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Analysis of Fenretinide and its metabolites in human plasma by liquid chromatography-tandem mass spectrometry and its application to clinical pharmacokinetics

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Abstract

A simple and accurate high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was developed for the determination of N-(4-hydroxyphenyl)retinamide (fenretinide, 4-HPR) and its metabolites, 4-oxo-N-(4-hydroxyphenyl)retinamide (4-oxo-4-HPR) and N-(4-methoxyphenyl)retinamide (4-MPR), in human plasma. Plasma samples were prepared using protein precipitation with ethanol. Chromatographic separation of the three analytes and N-(4-ethoxyphenyl)retinamide (4-EPR), an internal standard, was achieved on a Zorbax SB-C18 column (3.5 μ m, 50 $\times$ 2.1 mm) using gradient elution with the mobile phase of 0.1 % formic acid in water and acetonitrile (pH* 2.4) at a flow rate of 0.5 mL/min. Electrospray ionization (ESI) mass spectrometry was operated in the positive ion mode with multiple reaction monitoring (MRM). The calibration curves obtained were linear over the concentration range of 0.2–50 ng/mL with a lower limit of quantification of 0.2 ng/mL. The relative standard deviation of intraday and inter-day precision was below 7.64 %, and the accuracy ranged from 94.92 to 105.43 %. The extraction recoveries were found to be higher than 90.39% and no matrix effect was observed. The analytes were stable for the durations of the stability studies. The validated method was successfully applied to the analyses of the pharmacokinetic study for patients treated with 4-HPR in a clinical trial.

Graphical abstract

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Keywords

Fenretinide (4-HPR); LC-MS/MS; assay development, clinical pharmacokinetics

1. Introduction

Retinoids, the derivatives of vitamin A, have been known to modulate the embryonic development, differentiation, proliferation and apoptosis. N-(4-hydroxyphenyl)retinamide (Fenretinide or 4-HPR) is one of the most promising synthetic retinoids in cancer prevention and treatment with tolerable toxicity [1, 2]. 4-HPR has exhibited cytotoxic activity against various cancer cells *in vitro* and in xenografts, including neuroblastoma, leukemia, colorectal, prostate, breast, ovarian [3–9] and lung cancer [10]. 4-HPR has been also shown activity against cell lines resistant to all-trans-retinoic acid and 13-cis-retinoic acid [8, 11]. Clinically, 4-HPR has been extensively evaluated as a chemo-preventive agent for breast [1], bladder [12] and oral mucosal cancers [13], and more recently as a therapeutic agent for pediatric [2] and adult cancers [14].

4-HPR is metabolized into a diversity of analogues, such as N-(4-methoxyphenyl)retinamide (4-MPR) and 4-oxo-N-(4-hydroxyphenyl)retinamide (4-oxo-4-HPR), changed structurally at the hydroxyphenyl ring and cyclohexenyl ring, respectively [15, 16]. 4-MPR is reported to be the most abundant metabolite of 4-HPR in human plasma, which accumulate in fatty tissues including, breast, prostate, muscles, liver and intestines [16]. Although 4-MPR is non-cytotoxic against numerous malignant cell lines [17], the presence of this nonpolar metabolite might serve as a latent biomarker for providing important information about the effectiveness and metabolic pathways of 4-HPR because 4-MPR is known to be detected only in cells sensitive to 4-HPR [18]. 4-oxo-4-HPR, obtained by oxidation at the C4 position of 4-HPR, is a polar metabolite being present at lower concentrations in humans. 4-oxo-4-HPR is two- to four-fold more cytotoxic than 4-HPR in solid tumor cell lines and also active against some 4-HPR-resistant cell lines. Additionally, 4-oxo-4-HPR is able to act synergistically with 4-HPR [17].

In spite of their preclinical activities, the clinical trials with fenretinide are still limited by the low bioavailability due to poor solubility in aqueous body fluids [2, 4]. Moreover, 4-HPR has been known to bind extensively to diverse endogenous components such as plasma proteins and lipids [19]. Consequently, these physicochemical properties could not only have a negative impact on the therapeutic efficacy but also make the analysis of drugs in biological samples more challenging. Thus, a reliable and sensitive analytical assay of 4-

HPR and its metabolites in biological samples is essential to ensure accurate measurement of drug levels in patients that can be used to determine therapeutic levels.

Several high-performance liquid chromatographic (HPLC) methods have been previously reported for determination of 4-HPR in biological samples [20–23]. However, these methods do not include an important metabolite (4-oxo-4-HPR), have poor sensitivity (LLOQ>20 ng/mL), or require a relatively large amount of samples or a longer run time (20 min). Recently, a liquid chromatographic technique coupled with mass spectrometry based on atmospheric pressure chemical ionization (APCI) has been proposed to determine 4-HPR from mouse plasma [24]. However, this method called for a large injection volume of 40 μ L to reach an LLOQ of 0.5 ng/mL and was not applicable for the simultaneous analysis of polar metabolites in more complex human plasma. In addition to the APCI method, several electrospray ionization (ESI) methods were reported for quantification and identification of metabolites but the validation of the assay was not described [25, 26].

Even with the use of advanced instruments, the assay would suffer from interference caused by the inappropriate sample preparation. Conventional liquid-liquid extraction (LLE) and solid-phase extraction (SPE) technique require relatively larger volumes of solvents, lab-intensive and time-consuming cleanup procedures and often prohibit low extraction recovery and reproducibility. Alternatively, the protein precipitation with the organic solvent is a simple and fast sample preparation method which isolates the analytes of interest from the interfering compounds or drug-protein complex, and it simultaneously dissolves the analytes in a solvent. As regards protein precipitation, an important consideration is a choice of suitable solvent. Most methods previously reported in the literature involved the use of acetonitrile as precipitants. However, the use of acetonitrile would be unfavorable because of its relatively low dissolubility compared to alcohols [27].

In the present study, a simple protein precipitation method with ethanol without pH adjustment was investigated for sample clean up procedure, and a reliable and fast LC–MS/MS method for the determination of 4-HPR and its metabolites in human plasma was developed and fully validated. The validated method was successfully applied to the quantitation of 4-HPR and its metabolites in plasma samples from neuroblastoma patients treated with 4-HPR in a phase I clinical trial (NANT 2004-003, [ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT00646230) Identifier NCT00646230).

2. Experimental

2.1. Chemicals and reagents

4-HPR, 4-MPR, 4-oxo-4-HPR and the internal standard N-(4-ethoxyphenyl) retinamide (4-EPR) were supplied by the Developmental Therapeutics Program of the National Cancer Institute (NCI, Bethesda, MD, USA). HPLC-grade acetonitrile, methanol and LC-MS-grade formic acid were from Sigma-Aldrich (St Louis, MO, USA). HPLC-grade ethanol, acetone and 2-propanol were from Fisher Scientific (Pittsburgh, PA, USA). All other chemicals were the highest quality and were used without further purification. De-ionized water was prepared using a Milli-Q water purification system (Millipore, Bedford, MA, USA). The

different lots of drugfree (blank) heparinized human plasma from healthy donors were provided by Blood Bank, University Medical Center (Lubbock, TX).

2.2. Liquid chromatographic conditions

The HPLC system consisted of an Agilent 1200 series system with a quaternary pump, a vacuum degasser, a thermo-controlled column compartment and an autosampler (Agilent Technologies Inc., Santa Clara, CA, USA). Chromatographic separation was achieved on a Zorbax SB-C18 column (3.5 μ m, 50 $\times$ 2.1 mm i.d., Agilent) protected with an XBridge C18 guard column (3.5 μ m, 10 $\times$ 2.1 mm i.d., Waters, Milford, MA, USA) at 30°C. The mobile phases consisted of 0.1% formic in water (pH 2.4, A) and 0.1% formic acid in acetonitrile (B). The gradient elution was performed as follows: 0–2 min, 45–95% B (v/v); 2–7 min, 95% B; 7–7.5 min, 95–45% B; 7.5–10 min, 45% B at a flow rate of 0.5 mL/min. For the first 2 min and after 4 min, the column eluent was diverted to a waste line using a two position valve (Valco Instruments Co. Inc., Houston, TX, USA) to minimize contamination of the electrospray ion source and mass analyzer. The autosampler was maintained at 4°C and the injection volume was 3 μ L.

2.3. Mass spectrometer conditions

Mass spectrometric detection was carried out on a Sciex 4000 QTRAP system (AB Sciex, Concord, Canada) equipped with an electrospray ionization (ESI) source operating in positive ion mode. The drug and the metabolites were analyzed by multiple reaction monitoring (MRM) mode using ion transitions of m/z 406.3 $\rightarrow$ 297.2 for 4-oxo-4-HPR, m/z 392.3 $\rightarrow$ 283.2 for 4-HPR, m/z 406.3 $\rightarrow$ 283.2 for 4-MPR and m/z 420.3 $\rightarrow$ 283.2 for 4-EPR. Quadrupole 1 and quadrupole 3 were set at unit mass resolution and the dwell time was kept at 50 ms. The source temperature was 600 °C and the spray voltage was 5500 V. The nebulizer gas (Gas 1), heater gas (Gas 2), curtain gas (CUR) and collision activated dissociation gas (CAD) were 50, 50, 20 and high, respectively. Declustering potentials (DP) were 60 V for 4-oxo-4-HPR, 65 V for 4-HPR, 70 V for 4-MPR, and 50 V for 4-EPR. The optimal collision energy (CE) and collision exit potential (CXP) were 17 eV and 18 V, respectively.

All source parameters were optimized under LC conditions and the electrical parameters were optimized by direct infusion. The software package Analyst (Version 1.6.2, AB Sciex) was used for data acquisition and processing.

2.4. Preparation of standard and quality control (QC) samples

Stock solutions of 4-oxo-4-HPR, 4-HPR, 4-MPR and IS were prepared in ethanol at a concentration of 1 mg/mL. Standard working solutions were prepared by diluting the stock solutions with ethanol to desired concentrations. IS working solution was prepared by dilution with ethanol to concentration of 10 μ g/mL.

Calibration standard samples were prepared by spiking the working solutions into drug-free blank plasma (25 μ L) at final concentrations of 0.2, 0.5, 1.0, 2.0, 5.0, 10, 20 and 50 ng/mL. Quality control samples were prepared at four levels representing the lower limit of

quantification (LLOQ), low (LQC), medium (MQC) and high (HQC) quality controls at 0.2, 0.6, 4.0 and 40 ng/mL, respectively.

Silanized amber glass vials and amber polypropylene microcentrifuge tubes were used to store all solutions and unknown samples throughout the procedure. All of the procedures were carried out under reduced yellow lighting to prevent light-induced degradation of analytes. All solutions were stored at -20°C prior to use.

2.5. Sample preparation

Five μL of internal standard (4-EPR, 10 $\mu\text{g/mL}$) and 470 μL of cold ethanol were added to a 25 μL aliquot of plasma sample in a 1.5 mL amber microcentrifuge tube. The mixture was vortex-mixed for 3 min and centrifuged at $10,000 \times g$ for 5 min. The supernatants were collected and then diluted to 1:50 by volume with mobile phase B. The diluted supernatant was transferred into an amber autosampler vial and the 3 μL aliquot was injected into the LC-MS/MS system.

2.6. Method validation

The method was validated according to the FDA guidance for validation of bioanalytical methods [28].

2.6.1. Selectivity and carryover—The selectivity of the method was determined by analyzing six different lots of blank plasma for the presence of interfering peaks in the retention time of the analytes and IS. No interfering peaks were observed and the selectivity was considered to be acceptable. Carryover was assessed by injecting double blank sample after the injection of the upper limit of quantitation (ULOQ) sample.

2.6.2. Linearity and lower limit of quantification (LLOQ)—The linearity was evaluated by using eight point calibration curves between 0.2 to 50 ng/mL. The calibration curves were constructed by plotting the peak area ratio (y) of each analyte to IS versus the concentration of the analytes using a weighted ($1/x^2$) least squares linear regression analysis. The lower limit of quantitation (LLOQ) was the concentration at which the precision and accuracy were $\geq 20\%$, and the signal-to-noise ratio (S/N) was ≥ 5 . Relative standard deviation (RSD %) was used to assess precision. Percent recovery (%) was used to estimate accuracy, calculated as $(\text{measured conc.}/\text{normal conc.}) \times 100\%$.

2.6.3. Precision and accuracy—Intra-run accuracy and precision were determined using five QC samples of four concentration levels (LLOQ, low, medium and high) in the same run. Inter-run accuracy and precision were determined by analyzing the four levels of QC samples on five consecutive days. The criteria for acceptability of the data included accuracy within $\pm 15\%$ from normal values and a precision within $\pm 15\%$ RSD except for LLOQ, for which precision accuracy should not exceed $\pm 20\%$.

2.6.4. Recovery and matrix effect—The extraction recovery and matrix effect at six replicates of three QC concentrations (low, medium and high) of the three analytes and of IS at 2.0 ng/mL were measured by comparing peak areas of spiked samples of spiked samples

of plasma, processed as described in Section 2.4 to those of compounds diluted with mobile phase. The matrix effect was evaluated by comparing the peak areas of analytes spiked into post-extraction supernatants from six different lots of plasma with those at equivalent standards.

2.6.5. Dilution integrity—Dilution integrity was assessed to validate the measurements of higher concentrations of analytes (above the ULOQ), which could be encountered during real sample analysis.

The dilution integrity experiments were prepared by spiking analytes at high concentrations (80 ng/mL and 160 ng/mL) and diluting with blank matrix to make a 2- and 4-fold dilution, respectively. Dilution integrity was evaluated by analyzing five replicates per dilution factor to determine accuracy and precision in the measurements of diluted samples.

2.6.6. Stability—The stability of the analytes in plasma was tested by analyzing three replicates of QC samples at low, medium and high concentration levels after short-term (24 h at 4 °C) and long-term (2 months at –80 °C). In addition, freeze/thaw stability was determined by analysis of samples subjected to three freeze/thaw cycles (–80 to 25 °C). The stability of the processed samples in the autosampler was assessed after 24 h at 4 °C. A sample was considered to be stable in the biological matrix if the concentration variance was less than 15% of the freshly prepared samples.

2.7. Pharmacokinetic(PK) study—The validated method was applied to PK samples from a phase I clinical trial of 4-HPR conducted in the New Approaches to Neuroblastoma Therapy (NANT) consortium (NANT 2004-03) [29]. The study was approved by the Institutional Review Board at each site where patients provided blood samples for PK and by the Texas Tech University Health Sciences Center (TTUHSC), Lubbock, Texas. Informed consent was obtained from the all patients and/or their legal guardian(s). Patients received 4-HPR by a continuous intravenous infusion at doses of 1175 mg/day (565 mg/m²), 2025 mg/day (1131mg/m²) or 2217 mg/day (1131 mg/m²) for 120 hrs every 21 days according to the NANT 2004–03 Phase I study Protocol ([ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study?term=NCT00646230) Identifier NCT00646230). Blood samples (4 mL) were collected at 0, 6, 12, 18, 24, 36, 48, 72, 96, 120 hours during and end of infusion, +2 hours, +48 hours and +72 hours after the infusion stop. Blood samples were immediately protected from light and centrifuged at 3000×g for 10 min. Plasma was transferred to a foil-wrapped polypropylene tube to be stored at –80°C until analysis.

The 4-HPR plasma concentrations of individual patients were fitted with a one-compartment model with weighted least squares regression in the ADAPT 5 software (Biomedical Simulation Resource, University of Southern California, Los Angeles, CA) to estimate model parameters. The maximum blood concentration (C_{max}) areas under the concentration–time curves from 0 to +192 h after the infusion stop (AUC_{0-192h}) were determined according to the trapezoidal rule.

3. Results and Discussion

3.1. Method development

3.1.1. Mass spectrometry—Mass spectrometry parameters were optimized to obtain sensitivity and stable response during direct infusion of standard solutions into the electrospray ion source operated in positive ion mode at a flow rate 0.01 mL/min. The Q1 full scan mass spectra of 4-oxo-4-HPR, 4-HPR, 4-MPR and 4-EPR exhibited prominent protonated ion $[M+H]^+$ at m/z 406.3, 392.3, 406.3 and 420.3, respectively. The major fragment ions formed by the cleavage of amide bond were observed at m/z 297.2 for 4-oxo-4-HPR and 283.2 for all other compounds (Fig. 1). Another common fragment ion of 4-HPR, 4-MPR and 4-EPR was produced at m/z 161.0 corresponding to additional loss of trimethyl-cyclohexenyl from the primary fragment ion through the collision energy of 32 eV, while consecutive fragmentation from 4-oxo-4-HPR rarely occurred as C6–C7 bond was stabilized by resonance due to oxo substituent at C4 position. Thus, the ion transitions of m/z 406.3 $\rightarrow$ 297.2, 392.3 $\rightarrow$ 283.2, 406.3 $\rightarrow$ 283.2 and 420.3 $\rightarrow$ 283.2 were utilized for monitoring 4-oxo-4-HPR, 4-HPR, 4-MPR and 4-EPR, respectively.

3.1.2. Chromatography—Initially, methanol–water and acetonitrile–water systems with 0.1 % formic acid (pH 2.4), 0.5 % acetic acid (pH 2.7) or 5 mM ammonium acetate (pH 3.5) were investigated under the gradient elution. As the organic mobile phase, acetonitrile and methanol exhibited similar MS responses, but methanol showed tailing and partially overlapped peaks. Furthermore, the large pressure fluctuation by gradient elution with methanol required a long column equilibration time. Overall, different types of additives in mobile phase did not affect variations of peak symmetry, retention and separation. While low concentration of formic acid resulted in enhancement of the signal intensity. Retention of 4-oxo-4-HPR, which is a polar metabolite of 4-HPR, changed dramatically depending on the initial composition of mobile phase compared to those of other non-polar analytes. At the beginning of the gradient elution, aqueous mobile phase of less than 50% led to greater peak distortion and broadening.

Three different C18 columns were tested, including Kinetex C18 (2.6 μ m, 100 mm $\times$ 2.1 mm), XBridge C18 (3.5 μ m, 50 $\times$ 2.1 mm) and Zorbax SB-C18 column (3.5 μ m, 50 $\times$ 2.1 mm). Analytes and IS were effectively separated on all columns within 6 min but low response and greater background noise were detected from Kinetex C18 and XBridge C18. Zorbax SB-C18 column exhibited satisfactory sensitivity, chromatographic separation and retention and was selected for the method development.

Additionally, the effects of column temperature and flow rate on the separation efficiency were investigated at 25 to 50°C and at 0.3 to 0.6 mL/min, respectively. Briefly, the higher temperature consistently reduced elution time of all compounds and column backpressure. However, the high temperatures caused the slight band broadening and poor peak shape for 4-oxo-4-HPR. The flow rates below 0.4 mL/min contributed to ion suppression related to a late-eluting matrix component from previous injection while the higher flow rate allowed a faster analysis with a negligible loss of ionization efficiency. The flow rate at 0.5 mL/min with a column temperature at 30 °C resulted in a good separation, a symmetric peak shape and a shorter retention time. Under the optimized chromatographic conditions, 4-oxo-4-

HPR, 4-HPR, 4-MRR and 4-EPR were separated with suitable retention time of 3.1, 4.2, 5.1, and 5.5 min, respectively.

3.1.3. Sample preparation—Sample preparation is a fundamental step for accurate and reliable LC-MS/MS assays. In the present study, protein precipitation with organic solvents was chosen for a simple and economical process with improved extraction efficiency and reproducibility. 4-HPR and its metabolites have a high binding affinity to protein in plasma [19]. Thus, it was necessary to find suitable solvent for releasing the analytes from protein binding. Various solvents such as acetonitrile, methanol, ethanol, acetone or isopropanol were evaluated by comparing the peak areas of analytes from extracted samples with those from the neat standards, indicating extraction efficiency (Supplementary Fig. S1).

Acetonitrile is the most commonly employed protein precipitants, but was found to be inadequate for extraction of 4-HPR and metabolites. The extraction with acetonitrile was not reproducible and the extraction efficiency of the analytes except 4-oxo-4-HPR was less than 70%. As the hydrophobicity of analytes increased, extraction efficiency dramatically decreased. Extraction with acetone was consistent and compound-independent. However, despite high dissolving power for 4-HPR [27], acetone generated limited extraction efficiency of 88%. Methanol and 2-propanol yielded relatively low extraction efficiency as well as unfavorable variability in reproducibility compared to ethanol. Ethanol was selected as the optimal solvent which consistently produced the extraction efficiency of over 95% for all three analytes and the IS.

3.2. Method validation

3.2.1. Selectivity and carry-over—The selectivity was evaluated using six different lots of blank plasma. Typical chromatograms of blank plasma, blank plasma spiked with analytes at LLOQ and the IS, and a plasma sample drawn after intravenous administration of 4-HPR at 1810 mg/m²/day for 120 hrs are shown in Fig. 2. No endogenous interference derived from plasma was observed at the retention times of analytes or IS, indicating that this method is specific. Carry-over was evaluated by analyzing a blank sample following the ULOQ. No carry-over was found under the present condition.

3.2.2. Linearity and LLOQ—Calibration curves were constructed by plotting the peak-area ratios (y) of each analyte to the IS versus the nominal concentrations (x) using weighted ($1/x^2$) least-squared linear regression analysis. The calibration curves were linear over the concentration range of 0.2 – 50.0 ng/mL for three analytes. The deviations of the back-calculated concentrations were within 15% of the nominal values. The mean regression equations of the calibration curves were $y = (0.4151 \pm 0.0071)x - (0.00633 \pm 0.00364)$, $r = 0.9991$ for 4-oxo-4-HPR, $y = (0.3076 \pm 0.0057)x + (0.00089 \pm 0.00291)$, $r = 0.9990$ for 4-HPR, and $y = (0.5877 \pm 0.0042)x + (0.00784 \pm 0.00215)$, $r = 0.9998$ for 4-MPR. The LLOQ for all analytes confirmed at 0.2 ng/mL with the acceptable accuracy and precision, indicating that this method was considerably sensitive for the determination of 4-HPR and its metabolites.

3.2.3. Precision and accuracy—The accuracy and precision of the method are summarized in Table 1. Based on five replicates of QC samples at LLOQ, low, medium, and high levels, the intra-run accuracy for three analytes assay was 94.99–105.43% with a precision of 1.69–7.64%. The inter-run accuracy was 94.92–101.22% with a precision of 2.22–7.26%. The results clearly demonstrated that the proposed method was accurate and precise for the determination of these analytes in human plasma.

3.2.4. Recovery and matrix effect—Plasma samples were extracted by protein precipitation using ethanol and high dilution to ensure reproducibility and minimal matrix effects. The extraction recovery and matrix effect are presented in Table 2. The recovery was within the range 91.82–102.38% for three analytes at the three QC levels. The recovery of the IS was 98.71 % at single level of 2 ng/mL. The recoveries were consistent and precise for the analytes and the IS.

The ion suppression or enhancement is generally induced by the coeluting substances on the ionization process. The matrix effects of analytes and IS were 95.25–102.13% and RSD (%) from six lots of plasma were less than 7.79%. In short, plasma matrix interferences were almost eliminated through an appropriate sample preparation and chromatographic separation.

3.2.5. Dilution integrity—Dilution procedure was tested to ensure that samples could be diluted with blank matrix with no impact on the analytical result. The measured concentrations of analytes in human plasma following a 2 and 4 fold dilution were between 93.79% and 101.93% of their nominal values and the precisions (RSD %) were less than 6.33 % (data not shown). The results suggested that samples with concentrations above the upper limit of quantitation (ULOQ) could be quantified after appropriate dilution with blank matrix.

3.2.6. Stability—The stability of the analytes was assessed at three concentrations under various conditions used throughout the current study. The stability results presented in Table 3 show that the three analytes were stable in human plasma after three freeze-thaw cycles at room temperature for 24h and at -80 °C for two months. Moreover, no significant degradation occurred after postpreparation storage in the autosampler at 4 °C for 24 h.

3.3. Pharmacokinetic study

The established LC-MS/MS method was successfully applied to the analyses of human plasma samples collected after a continuous intravenous administration at 1175 mg/day (565 mg/m²), 2025 mg/day (1131 mg/m²) or 2217 mg/day (1131 mg/m²) of 4-HPR. The plasma concentration-time profiles of 4-HPR and its metabolites and their PK parameters are shown in Fig. 3 and supplementary Table S1. The PK parameters obtained from plasma concentrations assessed by the current assay were consistent with the values reported in the previous study (NANT 2004-003) that used the HPLC assay [30]. The PK profiling of 4-oxo-4-HPR was incomplete for the previous study in patient with lower doses of 4-HPR, mainly due to the LLOQ not being sensitive enough to quantitate 4-oxo-4-HPR levels at later time points. The current assay method provided sensitivity to quantitate 196h, the last

time point for PK measurement, and the PK profile for those patients with low doses are fully characterized using the current method. Overall, this LC/MC/MS method is suitable for the PK studies of 4-HPR clinical trials.

4. Conclusion

A LC–MS/MS method was developed and validated for the quantitative determination of 4-HPR, 4-oxo-4-HPR and 4-MPR in human plasma. The developed method includes simple and reliable sample pretreatment procedure with acceptable good linearity, precision, accuracy and recovery characteristics. The method was successfully applied to the analysis of 4-HPR in human plasma samples from 4-HPR clinical trial. To our knowledge, this is the first study reporting an LC–MS/MS method for the assay of 4-HPR in human plasma. The established method will be utilized to measure 4-HPR and its metabolites in plasma samples from the patients treated with 4-HPR.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Simultaneous determination of 4-HPR and its metabolites with different polarities
- Comparison and identification of the optimal extraction solvent for 4-HPR and its metabolites from human plasma
- Inclusion of a metabolite, 4-oxo-4-HPR (4-oxo-fenretinide), that is active in the analytes
- Application of the assay to the analyses of human samples from a fenretinide clinical trial for profiling fenretinide pharmacokinetics

Fig. 1.
Product ion mass spectra of 4-oxo-4-HPR (A), 4-HPR (B), 4-MPR (C) and 4-EPR (D)

Fig. 2.

Representative MRM chromatograms of 4-oxo-4-HPR, 4-HPR, 4-MPR and IS in human plasma (top to bottom): (A) blank plasma; (B) plasma spiked with analytes at 0.2 ng/mL (LLOQ) and IS at 2 ng/mL; (C) patient sample after intravenous administration of 4-HPR at dose of 2025 mg/day (1131 mg/m²) as a 120 hr infusion

Fig. 3. Individual observed and modeled plasma concentration-time profiles for 4-HPR and its metabolites following continuous intravenous administration. Observed data are indicated by the closed circles for 4-HPR, the open squares for 4-MPR and the open triangles for 4-oxo-4-HPR and modeled data by the solid lines for 4-HPR, the dashed lines for 4-MPR and the dotted lines for 4-oxo-4-HPR. AUC: based on estimated value, C_{max}: observed maximum value, Arrow: end of infusion

Table 1

Precision and accuracy for the analysis of 4-HPR and metabolites in human plasma

Analytes	Nominal conc. (ng/mL)	Within-run (n=5)			Between-run (n=5)		
		Observed conc. (ng/mL, Mean $\pm$ SD)	Accuracy (%)	Precision (RSD %)	Observed conc. (ng/mL, Mean $\pm$ SD)	Accuracy (%)	Precision (RSD %)
4-oxo-4-HPR	0.2	0.20 $\pm$ 0.01	99.96	5.84	0.20 $\pm$ 0.01	101.22	6.53
	0.6	0.63 $\pm$ 0.04	105.43	6.16	0.60 $\pm$ 0.01	100.10	2.27
	4	4.08 $\pm$ 0.14	102.05	3.40	4.00 $\pm$ 0.20	99.92	4.98
	40	41.49 $\pm$ 1.33	103.73	3.20	39.40 $\pm$ 1.08	98.51	2.73
4-HPR	0.2	0.19 $\pm$ 0.01	96.74	7.64	0.20 $\pm$ 0.01	98.60	5.50
	0.6	0.59 $\pm$ 0.03	97.67	5.97	0.59 $\pm$ 0.03	97.87	4.89
	4	4.01 $\pm$ 0.16	100.32	4.04	3.87 $\pm$ 0.25	96.77	6.43
	40	38.55 $\pm$ 0.87	96.38	2.26	37.97 $\pm$ 1.22	94.92	3.22
4-MPR	0.2	0.21 $\pm$ 0.02	105.41	7.30	0.20 $\pm$ 0.01	98.83	4.53
	0.6	0.62 $\pm$ 0.03	102.55	4.90	0.60 $\pm$ 0.02	99.62	3.07
	4	3.98 $\pm$ 0.10	99.41	2.39	3.95 $\pm$ 0.29	98.78	7.26
	40	38.00 $\pm$ 0.64	94.99	1.69	38.13 $\pm$ 0.85	95.33	2.22

Recovery and matrix effect of 4-HPR, its metabolites and 4-MPR (IS) from human plasma. (n=6)

Table 2

Compounds	Nominal conc. (ng/mL)	Recovery		Matrix effect	
		Mean $\pm$ SD (%)	RSD (%)	Mean $\pm$ SD (%)	RSD (%)
4-oxo-4-HPR	0.6	102.38 $\pm$ 10.77	10.52	95.64 $\pm$ 7.45	7.79
	4	101.40 $\pm$ 3.57	3.52	96.11 $\pm$ 4.04	4.21
	40	94.28 $\pm$ 5.74	6.09	101.08 $\pm$ 7.59	7.51
4-HPR	0.6	96.66 $\pm$ 4.50	4.66	96.44 $\pm$ 4.75	4.92
	4	100.37 $\pm$ 2.14	2.14	95.25 $\pm$ 1.30	1.36
	40	93.83 $\pm$ 3.46	3.68	102.13 $\pm$ 1.79	1.75
4-HPR	0.6	95.28 $\pm$ 4.88	5.12	98.72 $\pm$ 3.81	3.85
	4	99.95 $\pm$ 1.97	1.97	98.17 $\pm$ 2.01	2.04
	40	91.82 $\pm$ 4.14	4.51	101.91 $\pm$ 4.31	4.23
4-MPR (IS)	2	98.71 $\pm$ 1.98	2.01	99.49 $\pm$ 1.64	1.65

Table 3

Stability of 4-HPR and 4-MPR in human plasma at different conditions (n=3)

Storage Conditions	Nominal conc. (ng/mL)	4-oxo-4-HPR		4-HPR		4-MPR	
		Mean $\pm$ SD (%)	RSD (%)	Mean $\pm$ SD (%)	RSD (%)	Mean $\pm$ SD (%)	RSD (%)
Freeze-thaw stability ^a	0.6	94.56 $\pm$ 4.92	5.21	95.65 $\pm$ 5.64	5.90	100.73 $\pm$ 5.13	5.09
	4	89.84 $\pm$ 5.87	6.53	92.61 $\pm$ 4.21	4.55	94.95 $\pm$ 3.21	3.38
	40	96.66 $\pm$ 3.53	3.65	93.01 $\pm$ 1.67	1.80	92.34 $\pm$ 2.70	2.92
Short-term stability ^b	0.6	93.71 $\pm$ 5.74	6.12	92.18 $\pm$ 3.58	3.88	96.41 $\pm$ 2.24	2.33
	4	92.78 $\pm$ 2.81	3.03	91.99 $\pm$ 0.60	0.65	92.49 $\pm$ 0.82	0.89
	40	97.57 $\pm$ 2.56	2.63	94.38 $\pm$ 3.20	3.39	93.53 $\pm$ 1.39	1.49
Long-term stability ^c	0.6	102.31 $\pm$ 2.88	2.82	101.21 $\pm$ 4.48	4.43	96.67 $\pm$ 4.09	4.23
	4	90.94 $\pm$ 5.13	5.64	94.18 $\pm$ 3.99	4.23	93.32 $\pm$ 1.56	1.67
	40	101.35 $\pm$ 1.48	1.46	96.98 $\pm$ 1.89	1.94	99.20 $\pm$ 2.15	2.17
Post-preparative stability ^d	0.6	104.98 $\pm$ 6.97	6.64	100.77 $\pm$ 6.56	6.51	103.04 $\pm$ 8.49	8.24
	4	100.83 $\pm$ 3.98	3.95	100.36 $\pm$ 1.44	1.44	99.88 $\pm$ 2.51	2.51
	40	105.95 $\pm$ 0.68	0.64	101.00 $\pm$ 1.48	1.47	97.96 $\pm$ 1.51	1.54

^aStorage from -80°C to 25°C through three cycles.

^bStorage at room temperature for 24 h.

^cStorage for 2 months at -80°C .

^dStorage in processed samples in the autosampler at 4°C for 24 h.