized in Table 2. The percent error at the limit of detection was also calculated in the same manner to assess accuracy. The percent errors at the limit of detection for 4-oxo-all-*trans*-, 4-oxo-13-*cis*-, 13-*cis*-, 9-*cis*- and all-*trans*-retinoyl-β-glucuronide were 3.30, 0, 6.70, 3.30 and 6.70%, respectively.

3.2. Sensitivity

The limit of detection of 4-oxo-all-*trans*-, 4-oxo-13-*cis*-, 13-*cis*-, 9-*cis*- and all-*trans*-retinoyl-β-glucuronides in rat urine is 0.01 μg/ml using a 5-ml sample. The calibration curve range for rat urine is 0.01–0.50 μg/ml. All calibration curves were linear over this range with the correlation coefficient ≥0.99.

Table 2
Estimation of assay accuracy

Retinoyl-β-glucuronide	Error[a] (%)	
	0.03 μg/ml[b]	0.40 μg/ml[b]
4-Oxo-all-*trans*-	3.33	6.25
4-Oxo-13-*cis*-	1.11	3.83
13-*cis*-	5.56	3.42
9-*cis*-	1.10	5.50
All-*trans*-	12.22	1.67

[a] Values presented represent the percent error from theoretical value.
[b] QC concentrations.

3.3. Recovery

The recovery of the retinoyl-β-glucuronides was determined by comparing the mean of the peak heights from six replicate control urine samples fortified to 0.20 μg/ml each, with the five retinoyl-β-glucuronides before extraction, to the mean of the peak heights of six replicates consisting of extracts from control urine which were fortified with analytes at the same concentration as urine. The percent recoveries for 4-oxo-all-*trans*-, 4-oxo-13-*cis*-, 13-*cis*-, 9-*cis*- and all-*trans*-retinoyl-β-glucuronides from rat urine are 97.6, 98.5, 98.6, 88.8 and 91.6%, respectively.

3.4. Stability

The stability of the retinoyl-β-glucuronides in rat urine at room temperature was evaluated by analyzing samples fortified with all analytes (0.20 μg/ml) immediately, and after standing 3 h. The storage of the retinoyl-β-glucuronides at 4°, −20° and −70°C for 30 days was investigated by fortifying control rat urine (0.20 μg/ml) with all analytes followed by analysis at the appropriate time interval. A retinoyl-β-glucuronide was considered stable in rat urine if the amount of degradation observed was less than or equal to 20%. The results are summarized in Table 3. The data suggest that the retinoyl-β-glucuronides are unstable in rat urine at both room temperature and

Table 3
Stability of retinoyl-β-glucuronides in rat urine at room temperature and storage at 4°, −20°C and −70°C

Retinoyl-β-glucuronide	% Difference (from 0 h)									
	Ambient	4°C			−20°C			−70°C		
	3 h	3 days	13 days	30 days	3 days	13 days	30 days	3 days	13 days	30 days
4-Oxo-all-*trans*-	−59.82	−35.55	−78.99	−99.85	−6.36	16.68	17.61	−11.52	9.52	3.39
4-Oxo-13-*cis*-	−41.70	−34.09	−77.25	−99.87	−6.54	12.31	4.75	−11.09	7.18	−2.83
13-*cis*-	−34.43	−20.36	−35.43	−71.90	−10.70	4.66	−2.85	−10.10	−0.40	−3.95
9-*cis*-	−68.28	−39.64	−75.39	−88.37	−20.91	−3.97	−13.30	−21.30	−5.96	−12.68
All-*trans*-	−46.72	−29.70	−64.31	−85.34	−7.14	8.29	2.49	−10.83	−6.66	−9.61

storage at 4°C, but stable up to 30 days storage at either −20° or −70°C.

3.5. Determination of the retinoyl-β-glucuronides produced in rat urine after single and multiple dosing with all-trans-, 9-cis- and 13-cis-retinoic acids

The amount of the five retinoyl-β-glucuronides observed in the urine of rats multiply dosed with 13-*cis*-, 9-*cis*- and all-*trans*-retinoic acids is presented in Table 4. Each value represents an individual animal. For rats dosed with all-*trans*-retinoic acid, only 4-oxo-all-*trans*- and 4-oxo-13-*cis*-retinoyl-β-glucuronides were observed in the urine after single and multiple dosing. The data may also suggest that the amount of 4-oxo-13-*cis*-retinoyl-β-glucuronide observed in rat urine may slightly increase after multiple dosing.

For rats dosed with 9-*cis*-retinoic acid, no retinoyl-β-glucuronides were detectable in the urine after either single or multiple dosing with 9-*cis*-retinoic acid. After a single dose of 13-*cis*-retinoic acid, only 4-oxo-13-*cis*-retinoic acid was detected in the urine. However, after multiple dosing with 13-*cis*-retinoic acid, both 4-oxo-13-*cis*- and 13-*cis*-retinoyl-β-glucuronides were detectable in the urine with the amount of 4-oxo-13-*cis*-retinoyl-β-glucuronide slightly decreased with multiple dosing.

4. Conclusions

An accurate and specific method utilizing SPE to quantitate the intact glucuronide conjugates of 4-oxo-all-*trans*-, 4-oxo-13-*cis*-, 13-*cis*-, 9-*cis*- and all-*trans*-retinoic acids in rat urine was developed. This method was utilized to determine the amount of retinoyl-β-glucuronides produced in the urine of rats singly and multiply dosed with 13-*cis*-, 9-*cis*- and all-*trans*-retinoic acids. Overall, our data showed that the amount of retinoyl-β-glucuronide produced in rat urine after single and multiple dosing with these retiniods is insignificant compared to the dose. Therefore, urinary excretion of retinoyl-β-glucuronide conjugates does not appear to play an important

Table 4
Total amount of retinoyl-β-glucuronides in 24-h urine from rats singly and multiply dosed with 13-*cis*-, 9-*cis*- and all-*trans*-retinoic acids

Retinoyl-β-glucuronide	Total amount of retinoyl-β-glucuronides (μg)											
	Drug 13-*cis*-RA				Drug 9-*cis*-RA				Drug all-*trans*-RA			
	Day 1		Day 4		Day 1		Day 4		Day 1		Day 4	
4-Oxo-all-*trans*-	nm	nm	nm	nm	nm	nm	nm	nm	0.50	0.15	0.26	nm
4-Oxo-13-*cis*-	0.6	0.55	0.26	0.39	nm	nm	nm	nm	0.92	0.76	1.11	2.46
13-*cis*-	nm	nm	0.17	0.22	nm	nm	nm	nm	nm	nm	nm	nm
9-*cis*-	nm	nm	nm	nm	nm	nm	nm	nm	nm	nm	nm	nm
All-*trans*-	nm	nm	nm	nm	nm	nm	nm	nm	nm	nm	nm	nm

nm=nonmeasurable.
RA=retinoic acid.

role in the metabolism of 13-*cis*-, 9-*cis*- and all-*trans*-retinoic acids in the rat. Glucuronidation of all-*trans*-retinoic acid, solely, cannot account for the observed reduction in the AUC after multiple oral administration of all-*trans*-retinoic acid in the rat.

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