HPLC-MS/MS analysis of the products generated from all-trans-retinoic acid using recombinant human CYP26A

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Abstract Two mammalian hCYP26A expression systems have been used to analyze the metabolic products of CYP26A. Through the use of extensive HPLC, UV spectroscopy, and liquid chromatography/tandem mass spectrometry (LC-MS/MS) methodology, we have conclusively demonstrated that the complex mixture of products comprises 4-OH-all-trans-retinoic acid, 4-oxo-all-trans-retinoic acid, and 18-OH-all-trans-retinoic acid, and more polar products, partially identified as dihydroxy and mono-oxo, mono-hydroxy derivatives. These more polar products are presumed to result from multiple hydroxylations on the β-ionone ring. The inter-relationship of initial and polar metabolites was inferred from both gene-dose and time-course experiments. Both initial and secondary metabolic steps after 4-oxo-all-trans-retinoic acid are ketoconazole-sensitive, suggesting that steps in the production of water-soluble metabolites are cytochrome P450-dependent.—Chithalen, J. V., L. Luu, M. Petkovich, and G. Jones. HPLC-MS/MS analysis of the products generated from all-trans-retinoic acid using recombinant human CYP26A. J. Lipid Res. 2002. 43: 1133–1142.

Supplementary key words vitamin A metabolism • retinoids • cytochrome P450 • RAI-1

A more complete understanding of the mechanism of action of vitamin A in cell differentiation and gene expression has emerged since the discovery of the family of nuclear transcription factors known as the retinoic acid receptors (RARs) (1). Other levels of control, however, have been proposed for the regulation of the retinoid response. The enzymes involved in activating all-trans-retinol to all-trans-retinoic acid (atRA) and inactivating atRA to excretory products are important because they regulate the cellular concentration of the active ligand and therefore the biological response. The emergence of a family of inducible retinoic acid-metabolizing cytochrome P450 proteins (P450-RAIs or CYP26s) with high specificity for atRA across species simply reinforces the importance of maintaining a tight regulation of the levels of this highly potent ligand inside all cells (2–6). Over the past two decades, several catabolic steps have been suggested for reducing the biological activity of atRA, including: a) oxidation at the 4 position of the β-ionone ring (7–9); b) oxidation at C-18 (9, 10); c) 5,6-epoxidation (11–14); and d) glucuronidation (15–19).

The cloning from zebra fish, human, and mouse of the specific cytochrome CYP26A1 involved in retinoic acid metabolism (2–5, 12, 20) has allowed for a more detailed analysis of the exact catabolic properties of the overexpressed recombinant protein. Initial analysis of the catabolic products derived from atRA incubation with CYP26A has indicated that the main role of this enzyme is the 4-oxidation of atRA (3, 5, 21, 22), though there was one report suggesting that mCYP26A is involved in 5,6-epoxidation (12). All of these studies have generally relied upon the comparison of HPLC retention times of CYP26A metabolites to authentic reference standards, with no further rigorous physico-chemical techniques utilized. Furthermore, metabolite profiles from transient and stable CYP26A expression in a number of mammalian systems were quite complex and in need of more detailed analysis using state-of-the-art techniques.

Given the thermal instability of retinoids, the inherent complications of derivatization involved in gas chromatography-mass spectrometry (GC-MS) (23–25), and the lack of characteristic fragmentation using matrix-assisted laser desorption ionization- and laser desorption ionization-MS (26), we opted to characterize the products of CYP26A using the newly emerging technology of liquid chromatography-mass spectrometry (LC-MS) (27, 28). Recent improvements in this technology, particularly in the area of more efficient ionization techniques, have allowed for a

Abbreviations: atRA, all-trans-retinoic acid; ddH2O, deionized distilled water; ddH2O/A/GAA, ddH2O-acetonitrile-glacial acetic acid; DIA, daughter ion analysis; DPPD, 1,2-Dianilinoethane (N,N-diphenyl-ethylenediamine); H/I/M/GAA, hexane-isopropanol-methanol-glacial acetic acid; MS, mass spectrometry; TIC, total ion current.

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Manuscript received 2 October 2001 and in revised form 22 March 2002.
DOI 10.1194/jlr.M100343-JLR200

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greater range of compounds to be analyzed by direct on-line LC-MS, obviating the need for derivatization (29, 30). This paper describes the establishment of LC-MS conditions for all-trans-retinoic acid and its metabolites and the application of LC-MS procedures to the characterization of some of the products of all-trans-retinoic acid metabolism after stable expression of recombinant hCYP26A in two different mammalian host cell systems. The results confirm the chemical nature of the major products as 4-hydroxy- and 4-oxo-derivatives and clarify the role of the enzyme in the appearance of more polar products.

MATERIALS AND METHODS

Construction and maintenance of transfected cell lines

The cDNA for hCYP26A was isolated from the pBluescript SK+ vector (Stratagene) containing the hCYP26A gene (3) by excision with NotI and XhoI, or EcoRI and Sall restriction endonucleases. The 2.1 kb fragment was purified from a 0.8% (w/v) agarose gel and ligated into the multiple cloning site in a sense orientation of both the mammalian expression vectors pCEBV at the NotI/XhoI site, and pCl-neo at the EcoRI/Sall site, respectively. HeLa cells were transfected with either pCEBV-hCYP26A or the parental pCEBV mammalian expression vector using a calcium phosphate transfection method (31), and transfectants were selected for with 800 μg/ml G-418 sulfate. Cells designated HeLa-CYP26A (containing the hCYP26A gene) and HeLa-P (containing parental plasmid alone) were maintained in DMEM supplemented with 5% FBS, 200 μg/ml G-418 sulfate, 1X antibiotic-antimycotic, and 4.5 g/l glucose.

Incubation with retinoids and extraction of metabolites

All retinoids were handled under yellow lighting. Metabolic incubations were carried out in 4 ml basal media with the addition of 100 μM 1.2-Dianilinoethane (N,N'-diphenylethylenediamine) (DPPD) and 1.25 μM substrate (atRA or 4-oxo-atRA) for the durations indicated. Incubations to test the effects of the cytochrome P450 inhibitor ketoconazole were carried out as above with the addition of 100 μM ketoconazole or carrier alone (2% 0.05M HCl). At the end of the allocated time, 1.5% (v/v) glacial acetic acid was added to incubations with acidic substrates and all incubations were terminated by the addition of 4 ml ethanol and 1 μg internal standard (CD437; generous gift of Dr U. Reichert, Galderma). The ethanolic solution was transferred to an extraction tube, the cells washed with a second 4 ml aliquot of ethanol, and the ethanolic solutions combined. The media was then extracted twice with 4 ml ethyl acetate (33), once with 4 ml hexane, diluted with 4 ml distilled water, and centrifuged at 1000 g for 15 min to separate the phases. The organic phase was dried under a stream of nitrogen, resuspended in 200 μl ethanol, dried again, and resuspended in 60:40 acetonitrile-ddH₂O for analysis by HPLC, resulting in a minimum 98% recovery of all synthetic standards.

Northern analysis

Total RNA was extracted using Trizol reagent from V79-4, V79-CYP26A, V79-NEO, HeLa-CYP26A, and HeLa-P cells at 80% confluency in the medium described previously (32) containing 1.25 μM atRA or vehicle alone, size-fractionated on a 1% formaldehyde-agarose gel, blotted to a nylon membrane, and probed with [32P]dCTP by random priming (32). Hybridization was performed for 18 h at 65°C. The membrane was also probed for the related cytochrome hCYP26B and 18S rRNA expression using a similar procedure.

Fig. 1. Northern analysis of hCYP26A expression in V79-4 and HeLa stable transfectants. V79-4 and HeLa cells transfected with the cytochrome P450 hCYP26A (V79-CYP26A, HeLa-CYP26A) or the parental vector (V79-NEO, HeLa-P) were analyzed for: A: Expression of the gene product in their basal state (–) or after induction for 16 h with 1.25 μM all-trans-retinoic acid (atRA) (+). B: Blots were normalized using a probe for 18S rRNA.
Recovery was normalized by integration of the internal standard.

In some experiments [11,12-3H]atRA (New England Nuclear, specific activity = 49.3 Ci/mmol) was mixed with non-radioactive substrate to give a specific activity of 20 mCi/mmol. Metabolism to lipid-soluble and aqueous-soluble metabolites was measured by liquid scintillation counting using quench correction.

**HPLC analysis of retinoid metabolites**

Initial HPLC analysis was based upon a general method (7) using a Waters 2690 separations module, 996 photodiode array detector, and Zorbax-SB C18 column (150 × 4.6 mm, 3.5 μm). Separation of substrate and products was achieved using a gradient solvent system (34) consisting of ddH2O-acetonitrile-glacial acetic acid (ddH2O/A/GAA) at 1 ml/min in the proportions 74.9:25:0.1 to 0.9:99:0.1 over 35 min, with fractions (where indicated) collected using a Pharmacia Biotech SuperFrac fraction collector. Fractions were dried under a stream of nitrogen, resuspended in running solvent, and separated as above using a Zorbax-CN column (250 × 4.6 mm, 5 μm) and an isocratic solvent system consisting of 93.5:5:1:0.5 or 90.5:7:2:0.5 hexane-isopropanol-methanol-glacial acetic acid (H/I/M/GAA), where indicated. Fractions were collected as previously, and prepared for LC-MS/MS analysis. Analysis of the chromatograms was performed using Waters Millennium software. Metabolites of atRA were routinely monitored at a wavelength of 350 nm, while incubation of 4-oxo-atRA was analyzed at 360 nm. Peak area measurements were used to quantitate retinoid.

**Fig. 2.** Analysis of atRA metabolism in mammalian cells transfected with hCYP26A. A: V79-CYP26A (solid line) and V79-NEO (broken line) cells were incubated for 24 h with 1.25 μM atRA and analyzed by reverse-phase HPLC. B: HeLa-CYP26A (solid line) and HeLa-P (broken line) cells were incubated for 18 h with 1.25 μM atRA and analyzed by reverse-phase HPLC. Metabolites indicated are consistent with those listed in Table 2. Parental controls are displayed with a 1 min lag in retention time and a shift of 0.01 AU, for clarity. Standards ran as follows: atRA, 31.27 min; 4-OH-atRA, 17.90 min; 4-oxo-atRA, 19.59 min; internal standard CD437, 26.23 min.
TABLE 1. Time-course of the metabolism of all-trans-retinoic acid (1.25 μM)

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Incubation Time</th>
<th>Substrate Remaining</th>
<th>Lipid Soluble Metabolites</th>
<th>Aqueous Soluble Metabolites</th>
<th>Total Retinoids</th>
<th>Recovery^d</th>
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<tr>
<td></td>
<td></td>
<td>µmol</td>
<td>µmol</td>
<td>µmol</td>
<td>µmol</td>
<td>%</td>
</tr>
<tr>
<td>V79-NEO</td>
<td>4</td>
<td>4.75 ± 0.24</td>
<td>0</td>
<td>0.05 ± 0.08</td>
<td>4.80 ± 0.32</td>
<td>94 ± 7</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>4.78 ± 0.16</td>
<td>0</td>
<td>0.05 ± 0.09</td>
<td>4.83 ± 0.25</td>
<td>95 ± 5</td>
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<td>V79-CYP26A</td>
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<td>2.20 ± 0.19</td>
<td>2.39 ± 0.19</td>
<td>0.12 ± 0.04</td>
<td>4.71 ± 0.42</td>
<td>92 ± 9</td>
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<td>24</td>
<td>4.03 ± 0.06</td>
<td>3.74 ± 0.18</td>
<td>0.24 ± 0.09</td>
<td>4.41 ± 0.33</td>
<td>86 ± 7</td>
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<td>HeLa-P</td>
<td>4</td>
<td>4.99 ± 0.09</td>
<td>0</td>
<td>0.01 ± 0.08</td>
<td>4.99 ± 1.07</td>
<td>98 ± 21</td>
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<tr>
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<td>24</td>
<td>4.76 ± 0.81</td>
<td>0</td>
<td>0.02 ± 0.06</td>
<td>4.78 ± 0.87</td>
<td>94 ± 18</td>
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<tr>
<td>HeLa-CYP26A</td>
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<td>0.51 ± 0.03</td>
<td>1.54 ± 0.22</td>
<td>0.78 ± 0.15</td>
<td>2.83 ± 0.40</td>
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<tr>
<td></td>
<td>24</td>
<td>0.03 ± 0.02</td>
<td>0.40 ± 0.04</td>
<td>1.74 ± 0.13</td>
<td>2.17 ± 0.19</td>
<td>43 ± 9</td>
</tr>
</tbody>
</table>

^a Assessed in a 4 ml culture volume.
^b Amounts represent means of triplicate determinations ± SD, after normalization using recovery of the internal standard.
^c Measured using liquid scintillation counting of aliquots of the aqueous-soluble fraction from extraction.
^d Percent recovery is based upon 5.10^4 pmol, the amount added to the no-cell control and is arbitrarily set at 100%.

amounts using a standard curve based upon all-trans-retinoic acid and normalized using the internal standard.

HPLC-MS/MS analysis of metabolites

Standards and samples from initial fractionation were dried under a stream of nitrogen and resuspended in 60:40 acetoni-trole-ddH2O for introduction into the HPLC interface of the mass spectrometer. HPLC was performed using a Waters 2690 separation modules, a Zorbax-SB C18 column (150 × 2.1 mm, 3.5 μm), and a ddH2O-acetonitrile-glacial acetic acid gradient at 200 μL/min as follows; 74.99:25:0.01 to 0.99:99:0.01 over 35 min, with these conditions held for 5 min before returning to the initial conditions over 5 min, and equilibrating for 5 min. In-line MS/MS analysis was performed using a Micromass Quattro Ultima mass spectrometer with a Z-Spray electrospray interface in the negative mode, specifically with a capillary voltage of 3.10 kV and cone voltage of 3.10 mV for introduction into the HPLC interface of the mass spectrometer. HPLC was performed using a Waters 2690 separations module, a Zorbax-SB C18 column, and a Waters 2996 radioflow monitor (EG&G Berthold) following HPLC. Radioactivity profiles of the substrate and its lipid-soluble

RESULT

Cytochrome P450, hCYP26A mRNA expression

To address the effects of differential expression of hCYP26A, HeLa human cervical carcinoma cells and V79-4 Chinese hamster lung cells were transfected with either vector containing the gene encoding hCYP26A or the parental vector pCEBV and pCI-neo, respectively. Transfections were maintained by the presence of either hygromycin B (pCEBV), or G-418 sulfate (pCI-neo), creating the novel cell lines HeLa-CYP26A, HeLa-P, V79-CYP26A, and V79-NEO. The four cell lines, as well as the parental V79-4 cell line, were examined by Northern analysis to determine the degree of CYP26A mRNA expression in the basal state or after induction for 16 h with 1.25 μM atRA (Fig. 1A). No CYP26A mRNA was detectable in the V79-4 parental cell line, or in either of the cell lines transfected with the parental vector (V79-NEO or HeLa-P), under basal conditions or after induction with atRA. CYP26A mRNA was expressed to a much greater extent in the HeLa-CYP26A as compared with the V79-CYP26A cell type, and was not induced by atRA in either cell line. Expression of 18S rRNA was equivalent in all samples analyzed (Fig. 1B), allowing direct comparison between the expressions of CYP26A RNA across the cell lines. No expression of cytochrome P450 CYP26B (also known as P450RAI-2) mRNA was detectable after exposure for 10 days in any of the cell lines (data not shown).

Metabolism of atRA

Analysis of atRA metabolism in V79-CYP26A cells showed that the major metabolic products ran in the region of the chromatogram comigrating with the synthetic standards for 4-OH-atRA and 4-oxo-atRA (RT = 16–22 min) while other peaks accumulated to a much lesser extent in the 5–15 min region of the chromatogram (Fig. 2A). In time-course experiments, remaining substrate declined with time while lipid-soluble products correspondingly increased, whereas water-soluble products accumulating in the aqueous phase of the extraction began to plateau after the initial rise (Table 1). No traces of any of these metabolites were observed in incubations with V79-NEO cells (Fig. 2A, Table 1).

Incubation of 1.25 μM atRA with the HeLa-CYP26A cell type resulted in a slightly different metabolic profile from that seen with the V79-CYP26A cell type, with more metabolites spread across the 5–15 min region of the chromatogram (Fig. 2B). Time course analysis indicated only transient presence of products in the region of the chromatogram (RT = 16–22 min) comigrating with the 4-OH-atRA and 4-oxo-atRA synthetic standards. Substrate and lipid-soluble product peaks had decreased to minimal amounts by 24 h of incubation while each time point showed a significant increase in material segregating to the aqueous phase of the extraction (Table 1). No metabolism of atRA was evident using the same conditions with the HeLa-P cell line (Fig. 2B, Table 1).

Using both cell lines, and at all time points studied, results with UV detection were substantiated using a LB509 radioflow monitor (EG&G Berthold) following HPLC. Radioactivity profiles of the substrate and its lipid-soluble
products were qualitatively and quantitatively similar to the chromatograms shown in Fig. 2 (data not shown).

**Inhibition of metabolism with ketoconazole in HeLa-CYP26A cells**

Incubation medium containing 1.25 μM atRA with 100 μM ketoconazole or vehicle alone (0.05% HCl) was removed from a HeLa-CYP26A monolayer and extracted. The cells were then washed with PBS, and fresh medium containing 1.25 μM atRA was added to the monolayer and treated as a new incubation. No metabolites were detected in the extracted medium containing ketoconazole, although a wide range of metabolites were detected both in the medium with substrate alone (Fig. 3A), and after the ketoconazole washout (data not shown). With 4-oxo-atRA as substrate in HeLa-CYP26A cells, both the substrate consumption and product formation were significantly decreased in the presence of ketoconazole (Fig. 3B). The
presence of ketoconazole was seen to inhibit both the consumption of substrate (92% inhibition) and formation of metabolic products (none detected) with each of the substrates analyzed. Cells treated with medium containing ketoconazole for the duration of the initial analysis and then changed to basal medium with no ketoconazole were able to metabolize atRA, albeit to a smaller extent than untreated cells (data not shown).

Identification of metabolites by LC-MS/MS

Synthetic standards of atRA, 4-OH-atRA, 4-oxo-atRA, and 18-OH-atRA were used to establish the most sensitive and specific conditions to use for LC-MS/MS analysis. The results shown in Table 2 and Fig. 4A–D illustrate typical data for LC retention, diode-array spectrophotometry, and full scan mass spectrum (LC-MS/MS) of all-trans-retinoic acid and its three major metabolites. Under the electrospray negative conditions employed atRA, 4-OH-atRA, 4-oxo-atRA, and 18-OH-atRA each gave a prominent [M-\text{H}]^{-} ion (one mass unit lower than their molecular mass) at m/z 299, 315, 313, or 315 respectively, which was selected in MS1 and further used to determine characteristic fragmentation patterns summarized in Table 2. Full mass spectra showed ions that can be attributed to dehydration and decarboxylation products consistent with mass spectra obtained by GC/MS using derivatized retinoids (24, 35). Though most of the LC-MS/MS fragmentation patterns were not sufficiently detailed to differentiate
retinoid isomers, the combination of the three parameters (retention time, UV spectrum, and molecular ion) allowed for the identification of most of the biologically produced metabolites under experimental conditions.

Extracts of V79-CYP26A and HeLa-CYP26A cells incubated with 1.25 μM atRA were analyzed by LC-MS/MS in daughter ion analysis mode following fractionation over two different LC methods (Table 2). The expected [M-H]-ions used for daughter ion analysis were determined from ES+ full scan LC-MS of metabolite fractions and standards at m/z 313, 315 as well as [M-H]-ions detected in MS1, which might be expected for further hydroxylated metabolites such as m/z 329 and 331 (Table 2). The UV350 trace of the V79-CYP26A extract revealed two major product regions by straight-phase LC, which separated to four unique products, each with a characteristic retinoid spectrum after analysis by straight-phase LC. Three of these products comigrated and showed similar fragmentation by LC-MS/MS as the synthetic standards for 4-OH-atRA, 4-oxo-atRA, and 18-OH-atRA (Table 2, Fig. 4), while the fourth was determined as novel (Fig. 5A).

The UV350 trace of the HeLa-CYP26A extract shown in Fig. 2 revealed a much more complex pattern with very polar peaks in the 10–18 min region of the reverse-phase LC system. The more polar metabolites present in the HeLa-CYP26A extract were purified using straight-phase LC and analyzed by DIA (Table 2). The determined retention times, mass ions, and fragmentation patterns were mainly indicative of mono-oxo, mono-hydroxy-atRA, and dihydroxy-atRA metabolites, although no synthetic standards of these were available. Mass spectra for metabolite 4 from incubation in V79-CYP26A cells, as well as metabolites 8 and 9a from incubation in HeLa-CYP26A cells, are shown in Fig. 5.

### DISCUSSION

In this paper we describe the stable expression of hCYP26A (P450RAI-1) in different expression systems, allowing for the characterization of its enzymatic properties in atRA metabolism. Our results indicate that CYP26A is not only capable of 4-hydroxylation of atRA but is also capable of multiple hydroxylations of the β-ionone ring at C-4, C-18, and possibly C-3 as well. Characterization of retinoid metabolites is based upon three different criteria: LC retention times, UV spectra, and mass spectra. As earlier attempts to identify the products of CYP26A have lacked physico-chemical approaches, the application of LC-MS and tandem MS conditions to detect and characterize these highly labile retinoids has provided an additional level of confidence to the identification work. While many of the hydroxylations we ascribe to CYP26A have been previously reported in various biological systems (7–14), this is the first demonstration that all such metabolites can be produced by CYP26A alone. Furthermore, the gene dose and time course experiments indicate that atRA can be repeatedly hydroxylated to produce a series of increasingly water-soluble products, thereby giving credence to the theory that CYP26A is a catabolic system for protecting the cell from further stimulation by a potentially biologically active signaling molecule.

### TABLE 2. Chromatographic and LC-MS/MS properties of atRA metabolites from V79-CYP26A and HeLa-CYP26A cells

<table>
<thead>
<tr>
<th>Cell Type/ Metabolite</th>
<th>Z-CN</th>
<th>UV max</th>
<th>Z-CN</th>
<th>UV max</th>
<th>LCMS</th>
<th>UV max</th>
<th>[M-H]-</th>
<th>Major Fragments</th>
<th>Putative Identity</th>
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### Notes

- Retention time with gradient system of 74.99:25:0.1 neighborhood 35 minutes.
- Retention time with solvent composition 90.5:7:2:0.5 H/I/M/GAA.
- Retention time with solvent composition 93.5:5:1:0.5 H/I/M/GAA.
- Retention time with gradient system of 74.9:25:0:1 neighborhood 35 minutes.
- UV max determined using corresponding solvent.
- nd, not determined.

Chithalen et al. HPLC-MS/MS of hCYP26A products 1139
To obtain a detailed analysis of CYP26A catabolic function, we stably expressed hCYP26A1 cDNA in two cell culture systems, V79-4 and HeLa. Northern analysis indicated that both of the systems expressed cytochrome hCYP26A, albeit to different extents. We could not detect hCYP26B expression (Fig. 1), indicating that any atRA metabolism over the parental cell type was due solely to the presence of CYP26A. The distinct, fixed degree of hCYP26A expression in the HeLa-CYP26A and V79-CYP26A cells allowed us to observe the effect of CYP26A

Fig. 5. Daughter ion fragmentation analysis of unknown atRA metabolites. A: V79-CYP26A metabolite 4 eluting at 17.89 min on reverse-phase HPLC. B: HeLa-CYP26A metabolite 8 eluting at 13.07 min on reverse-phase HPLC. C: HeLa-CYP26A metabolite 9a eluting at 12.55 min on reverse-phase HPLC. Parent ions were selected from intense peaks observed from ES− MS1 analysis. Metabolite numbering is consistent with Table 2. Inset structures are of known synthetic standards with identical mass ions.
on atRA metabolism in a stable and reproducible manner at two different degrees of expression. Analyzing different levels of CYP26A expression allows for insight into different cellular physiological events due to the presence of a retinoic acid response element (RARE) in the gene promoter, and thus inducible and variable expression of this enzyme in many cell lines (e.g., MCF-7) (6).

Most natural retinoids are sensitive to heat, light, and oxidants, making their analysis difficult. Our successful development of LC-MS and LC-MS/MS conditions has allowed a rigorous physico-chemical analysis of CYP26A products and indeed all natural retinoids without risk of destroying the analyte. Popular techniques in small molecule analysis that employ excessive heat, such as GC-MS, can be used for retinoids but require extreme care (7, 23, 24). The emergence of efficient LC-MS interfaces has made possible direct introduction of even labile molecules such as natural retinoids into the MS. The exact MS conditions worked out here for retinoids provide sensitivity into the picogram range. Though current LC-MS techniques do not distinguish between different retinoid isomers, when used in conjunction with the other criteria of modern LC analysis (accurate retention times and full UV spectra), the addition of mass spectral data provides fairly conclusive characterization for most natural retinoids.

Despite remaining uncertainties about the nature of some of the mono- or dihydroxy-metabolites that were formed from CYP26A, we learned a lot in this work about the role of this cytochrome P450. It is clear that the site of hydroxylation by CYP26A is not confined to C-4 of the β-ionone ring or that it performs only one hydroxylation per retinoid molecule. This lack of regio-specificity is a hallmark of cytochromes P450. The evidence suggests that CYP26A repeatedly hydroxylates the β-ionone ring at C-4, C-18, and possibly C-3 (36) to make multi-hydroxylated products with much increased aqueous solubility. In this sense CYP26A resembles CYP24, the vitamin D-inducible cytochrome P450 thought to be responsible for the 5-step catabolism of calcitriol, the active form of vitamin D (37). Indeed, both CYP26A and CYP24 are highly efficient at catabolism of calcitriol, the active form of vitamin D (37). cDNA cloning of human retinoic acid-metabolizing enzyme (hP450RAI) identifies a novel family of cytochromes P450 (CYP26). J. Biol. Chem. 272: 18538–18541.

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**23rd Annual Meeting of the American Society of Bone and Mineral Research, Phoenix AZ to be held October 12–16, 2001.** (abstract) 


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