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Liquid chromatography method for quantifying D-*threo*-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (D-*threo*-PPMP) in mouse plasma and liver

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Abstract

A high-performance liquid chromatography (HPLC) method was developed to measure levels of D-*threo*-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (D-*threo*-PPMP) in mouse plasma and liver. D-*threo*-PPMP was measured by HPLC with a Luna Pheny-Hexyl column (5 μm, 250 mm × 4.6 mm) employing UV detection at 210 nm using a mobile phase of potassium phosphate buffer (20 mM, pH 3.0)–acetonitrile in a 45:55 (v/v) ratio. D-*threo*-1-phenyl-2-pentadecanoylamino-3-morpholino-1-propanol (PC15MP) was employed as an internal standard (IS). The lower limit of quantitation (LLOQ) was 0.3 μg/ml. The assay was linear over a concentration range of 0.3–10 μg/ml, with acceptable precision and accuracy. Assayed in plasma, the intra- and inter-day validation for all coefficients of variation (R.S.D.%) were found less than 15%. The method was applied to samples from athymic (nu/nu) mice treated with D-*threo*-PPMP by intraperitoneal injection. D-*threo*-PPMP levels of ∼10–20 μg/ml (∼20–40 μM) in plasma and ∼45 μg/g in liver were obtained. The present method can be used to quantify D-*threo*-PPMP in mice for bioavailability and dose-response studies.

Keywords: D-*threo*-PPMP; HPLC

1. Introduction

Various isomers of 1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP) are inhibitors of glucosylceramide synthase and/or acylceramide synthase, enzymes that impact on the cellular catabolism of the sphingolipid, ceramide [1–3]. Ceramides, exogenous and endogenous, have been implicated as cell death signaling messengers in a variety of model systems [4]. Previously, we reported that the cytotoxic retinoid, *N*-(4-hydroxyphenyl)retinamide (fenretinide, 4-HPR) increased various intracellular ceramides in a time- and dose-dependent manner [5,6] in vitro, and that co-treatment with D,L-*threo*-PPMP further increased fenretinide-induced ceramides and cytotoxicity in multiple human cancer cell lines, including those of acute lymphoblastic leukemia (ALL) [7,8].

Recently, we have determined that D-*threo*-PPMP (Fig. 1) is a preferred isomer for synergizing fenretinide cytotoxicity [9]. Further, small molecule glucosylceramide synthase inhibitors may be useful for substrate reduction therapy in the treatment of patients with Gaucher disease, and other glycosphingolipidoses and/or ganglioside storage disorders, in whom enzyme replacement therapy may be unavailable or ineffective [10,11]. However, there are no reported methods for the determination of D-*threo*-PPMP in biological samples. Here we report the development, and validation [12–14], of a HPLC methodology for the

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analysis of D-*threo*-PPMP in mouse plasma, and an extraction method suitable for mouse liver. We are using these methodologies in the pre-clinical development of D-*threo*-PPMP as a modulator of ceramide metabolism for use with fenretinide.

2. Experimental

2.1. Chemicals

D-*threo*-1-phenyl-2-palmitoylamino-3-morphplino-1-propanol (D-*threo*-PPMP) and D-*threo*-1-phenyl-2-pentadecanoylamino-3-morpholino-1-propanol (PC15MP, or internal standard, or IS) (Fig. 1), were from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). D-*threo*-PPMP (as base, M.W. = 474.7) was prepared under Good Manufacturing Practice (GMP) conditions under NIH NCI FLAIR grant 1-R41-CA102842-01. *n*-Hexanes and ethyl acetate were HPLC grade and purchased from Aldrich Co. (Milwaukee, WI, USA). Cremophor El (Product C-5135) [CAS #61791-12-6] was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). All other chemicals (HPLC grade) were purchased from Fisher Scientific (Pittsburgh, PA, USA). Mouse plasma was purchased from Bioreclamation Inc. (Hicksville, NY, USA) and stored at −20 °C. Clinical-grade human albumin solution (5%) was obtained from the hospital pharmacy.

2.2. HPLC conditions

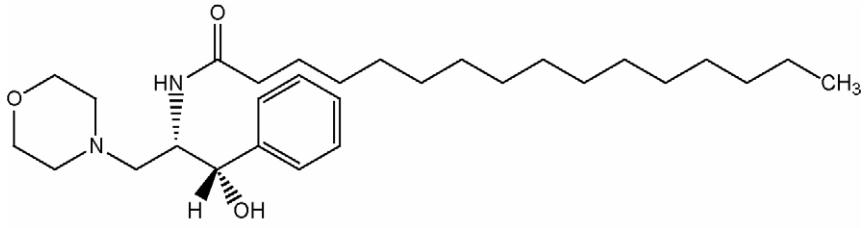
The samples were analyzed with a high-performance liquid chromatograph, consisting of a Waters 2695 Separation Module with sample heater/cooler and column heater (Alliance), Waters 515 HPLC pump, and Waters 717 plus autosampler. The Waters 2996 (Photodiode Array Detector) was set at 210 nm. A Luna Pheny-Hexyl column (5 μm, 250 mm × 4.6 mm) with a Phenyl (phenylpropyl) guard column (4 mm L × 3.0 mm ID) was used. Isocratic elution with potassium phosphate buffer (20 mM, pH 3.0): acetonitrile in 45:55 (v/v) was employed at a flow rate of 1.0 ml/min. Volume of injection was 100 μl. All samples were analyzed at room temperature. Waters Empower™ software was used for data processing.

2.3. Standard solutions

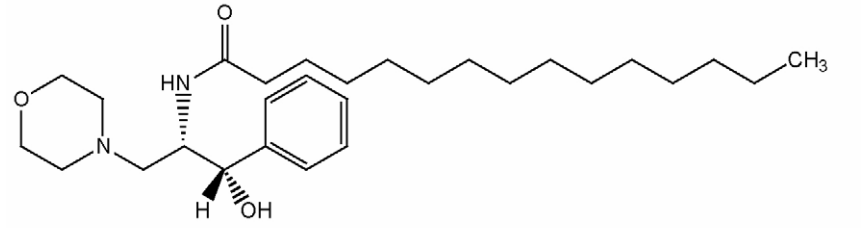
D-*threo*-PPMP (500 μg/ml) and PC15MP (1000 μg/ml) stock standard solutions were prepared in methanol. Stock standard solutions were stable at 4 or −20 °C for at least 2 months. Working standard solutions for D-*threo*-PPMP were prepared at 15.6–500 μg/ml from the stock solution. Working internal standard (IS) solution was prepared at 500 μg/ml from the stock solution.

2.4. Extraction procedures

Mouse plasma samples (0.1 ml, room temperature) were spiked with 10 μL of working internal standard (IS) solution (PC15MP, 500 μg/ml, in methanol), then vortexed vigorously with 2 ml of *n*-hexanes:ethyl acetate, 3:1 (v/v), for 1 min, followed by centrifugation at 2200 × *g* for 6 min. The upper (organic) layer was transferred to a silanized vial. The lower (aqueous) layer was extracted a second time, and the organic layers combined and dried under a stream of nitrogen gas. The residue was then dissolved in 1 ml of mobile phase to allow for multiple HPLC injections for determinations of precision although residue could be dissolved in smaller volumes of



D-threo-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (D-threo-PPMP)



D-threo-1-phenyl-2-pentadecanoylamino-3-morpholino-1-propanol (PC15MP)

Fig. 1. Structures of D-*threo*-1-phenyl-2-palmitoylamino-3-morphplino-1-propanol (D-*threo*-PPMP) and D-*threo*- 1-phenyl-2-pentadecanoylamino-3-morpholino-1-propanol (PC15MP, Internal Standard, IS).

mobile phase to enhance sensitivity. Samples for quality control (QC samples) were freshly prepared by spiking drug-free plasma with D-*threo*-PPMP from working solutions to final concentrations of 0.5, 4.5 and 9 μg/ml of D-*threo*-PPMP.

Mouse livers were homogenized with water (1:3, w/v) using a Tissue Tearor™ (Biospec Inc., Bartlesville, OK). Duplicate aliquots (250 μl) of the homogenates were then spiked with 10 μl of working IS solution, ice cold methanol (750 μl) was added, and then the samples were vortexed for 1 min and placed in an ice cold ultrasonic water bath for 10 min. Following sonication, the mixtures were kept on ice for 10 min, and then centrifuged (10,000 × *g*, 10 min, 4 °C). The supernatants were then transferred to a silanized vial for HPLC analysis.

2.5. Validation of analytical method

2.5.1. Linearity

Standard calibration curves were generated by spiking drug-free mouse plasma and mouse liver samples with D-*threo*-PPMP to produce six concentration levels ranging between 0.3 and 10 μg/ml. Plasma extraction and analysis were carried out as described above. The calibration curve was constructed by plotting the ratios of peak areas against concentration and analyzed by linear regression analysis.

2.5.2. Accuracy and precision

Accuracy and precision were determined by analyzing five separate measurements of QC plasma samples (0.5, 4.5 and 9 μg/ml of D-*threo*-PPMP). Intra-day variation was determined by analyzing five separate measurements of QC samples (0.5, 4.5 and 9 μg/ml of D-*threo*-PPMP) on three different days.

2.5.3. Extraction efficiency

The % recovery of D-*threo*-PPMP and IS extraction from mouse plasma was determined for D-*threo*-PPMP in QC plasma samples (0.5, 4.5 and 9 μg/ml), and for IS in plasma (5 μg/ml), by extracting five independent replicates and comparing the results to that of triplicate samples of D-*threo*-PPMP and IS stock solutions diluted into 1 ml of mobile phase and injected directly into the HPLC. The recovery was calculated using the following equation: % recovery ≡ [(mean extracted response)/(mean unextracted response)] × 100.

2.5.4. Specificity

The specificity for D-*threo*-PPMP in mouse plasma was determined by analysis of blank mouse plasma compared to mouse plasma spiked with D-*threo*-PPMP and IS. The specificity for D-*threo*-PPMP in mouse liver was determined by analysis of blank mouse liver homogenates compared to mouse liver homogenates spiked with D-*threo*-PPMP and IS.

2.5.5. Stability

The stability of D-*threo*-PPMP spiked into mouse plasma samples was evaluated in replicate spiked samples, in replicate samples frozen at −80 °C overnight and thawed to room temperature for 4 h before analysis, in replicate samples after 10 weeks of storage at −80 °C, and lastly, in replicate samples subjected to three daily freeze/thaw cycles at −80 °C.

2.6. Method application

Balb/C mice (female, 18–20 g) (Harlan, Indianapolis, IN, USA) were administered 1.5 mg of D-*threo*-PPMP by intraperitoneal injection, twice a day, ×3.5 days. D-*threo*-PPMP for injection was dissolved in ethanol and sterile filtered, then made either: (a) 15 mg/ml in NCI Diluent-12 (50% Cremophor EL/50% ethanol), then diluted 1:3 in normal saline and sterile filtered; or (b) slurried to 5 mg/cc in 0.5% clinical-grade human albumin in normal saline). Control animals received empty vector. Animals were treated, and then humanely sacrificed by CO_2 narcosis at 4 h after the last drug dosing following a protocol approved by the Childrens Hospital Los Angeles Institutional Animal Care and Use Committee (IACUC) in accordance with national guidelines. Mouse plasma and tissues were collected and stored at −80 °C until analysis.

2.7. Statistics

Data analysis was performed using Waters Empower™ software. Data variability expressed as the coefficient of variation, CV (%) ≡ R.S.D. × 100, where the relative standard deviation, R.S.D. ≡ standard deviation/mean.

3. Results

Selectivity in mouse plasma was evaluated using plasma from six different lots. The resulting chromatograms were free from endogenous interference (Figs. 2 and 3). Peak identification was performed via comparison of retention times of standards and spiked samples. The retention times of D-*threo*-PPMP and internal standard (IS) were about 12 and 9 min, respectively. The peak shapes and the resolution between D-*threo*-PPMP and IS were satisfactory.

The calibration curve was determined by plotting the drug concentration against the ratio of drug/internal standard peak area over the range of concentration examined (0.3–10 μg/ml). Linearity was confirmed by calculating calibration curve parameters from three different runs (Table 1). The lower limit of

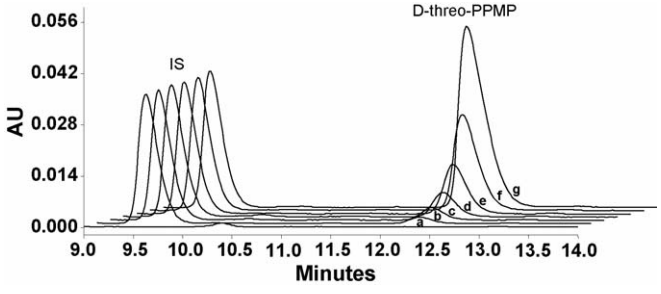


Fig. 2. Chromatogram of calibration curve standards. D-*threo*-PPMP in mouse plasma at, (a) 0.0 μg/ml; (b) 0.3 μg/ml; (c) 0.6 μg/ml; (d) 1.25 μg/ml; (e) 2.5 μg/ml; (f) 5 μg/ml; and (g) 10 μg/ml, respectively. Left peak represents the internal standard (IS), right peak represents D-*threo*-PPMP.

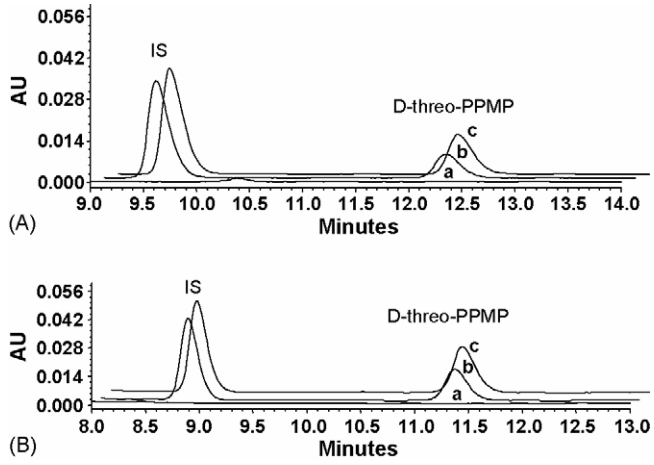


Fig. 3. Panel (A) representative chromatograms of: (a) plasma blank; (b) plasma from an animal treated with D-*threo*-PPMP slurried in albumin and delivered intraperitoneally, (c) a plasma spiked with D-*threo*-PPMP (2.5 μg/ml) and IS. Panel (B) Representative chromatogram of: (a) liver blank; (b) liver spiked with D-*threo*-PPMP (5 μg/ml) and IS; (c) liver from an animal treated with D-*threo*-PPMP dissolved in NCI Diluent-12 and delivered intraperitoneally. Left peak represents the internal standard (IS), right peak represents D-*threo*-PPMP.

Table 1
Calibration curve parameters in plasma

Parameter[a]	
Linear range (μg/ml)	0.3–10.0
$y = ax + b$	$0.917x + 0.023$
r	0.999698
Sa	0.022
Sb	0.021

r, correlation coefficient; a, slope; b, intercept; Sa, standard deviation of slope; Sb, standard deviations of the intercept.
[a] $n = 3$.

quantitation (LLOQ), defined as the lowest concentration in the calibration curve with an accuracy of a 20% systemic error and a precision of 20% coefficient of variation (CV) [14], was calculated from plasma blank noise, and directly measured, as 0.3 μg/ml (CV = 7.6%). The limit of detection (LOD), defined as the concentration at which the signal–noise ratio (blank plasma noise) was 3 [13], was estimated as ∼0.03 μg/ml. The determination coefficients (r^2) of liver calibration curves were 0.999.

The precision and accuracy of the methodology were evaluated in five dependent replicates of the three QC samples at nominal D-*threo*-PPMP concentrations of 0.5, 4.5 and 9 μg/ml on three separate days (Tables 2–4). The present method was

Table 2
Intra-day variation of D-*threo*-PPMP in plasma

Concentration (μg/ml)	Measured (μg/ml) (mean ± S.D.)[b]	CV (%)[a]
0.5	0.51 ± 0.01	1.41
4.5	4.77 ± 0.08	0.34
9.0	9.07 ± 0.11	1.27

[a] Coefficient of variation (CV) ≡ (S.D./mean) × 100.
[b] $n = 5$.

Table 3
Inter-day variation of D-*threo*-PPMP in plasma

Concentration (μg/ml)	Measured (μg/ml) (mean ± S.D.)[a]	CV (%)[b]
0.5	0.49 ± 0.02	3.39
4.5	4.80 ± 0.03	0.52
9.0	9.09 ± 0.05	0.53

[a] $n = 3$.
[b] Coefficient of variation (CV) ≡ (S.D./mean) × 100.

Table 4
Accuracy of method in plasma

Concentration (μg/ml)	Measured[a] (mean ± S.D.)	CV (%)[b]	Accuracy[c] (mean ± S.D.)
0.5	0.49 ± 0.02	3.32	97.4 ± 3.3
4.5	4.80 ± 0.03	0.53	106.6 ± 0.6
9.0	9.09 ± 0.05	0.53	100.5 ± 0.2

[a] $n = 5$.
[b] Coefficient of variation (CV) ≡ (S.D./mean) × 100.
[c] Accuracy (%) = (calculated concentration/nominal concentration) × 100.

found to be highly precise, with a R.S.D. < 3.5%, and accuracy in the range of 97.4–106.6% at each concentration tested.

The percent (%) recovery of D-*threo*-PPMP and IS from spiked plasma was evaluated. The extraction procedure was found to be efficient and reproducible with the percent recovery for the three QCs in plasma ranged from 88.9 to 92.4% for D-*threo*-PPMP, and 96.4 to 98.4% for IS (Table 5).

The stability of D-*threo*-PPMP was assessed in mouse plasma spiked with D-*threo*-PPMP at 1 μg/ml, and at 9 μg/ml. No significant reduction in measured D-*threo*-PPMP concentration was observed under the following conditions: after thawing to room temperature for 4 h (CV = 1.4 and 2.3%, respectively), after three −80 °C freeze/thaw cycles (CV = 0.6 and 3.3%, respectively), and after storage at −80 °C for 10 weeks (CV = 2.9 and 6.1%, respectively).

The present method was applied to plasma and liver samples of mice treated with D-*threo*-PPMP (1.5 mg, BID, ×7 doses) delivered by intraperitoneal injection in two different vectors (Fig. 3). Animals tolerated D-*threo*-PPMP well. Results showed that plasma levels of ∼10–20 μg/ml (∼20–44 μM) were obtained at 4 h after the last dose using the dose and delivery schedule described (Table 6). Liver levels obtained at 4 h after the last dose ranged from 23 to 89 μg/g tissue (Table 7). In con-

Table 5
Extraction efficiency in plasma

D-*threo*-PPMP (μg/ml)	Recovery (%)[a] (mean ± S.D.)	IS (μg/ml)	Recovery (%)[a] (mean ± S.D.)
0.5	88.9 ± 3.9	5	97.1 ± 0.8
4.5	90.8 ± 2.9		C.V. (%)[b] 0.85
9.0	92.4 ± 0.7		
Mean	90.7 ± 1.8		
C.V. (%)	1.95		

[a] $n = 5$.
[b] Coefficient of variation (CV) ≡ (S.D./mean) × 100.

Table 6
D-*threo*-PPMP plasma levels obtained in mice

Animal number	D-*threo*-PPMP (μg/ml)
Group one	
Control	ND
1	16.3
2	8.6
3	10.6
Group two	
Control	ND
1	22.4
2	11.9

Group one animals received D-*threo*-PPMP intraperitoneally as an albumin slurry. Group two animals received D-*threo*-PPMP intraperitoneally in NCI Diluent-12. ND, none detected.

Table 7
D-*threo*-PPMP levels obtained in liver in mice

Animal number	D-*threo*-PPMP (μg/g ± S.D.)		
1	42.3 ± 0.13		
2	23.6 ± 0.14		
3	89.2 ± 1.00		
4	34.7 ± 0.08		
5	44.7 ± 0.08		
Mean	46.9 ± 25.1	R.S.D.[a]	53.5

Animals received D-*threo*-PPMP intraperitoneally in NCI Diluent-12. Homogenates of individual livers were analyzed in duplicate.
[a] R.S.D., relative standard deviation.

trast, plasma and liver levels of D-*threo*-PPMP in animals that received 1 mg of D-*threo*-PPMP, BID, ×5 doses, by oral gavage, were below the level of detection (data not shown).

4. Discussion

An HPLC method for the determination of D-*threo*-PPMP in mouse plasma and liver is presented. A chromatographic method suitable for the separation of the salts of pure PPMP homologs (acyl chains C6-C18) has been reported which utilized detection at 254 nm [2]. However, we experienced unacceptable interference in various biological matrices at 254 nm and so developed the present methodology. The present method has been shown to be selective, and the linearity, precision, accuracy, and recovery from plasma were excellent. The method has been applied to preliminary formulation and bioavailability studies of D-*threo*-PPMP in the mouse. D-*threo*-PPMP is our preferred isomer of PPMP for the inhibition of glucosylceramide synthase (GCS) [9]. The conversion of ceramides into glucosylceramides has been reported to decrease ceramide-associated cytotoxicity in various tumor cell lines [15,16]. We have previously demonstrated that inhibitors of GCS can increase the cytotoxicity of ceramide-generating retinoids, such as fenretinide and, in particular, that D,L-*threo*-PPMP (3–12 μM) syngerized fenretinide (3–12 μM) cytotoxicity in multiple cancer cell lines in vitro [7,8]. However, at present, there are no GCS inhibitors that are effective at clinically tolerable dosing [15]. Using the present method, we have demonstrated that D-*threo*-PPMP was both well-tolerated and obtained plasma and tissue levels in mice when delivered intraperitoneally that equaled or exceeded concentrations in tissue culture medium needed to synergize fenretinide cytotoxicity. The present method will be useful for the further preclinical development of D-*threo*-PPMP, including both formulation/bioavailability studies and dose-response studies, in murine tumor xenografts.

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