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Liquid chromatography method for quantifying *N*-(4-hydroxyphenyl) retinamide and *N*-(4-methoxyphenyl)retinamide in tissues

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Abstract

A simple and accurate high-performance liquid chromatography (HPLC) method was developed to measure levels of *N*-(4-hydroxyphenyl) retinamide (fenretinide, 4-HPR) and its main metabolite *N*-(4-methoxyphenyl)retinamide (4-MPR) in tissue. Following ultrasonic extraction of fresh tissue in acetonitrile (ACN), 4-HPR and 4-MPR were measured by HPLC with UV absorbance detection at 340 nm, using isocratic elution with ACN, H_2O, and acetic acid. *N*-(4-ethoxyphenyl)retinamide (4-EPR) was employed as an internal standard. The 4-HPR and 4-MPR recovery in bovine liver or bovine brain tissue samples spiked with known amounts of 4-HPR and 4-MPR ranged from 93 to 110%. The detection limit of the method was 50 ng/ml. The method was tested on actual samples from an athymic (nu/nu) mouse carrying a subcutaneous tumor xenograft originating from SMS-KCNR neuroblastoma cells. The tissues were harvested and analyzed following a 3 day long treatment with intraperitoneal injections of 4-HPR/Diluent-12. 4-HPR and the metabolite 4-MPR were detected and quantitated in the tested tissues including tumor, liver, and brain. This method can be used to quantify 4-HPR and 4-MPR in different tissues to determine the bioavailability of 4-HPR.

Keywords: *N*-(4-hydroxyphenyl)retinamide; *N*-(4-methoxyphenyl)retinamide

1. Introduction

N-(4-hydroxyphenyl)retinamide or fenretinide (4-HPR) is a synthetic amide of all-*trans*-retinoic acid first produced in the late 1960s (Fig. 1). 4-HPR has been reported to inhibit the growth of neuroblastoma, colorectal, prostate, breast, ovarian, and small-cell lung cancer, and both lymphoid and myeloid leukemia cell lines in vitro at 1–10 μM concentrations [16,26]. 4-HPR has shown activity in vitro against cell lines resistant to all-*trans*-retinoic acid and 13-*cis*-retinoic acid [6,15,27,30,35]. 4-HPR has inhibited carcinoma formation in rat mammary glands, hamster lung, rat and mouse prostate, mouse bladder and liver [16], leading to 4-HPR being studied as a chemopreventive agent [8]. Toxicity of 4-HPR in chemoprevention clinical trials has been minimal, and no hematological toxicity has been reported, with the major clinical toxicity of fenretinide being decreased night vision, due to decreased plasma retinol levels [8,26]. Recently completed phase I studies have established that tolerated doses for future therapeutic studies are higher than those used in chemoprevention trials [26].

The mechanism of 4-HPR anti-tumor toxicity remains to be fully understood. One reported mechanism of 4-HPR cytotoxicity involves generation of reactive oxygen species (ROS) [7,24]. Another mechanism, which may account for the ability of 4-HPR to cause tumor cell cytotoxicity by both apoptosis and necrosis, is the ability of 4-HPR to induce increases of ceramide via de novo synthesis in transformed cells [17,18,23]. The latter observation has led to pre-clinical studies employing ceramide modulators in combination

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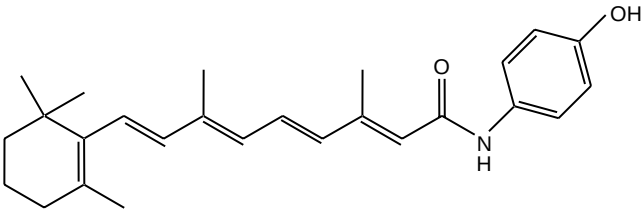

N-(4-hydroxyphenyl)retinamide (4-HPR)

Fig. 1. Structure of *N*-(4-hydroxyphenyl)retinamide = fenretinide = 4-HPR.

with 4-HPR. These in vitro studies suggest that high 4-HPR levels will need to be achieved in tumor tissue for an optimal anti-tumor effect [17]. Thus, methods to accurately determine 4-HPR levels in tissues are essential for pre-clinical development of 4-HPR. Several methods employing HPLC to measure levels of retinoids in tissues have been previously reported [1–4,8–11,13,14,19–22,28,31,34,36–39]. However, when measuring multiple samples at a time, most of these methods are too elaborate or time-consuming. We previously adapted the method of Formelli et al. [8] to the high throughput analysis of both 4-HPR and its metabolite *N*-(4-hydroxyphenyl)retinamide (4-MPR) in plasma samples. We have now extended this work to an easy and reproducible high-performance liquid chromatography (HPLC) method for separating and quantifying 4-HPR and its metabolites in various tissues.

2. Experimental

2.1. Chemicals

4-HPR, 4-MPR, and the internal standard *N*-(4-ethoxyphenyl)retinamide (4-EPR) were supplied by the National Cancer Institute. All chemicals were HPLC grade and were purchased from Sigma–Aldrich Co (St. Louis, MO, USA). Bovine tissues were purchased at a local market and stored at −80 °C.

2.2. HPLC conditions

The samples were analyzed with a high-performance liquid chromatograph, consisting of a Waters 2690 Separation Module with sample heater/cooler and column heater, Waters 515 HPLC pump, and Waters 717 plus autosampler. The Waters 2487 UV absorbance detector was set at 340 nm. A Symmetry C_{18} column (3.5 μm, 150 mm × 4.6 mm) with a Symmetry C_{18} guard column (5 μm, 3.9 mm × 20 mm) was used. Isocratic elution with acetonitrile:water:glacial acetic acid (80:18:2, v/v/v) was employed at a flow rate of 0.7 ml/min. Volume of injection was 20 μl. All samples were analyzed at room temperature.

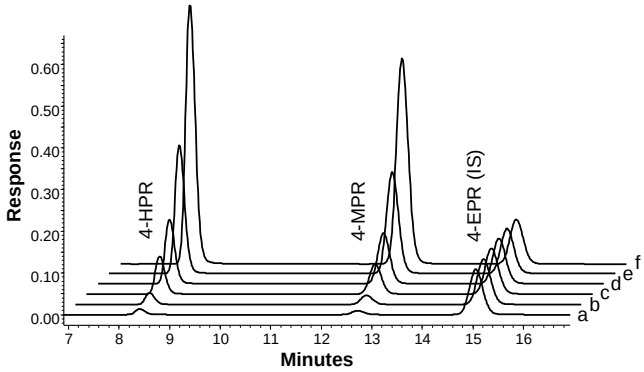

Fig. 2. Chromatogram of calibration curve standards in acetonitrile (ACN) at concentrations 0.5 μg/ml (a); 1 μg/ml (b); 3 μg/ml (c); 5 μg/ml (d); 10 μg/ml (e) and 20 μg/ml (f), respectively. First peak represents the 4-HPR, second peak the 4-MPR, third peak the internal standard (IS) 4-EPR.

2.3. Standard solutions

4-HPR, 4-MPR, and 4-EPR stock standards were prepared at a concentration of 500 μg/ml in acetonitrile:methanol (ACN:MeOH, 1:1, v/v) with an ultrasonic bath employed to aid in the dissolution of the drugs. Working solutions (50 and 25 μg/ml) of the 4-HPR and 4-MPR standards were prepared for low concentration measurements prior to each analysis. The stock standards were mixed fresh every 3 weeks and stored in a −80 °C freezer. Silanized amber glass vials and silanized amber polypropylene microcentrifuge tubes were used to store all standards and unknown samples throughout the procedure.

Calibration curves ranged from 0.5 to 20 μg/ml of 4-HPR and 4-MPR. Six concentration levels were prepared for each analyte (Fig. 2). 4-EPR was employed as an internal standard. Calibration standards were prepared by adding known volumes of the 4-HPR, 4-MPR, and 4-EPR standard solutions into ACN to achieve specified concentrations. The mixture was vortexed briefly and injected directly into the HPLC system.

2.4. Extraction procedure

Bovine tissue samples were minced with two scalpels to a slurry and approximately 200 mg of tissue homogenate was transferred into a silanized amber microtube and weighed. Sample weight was determined on a fresh basis. To estimate analyte recovery, known volumes of both 4-HPR and 4-MPR standards were added to each tissue sample. After adding 6 μl of the internal standard 4-EPR (final concentration 3 μg/ml) and 1.0 ml of ice cold acetonitrile (ACN), the samples were vortexed for a few seconds and placed in an ultrasonic bath for 10 min. To protect against any possible loss of 4-HPR due to heating, the ultrasonic bath was cooled periodically with crushed-ice.

Following sonication, the samples were centrifuged at 1100 × *g* for 10 min. The supernatant was transferred into

amber glass vials and kept in the dark at room temperature before injection into the HPLC system.

2.5. Tissue samples

To test the method on actual samples, an athymic (nu/nu) mouse carrying a subcutaneous tumor xenograft originating from SMS-KCNR neuroblastoma cells [25] was treated with intraperitoneal injections of 4-HPR/Diluent-12 at a dose of 180 mg/kg per day divided into two daily doses. The animal was treated for a period of 3 days and sacrificed 3 h after the last injection. Tumor samples 1–3 were taken from three different parts of the identical tumor, and liver, brain, and bladder were also harvested for 4-HPR and 4-MPR measurement. All tissue samples were analyzed immediately following the sacrifice. For brain and bladder analysis the complete organ was used. From the liver and tumor tissue a portion of approximately 100 mg per sample was collected for the analysis (Table 2, Fig. 4).

2.6. Statistics

Data analysis was performed using the Waters Millennium 32® Chromatography Manager software and the method of internal standard calibration [29].

Data variability was expressed as relative standard deviation (R.S.D., also called coefficient of variation, [5]), computed from the formula: R.S.D. = 100* (standard deviation/|mean|).

Difference of means was analyzed by two-sided Student's *t*-test using Microsoft Excel© and the level of significance was expressed as a *P*-value.

3. Results

Peak identification was performed via comparison of retention times of standards and spiked samples. All analytes were clearly separated (Figs. 2–4) and eluted with a retention time of approximately 8.2 min for 4-HPR, 12.4 min for its metabolite 4-MPR, and 14.6 min for the internal standard 4-EPR (Fig. 3). Relative standard deviation (R.S.D.) of internal standard levels ranged from 1.85% through 2.55% during the various measurements, indicating the reproducibility of the method.

Specificity, linearity, precision, accuracy, and recovery of this HPLC method for 4-HPR and 4-MPR detection in tissue were evaluated. The specificity of the assay was demonstrated as the analyte peak had a chromatographic baseline resolution of at least 1.5 from all other sample components (Figs. 2–4). The assay had a linear range of 0–20 μg/ml for both 4-HPR and 4-MPR (data not shown).

To determine the intra- and inter-assay precision and accuracy of the assay we measured 4-HPR and 4-MPR recovery in bovine liver tissue samples spiked with 4-HPR and 4-MPR at four different concentration levels (0.5, 1, 5 and 20 μg/ml, respectively) on successive days. Results including the average, the standard deviation (S.D.), and relative standard deviation (R.S.D.) values are presented in Table 1 The recovery on each concentration level represented an average of six individual tissue sample analyses. On the first day the recovery ranged from 93 to 110%; the R.S.D. ranged from 0.79 to 10.93% for both 4-HPR and 4-MPR (see Table 1 for detailed values on each of the presented levels). On the second day the recovery ranged from 99 to 106%; the R.S.D. reached from 0.78 to 7.90% between both analytes.

The inter-assay precision data were calculated and expressed as average, S.D., and R.S.D. values of measurements performed on two different days. The relative standard deviation values for both analytes ranged from 0.80 to 9.63% (Table 1).

The detection limit of the presented method was determined as 50 ng/ml (data not shown).

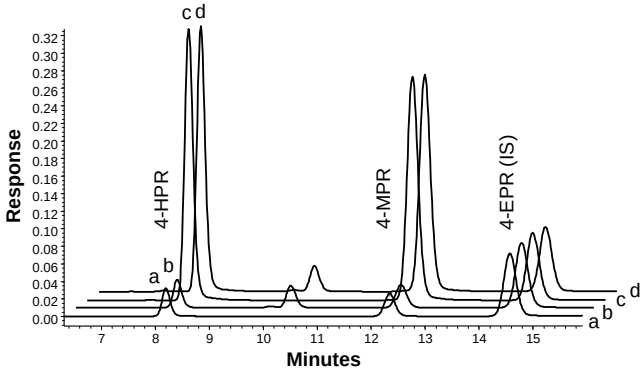

Fig. 3. Chromatogram of bovine brain (a, c) and bovine liver (b, d) samples spiked with 4-HPR and 4-MPR: (a) bovine brain; (b) bovine liver; both spiked with 4-HPR and 4-MPR at concentration level 1 μg/ml; (c) bovine brain; (d) bovine liver; both spiked with 4-HPR and 4-MPR at concentration level 10 μg/ml. First peak represents the 4-HPR, second peak represents retinol (detectable in bovine liver, not in bovine brain samples), third peak represents the 4-MPR, fourth peak the internal standard (IS) 4-EPR.

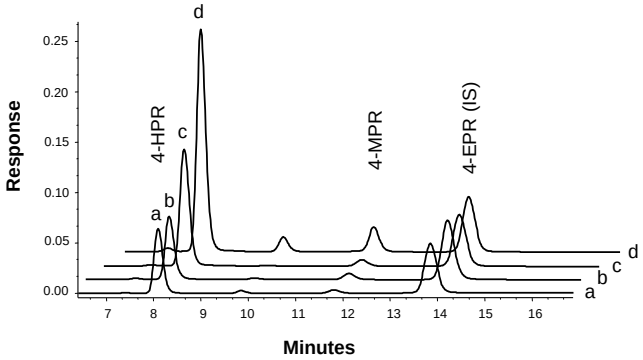

Fig. 4. Chromatogram of mouse tissue samples. Athymic mouse with a subcutaneous tumor xenograft treated with 4-HPR/Diluent-12 intraperitoneal injections: (a) mouse brain; (b) mouse tumor; (c) mouse bladder; (d) mouse liver. First peak represents the 4-HPR, second peak represents retinol (not labeled), third peak represents the 4-MPR and fourth peak the internal standard (IS) 4-EPR.

Table 1
Intra- and inter-assay precision and accuracy based on 4-HPR and 4-MPR recovery and final concentrations in bovine liver tissue samples spiked with 4-HPR and 4-MPR at four different concentration levels (0.5, 1, 5 and 20 μg/ml, respectively)

Concentration level (μg/ml)	First day				Second day			
	Concentration (μg/ml)		Recovery (%)		Concentration (μg/ml)		Recovery (%)	
	4-HPR	4-MPR	4-HPR	4-MPR	4-HPR	4-MPR	4-HPR	4-MPR
Intra-assay data								
0.5 (μg/ml)								
Mean	0.55	0.53	110.10	106.10	0.52	0.53	104.13	106.20
S.D.	0.06	0.04	12.03	7.84	0.04	0.02	8.23	4.87
R.S.D. (%)	10.93	7.39	10.93	7.39	7.90	4.58	7.90	4.58
1 (μg/ml)								
Mean	0.93	0.93	92.70	92.97	1.00	1.02	99.93	102.49
S.D.	0.05	0.05	5.13	4.94	0.01	0.05	1.25	5.23
R.S.D. (%)	5.53	5.31	5.53	5.31	1.25	5.10	1.25	5.10
5 (μg/ml)								
Mean	4.97	5.00	99.30	100.00	5.00	5.01	99.95	100.16
S.D.	0.04	0.04	0.79	0.90	0.05	0.04	0.98	0.78
R.S.D. (%)	0.79	0.88	0.79	0.90	0.98	0.78	0.98	0.78
20 (μg/ml)								
Mean	19.12	19.57	95.60	97.85	19.72	19.76	98.62	98.82
S.D.	0.70	0.73	3.51	3.64	0.40	0.67	1.99	3.34
R.S.D. (%)	3.69	3.72	3.67	3.72	2.02	3.38	2.02	3.38
Inter-assay data								
4-HPR concentration level (μg/ml)	0.5	1	5	20				
Mean (μg/ml)	0.54	1.03	4.96	19.13				
S.D.	0.05	0.05	0.05	0.63				
R.S.D. (%)	9.63	5.02	0.91	3.29				
4-MPR concentration level (μg/ml)	0.5	1	5	20				
Mean (μg/ml)	0.53	0.98	5.00	19.67				
S.D.	0.03	0.07	0.04	0.67				
R.S.D. (%)	5.86	7.11	0.80	3.43				

For intra-assay evaluation the average, S.D., and R.S.D. were calculated from six separate measurements on each presented day. Inter-assay data were calculated and expressed as average, S.D., and R.S.D. values of measurements performed on two different days. Concentration of internal standard 4-EPR was 3 μg/ml in each of the spiked samples. S.D.: standard deviation, R.S.D.: relative standard deviation.

In the present method, analyses were performed at an absorbance of 340 nm. Additionally, analysis was also conducted at 254 nm [12,32,33]. No marked difference between the two wavelengths was observed when comparing the relative standard deviations and recoveries of bovine liver samples spiked with 4-HPR and 4-MPR. With 4-HPR the R.S.D. reached 0.79% at 340 nm versus 1.11% at 254 nm. A similar trend was seen with 4-MPR, where the R.S.D. was 0.88% in samples measured at 340 nm versus 1.20% in samples measured at 254 nm. The recovery reached 97–01% in both experiments. However, when 4-HPR peak area values obtained were compared at the two wavelengths, the ratio was 6.2 times higher in favor of the analysis at 340 nm.

The standard calibration curves measured at 254 and 340 nm were nearly identical. At 254 nm, the correlation coefficient was 0.9991 for 4-HPR, and 0.9992 for 4-MPR; the internal standard R.S.D. was 2.14%. At 340 nm the correlation coefficient reached 0.9992 for 4-HPR, and 0.9991 for 4-MPR; the internal standard R.S.D. was 2.90%.

These observations supported routine analyte detection at an absorbance of 340 nm. In samples with high concentrations of 4-HPR and 4-MPR, analysis at 254 nm presents an alternative to sample dilution.

The effect of ultrasonication time on extraction efficiency was tested. No significant difference was observed in analyte recovery from six liver tissue samples spiked with 4-HPR ($P = 0.97$) and 4-MPR ($P = 0.52$) measured following a 10 min versus a 15 min long sonication. The recovery ranged from 93 to 103% in both experiments. Relative standard deviations after the 10 min ultrasonication reached 3.69% (4-HPR) and 3.72% (4-MPR). Following the 15 min sonication, the R.S.D. values were 2.68% (4-HPR) and 2.85% (4-MPR). Based on these results, a 10 min ultrasonication period was deemed adequate to obtain the desired analytes from a sample matrix. In contrast, a shorter 5 min ultrasonic extraction proved insufficient, as the tissue was not adequately resuspended in the extraction solution (data not shown).

Repeated extractions were performed to test the efficacy of the extraction procedure. A second extraction of a pre-

Table 2
Example of 4-HPR and 4-MPR levels in tissues from a mouse treated with 4-HPR recalculated to μg/g of the fresh sample weight

Athymic mouse tissue type	4-HPR concentration (μg/ml)	4-HPR tissue level (μg/g)	4-MPR concentration (μg/ml)	4-MPR tissue level (μg/g)	Sample weight (mg)
Tumor # 1	2.6	26.1	0.9	9.0	101
Tumor # 2	2.5	24.3	1.0	9.5	101
Tumor # 3	2.6	24.9	0.6	5.7	104
Brain	1.3	12.8	<LOD	<LOD	98
Liver	5.1	51.6	1.1	11.2	98
Bladder	2.4	78.7	2.0	66.6	30

The athymic mouse carrying a subcutaneous tumor xenograft was treated with intraperitoneal injections of 4-HPR/Diluent-12 at a dose of 180 mg/kg per day (divided into two daily doses) for a period of 3 days and sacrificed 3 h following the last injection. Tumor samples 1–3 were taken from three different parts of the identical tumor. All tissue samples were analyzed immediately following the sacrifice of the animal. LOD: limit of detection.

viously analyzed tissue sample homogenate presented only trace amounts of 4-HPR and 4-MPR, well below the limit of detection (data not shown).

Tissue samples from an athymic (nu/nu) mouse carrying a subcutaneous tumor xenograft originating from SMS-KCNR neuroblastoma cells were analyzed following treatment with intraperitoneal (i.p.) injections of 4-HPR/Diluent-12. The animal was treated at a dose of 180 g/kg per day divided into two daily i.p. injections for a period of 3 days. The animal was sacrificed 3 h after the last injection and all tissue samples were analyzed immediately following the sacrifice. 4-HPR was detected in all tested tissues including tumor, liver, and brain (Table 2, Fig. 4). The metabolite 4-MPR was detected in all tissues except the brain, where the 4-MPR level did not reach the level of detection (Table 2, Fig. 4).

4. Discussion

The present method differs from previously published HPLC methods for detection of 4-HPR in tissue in its distinct manner of sample preparation and analyte extraction. Previously, lyophilization has been used in some methods to remove water from fresh samples [14]. Another approach to dehydration of the tissue is the use of anhydrous sodium sulfate [11,28]. Other methods work with fresh tissue samples without dehydration [8,37]. Specifying whether the final calculations were based on fresh tissue weight or material handled on a dried basis remains an essential part of the data presentation. To enable a high throughput, we chose to extract with organic solvents without dehydration steps.

The extraction with acetonitrile was easy and our data demonstrate that the reproducibility of the method was satisfactory (Table 1). No clean-up step was needed in the present method since no impurities or interferences were detected at the retention times attributed to the desired analytes.

Retinoids are reported to be sensitive to light and oxidation [16,30,37]. Although some of the methods published earlier do employ and recommend addition of butylated hydroxytoluene to reduce oxidation [8], the recovery rate of our method indicates that addition of antioxidants during sample extraction was not necessary (Table 1) [30]. Additionally, use of the amber glass vials and amber microtubes throughout the procedure afforded sufficient light protection to the samples and the analytes, as no difference was observed between samples analyzed under fluorescent white light versus indirect yellow light (data not shown).

To facilitate high throughput of samples, a rapid extraction method was crucial. In the present study, an ultrasonic bath was found to be a more efficient and time-conserving technique than the use of a shaker as employed in other methods [13]. Avoiding evaporation of samples during extraction, and analyte transfers to another medium, helped to preserve both a high recovery of 4-HPR and 4-MPR, and a high throughput. Additionally, derivatization was not required for this method due to a well-defined 4-HPR peak in the UV spectrum and the high sensitivity of the method. Although the recovery was comparable at 340 and 254 nm, the 340 nm wavelength was selected for our analyses due to the greater sensitivity of detection. Also, extraction solution interference with the analyte peaks was higher at the 254 nm wavelength.

The present study defines a simple and reproducible HPLC method suitable for analysis of 4-HPR and its metabolite 4-MPR in various tissues. The levels of 4-HPR and 4-MPR that accumulate in tissues during treatment will be affected by the bioavailability of the 4-HPR formulation employed and biological features of each tissue, including the ability of each tissue to metabolize 4-HPR. Understanding the pharmacokinetics and pharmacodynamics of 4-HPR is necessary to optimize clinical trial design. A rapid, sensitive, and accurate method for measuring analyte levels in tissues along with the plasma drug levels should facilitate such studies in both in vivo and in vitro models.

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