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Determination of plasma and brain levels of isotretinoin in mice following single oral dose by high-performance liquid chromatography☆

Yongsheng Yang[a], Patrick J. Faustino[a,*], P. Scott Pine[b], Hirsch Davis[b], Neil Grunberg[c], Jennifer Phillips[c], Robbe C. Lyon[a], Lawrence X. Yu[d], Anthony B. Ciavarella[e], Alfred V. Del Grosso[e], Joseph P. Hanig[b]

[a] *Food and Drug Administration, Center for Drug Evaluation and Research/Division of Product Quality Research, 10903 New Hampshire Avenue, Silver Spring, MD 20993, USA*
[b] *Food and Drug Administration, Center for Drug Evaluation and Research/Division of Applied Pharmacology, 10903 New Hampshire Avenue, Silver Spring, MD 20993, USA*
[c] *Uniformed Services University of the Health Sciences, Department of Medical and Clinical Psychology, 4301 Jones Bridge Road, Bethesda, MD 20814, USA*
[d] *Food and Drug Administration, Center for Drug Evaluation and Research/Office of Generic Drugs, 7500 Standish Place, Rockville, MD 20855, USA*
[e] *Food and Drug Administration, Center for Biologics Evaluation and Research/Laboratory of Analytical Chemistry, 1401 Rockville Pike, Rockville, MD 20852, USA*

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Abstract

An isocratic reversed-phase high-performance liquid chromatographic method was established and validated according to FDA's Guidance for Industry, "Bioanalytical Method Validation", for the determination of isotretinoin in plasma and brain tissue from mice following single and multiple oral doses of Accutane®. Plasma sample preparation included deproteination with acetonitrile-perchloric acid followed by centrifugation. Brain tissue was homogenized and extracted with acetonitrile-perchloric acid followed by centrifugation. The supernatants were analyzed by high-performance liquid chromatography (HPLC). Benz[α]anthrancene-7,12-dione was used as the internal standard. Chromatographic separation was achieved on a C_{18} column using an acetonitrile–aqueous 0.5% acetic acid (85:15, v/v) elution. The average extraction efficiency was >95% for plasma and >82% for brain. The lower limit of quantification was 30 ng/mL for plasma and was 30 ng/0.1 g for brain tissue, respectively. The linear range for plasma was 30–600 ng/mL, and 15–300 ng/0.1 g for brain. Maximum concentrations of isotretinoin in both plasma and brain were observed at 1 h after single oral dosing (25 mg/kg). The maximum concentrations in plasma and brain were 2.36 μg/mL and 0.34 μg/g, respectively. The mean area under curve (AUC) in plasma was 6.13 μg h/mL. The mean eliminate half-life in plasma was estimated as 46 min.

Keywords: Accutane; Isotretinoin; HPLC; Pharmacokinetics; Mice

1. Introduction

Accutane® is clinically used to treat severe recalcitrant nodular acne. The active pharmaceutical ingredient, isotretinoin (13-*cis*-retinoic acid) shown in Fig. 1A, is a retinoid which is a naturally occurring metabolite of vitamin A [1,2]. In dermatology, isotretinoin proved to be efficacious in the treatment of severe cystic acne and related

☆ This scientific contribution is intended to support regulatory policy development. The views presented in this article have not been adopted as regulatory policies by the Food and Drug Administration at this time.

* Corresponding author. Tel.: +1 301 796 0021; fax: +1 301 796 9816.
E-mail address: faustinop@cder.fda.gov (P.J. Faustino).

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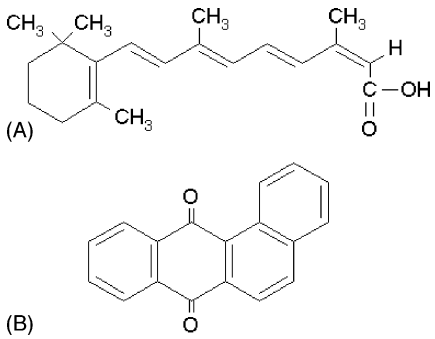

Fig. 1. Chemical structure of isotretinoin (A), and internal standard benz[α]anthrancene-7,12-dione (B).

disorders by oral administration [3–7]. It was also tested for topical application in the same indication. Isotretinoin has been extensively studied in animals and humans to evaluate the pharmacokinetics, safety and efficacy of this drug [7–10].

Several different chromatographic methods used to determine the retinoids in biological samples have been reported over the last few years [11–18]. However, most of the published methods are cumbersome and complex for routine use. Lengthy sample preparation schemes including extractions with an organic solvent, evaporation of solvent and reconstitution prior to HPLC analysis were used in several published procedures [12–14]. Lyophilization of the sample before extraction is required in other methods [15]. The use of HPLC-UV with column switching technique (on-line solid-phase extraction) appeared to be the most promising. However, a complicated processing technique and the requirement for a large amount of biological sample may limit its wide application [16,17]. Gadde and Burton [18] reported a simple reversed-phase (RP) HPLC method which is favorable for the analysis of biological fluids. Since larger sample volumes are required, this method was unsuitable for our study. Moreover, most of the previously cited methods were not validated.

The purpose of this study was to establish a simple and rapid procedure for the analysis of isotretinoin in murine plasma and brain tissue. The HPLC method was established and validated according to FDA's "Guidance for Industry"—Bioanalytical Method Validation [19] by determining its accuracy, precision, sensitivity, specificity and stability. The method has been successfully applied to the determination of isotretinoin time–concentration profiles in plasma and brain from mice following single and multiple oral dosing with Accutane®.

2. Experimental

2.1. Chemicals, reagents and product

USP Standard Reference Isotretinoin (lot I-353501) was obtained from USP (Rockville, MD). Internal standard: benz[α]anthrancene-7,12-dione and perchloric acid were purchased from Aldrich Chemical Co. (Milwaukee, WI). EDTA (tetrasodiumsalt, dihydrate) was obtained from J.T. Baker Inc. (Phillipsburg, NJ). Acetic acid and L-ascorbic acid were purchased from Sigma Chemical (St. Louis, MO). Methanol, acetonitrile and methylene chloride were purchased from Burdick & Jackson (Muskegon, MI). All other chemicals were of reagent grade. Accutane® (lot U0607) was supplied by the Division of Applied Pharmacology (Washington Drug Wholesale, Savage, MD).

2.2. Animal experiments

Blank murine plasma and brain tissues: Plasma (lot 0997-1323L) and brains (lot 0103-3343L) from male Swiss mice were purchased from Hilltop Lab Animals Inc. (Scottsdale, PA). Male Swiss Webster mice, age 21 days were purchased from Taconic Farms, and housed singly in standard polypropylene cages located in a limited access area maintaining a reverse 12 h light–dark cycle and controlled temperature (23 °C) and 50% relative humidity. Mice had free access to water and standard laboratory mouse food (Teklad Mouse Diet 7001). The dosing solution was freshly prepared from Accutane® capsules and diluted with soybean oil at varying concentrations while being maintained at 35 °C so as to deliver 25 mg/kg of isotretinoin in 0.2 mL. Doses were administered by intragastric intubation. Groups of 3 to 5 mice were sacrificed at 0.25, 1, 2, 4, and 24 h after orally dosing. The blood was drawn from the heart under pentobarbital anesthesia, and the brains were removed from the euthanized animals. Plasma was obtained after centrifugation of heparinized blood for 10 min at 3500 rpm for 10 min. All samples were stored at −20 °C until analysis.

2.3. Preparation of standard and plasma samples

The weighing of the compounds and samples, the preparation of calibration standards, as well as handling and analysis of all samples was performed under diffuse light conditions.

2.3.1. Preparation of standards

Two stock solutions (I and II) of isotretinoin (100 μg/mL) were prepared in low actinic volumetric flasks by dissolving 10 mg of isotretinoin in 100 mL of methanol. Stock solution I was for the calibration standards, and stock solution II for quality control samples. The stock solution of internal standard, as shown in Fig. 1B, benz[α]anthrancene-7,12-dione (200 μg/mL) was also prepared in methanol. Ultrasonication was used for complete dissolution. Eight different spiking standard solutions were prepared from the stock solution I to yield 1.2–24 μg/mL of isotretinoin. Plasma calibration standards (30–600 ng/mL) were prepared by pipeting 100 μL of blank plasma, 10 μL of standard solution, 10 μL of internal standard, 20 μL of 0.1 M perchloric acid solution, and 260 μL of acetonitrile into 1.5 mL plastic centrifuge vials. The vials were capped, vortexed and then centrifuged at 6000 rpm for 10 min at 10 °C. The clear supernatant was transferred to 0.5-mL amber conical vials placed inside white teflon holders and

then transferred to a temperature controlled automatic injector for HPLC analysis.

2.3.2. Preparation of quality control (QC) standards

Four quality control standards were prepared from the isotretinoin stock solution II to yield concentrations of 1.2, 3.6, 10 and 20 μg/mL. To 100 μL of blank plasma, 10 μL of standard solution, 10 μL of internal standard solution (200 μg/mL) were added and then treated with 20 μL of 0.1 M perchloric acid solution and 260 μL of acetonitrile in 1.5 mL plastic centrifuge vials. The vials were capped, vortexed and then centrifuged at 6000 rpm for 10 min at 10 °C. The clear supernatant was transferred to 0.5-mL amber conical vials placed inside white teflon holders and then transferred to a temperature controlled automatic injector for HPLC analysis.

2.3.3. Preparation of samples

A 100 μL aliquot of plasma sample was spiked with 10 μL of internal standard solution (200 μg/mL) and then treated with 20 μL of 0.1 M perchloric acid solution and 270 μL of acetonitrile. The vials were capped, vortexed, centrifuged and the supernatant was loaded into the HPLC automatic injector for analysis as noted above.

2.4. Preparation of standard and brain samples

Isotretinoin stock solutions, working standard solutions, QC standard solutions and internal standard solution were prepared as described above. Brain standards were prepared by adding 50 μL of working standard solution, 50 μL of internal standard solution and 500 μL of homogenization solution containing 0.5 mg/mL each of EDTA and ascorbic acid to 0.2 g of blank brain tissue. The brain was then homogenized at 0 °C using a small tissue homogenizer. Homogenates were immediately extracted with 80 μL of 0.1 M perchloric acid solution and 1320 μL of acetonitrile. Samples were vortexed and centrifuged at 6000 rpm for 10 min at 10 °C. The clear supernatant was loaded into the HPLC automatic injector for analysis.

2.4.1. Preparation of QC standards

To a 0.2 g of blank brain, 50 μL of QC standard solution, 50 μL of internal standard solution and 500 μL of homogenization solution were added. The brain was then homogenized and extracted with 80 μL of 0.1 M perchloric acid solution and 1320 μL of acetonitrile. Samples were vortexed, centrifuged and analyzed.

2.4.2. Preparation of samples

A 0.2 g of brain tissue sample was spiked with 50 μL of internal standard solution and added 500 μL of homogenization solution. The brain was homogenized and treated with 80 μL of 0.1 M perchloric acid solution and 1370 μL of acetonitrile. Samples were vortexed, centrifuged and analyzed.

2.5. Analytical method

All standards and samples were analyzed on Agilent 1100 (Wilmington, DE) high performance liquid chromatography system equipped with a 1311A series quaternary pump, 1315 series multiple wavelength detector, a 1316A series thermostated column compartment, and a 1329A series thermostated automated injector and 1322A series solvent degassor modules. Separation was obtained on a Phenomenex C_{18} (5 μM, 250 mm × 4.6 mm), Hydro-RP 80A (Torrance, CA) reverse-phase HPLC column with a Phenomenex C_{18} Security Guard cartridge (4.0 mm × 3.0 mm). The mobile phase was acetonitrile–aqueous 0.5% acetic acid (85:15, v/v) delivered isocratically. The flow rate was 1.5 mL/min. The injection volume for samples and standards was 75 μL. The UV detection wavelength was 360 nm.

2.6. Pharmacokinetic analysis

The following pharmacokinetic parameters were calculated for isotretinoin. The time (T_{max}) of the maximum observed blood and brain concentrations and the maximum observed blood and brain concentrations (C_{max}) were read directly from the observed concentration–time data. The area under the blood concentration–time curves (AUC) from time zero to infinity was calculated by trapezoidal summation from time zero to the time (TF) of the final measurable concentration (CF), then extrapolated to time infinity by adding the quotient of CF/β to the corresponding $AUC_{0–TF}$. The disposition rate constant β and clearance $t_{1/2\beta}$ were calculated from least-squares fits of the last three drug concentrations.

2.7. Mass spectrometry

LC/MS analysis was performed on an Agilent 1100 LC/MSD TRAP SL (Wilmington, DE). Separation was obtained on a Phenomenex $C_{18(2)}$ (3 μM, 100 mm × 4.6 mm, LUNA), RP-HPLC column with a Phenomenex C_{18} Security Guard cartridge (4.0 mm × 3.0 mm). The column temperature was maintained at 18 °C. The mobile phase was acetonitrile–aqueous 0.5% acetic acid (60:40, v/v) delivered isocratically. The flow rate was 1.0 mL/min. The injection volume was 5 uL with the samples maintained at 4 °C.

Ion trap mass spectrometry was carried out using electrospray ionization in the negative mode. The mass of the molecular ion for the accutane glucuronide (*m/z* 475) was isolated and fragmented using collision induced dissociation (CID) with helium. Nitrogen was used as the nebulizing gas at 65 psi. Nitrogen was also used as the drying gas at 9.5 L/min and 350 °C. In addition, the following parameters were set: capillary voltage, 4803 V; capillary exit voltage, −126.6 V; skimmer, −40.0 V; octopole 1 dc, −12.00 V; octopole 2 dc, −1.70 V; lens 1, 5.0 V; lens 2, 60.0 V; trap drive, 51.5; fragmentation amplitude, 0.6.

3. Results and discussion

3.1. Analytical method and validation

Owing to the photolability and sensitivity to heat and oxidation of the retinoids, extreme precautions to protect isotretinoin from white light and to minimize exposure to oxygen need to be taken when handling isotretinoin and respective solution. The assay method for biological fluids and tissues analysis should therefore be simple with few sample handling manipulations, yet specific to resolve isotretinoin from its metabolites, degradation products and matrix components. Our efforts were focussed on simplifying the sample preparation process and developing a sensitive and specific method for isotretinoin. By modifying Gadde and Burton's [18] method for plasma sample preparation and analysis and Kalin et al.'s [9] method for brain sample preparation, an isocratic reversed-phase high-performance liquid chromatographic method was established and validated for the determination of isotretinoin in plasma and brain tissue from mice. Modifications included utilizing different mobile phase, internal standard and new HPLC column and temperature control technology that allowed for the development of time efficient methods while enhancing the resolution of isotretinoin from other highly polar metabolites such as 4-oxo-13-*cis*-retinoic acid, 4-oxo-all-*trans*-retinoic acid and/or 13-*cis*-retinoyl-β-glucuronide. Plasma sample preparation included deproteination with acetonitrile-perchloric acid followed by centrifugation. Brain tissue was homogenized and extracted with acetonitrile-perchloric acid. Details of samples and standard preparation are given in Section 2.

3.1.1. Stability

The response factor of isotretinoin standard and internal standard solutions was found to be unchanged for up to 15 days under temperature of 4 °C. Less than 0.3% concentration difference was found between the solutions freshly prepared and those aged 15 days. The solutions can therefore be used within this period without the results being affected. The extracts of plasma and brain were found to be stable for up to 12 h under the present analysis condition (10 °C) controlled at both the autosamplier and column modules. Therefore, the samples were analyzed within this period.

3.1.2. Selectivity

To obtain the selectivity for retinoic acids and their metabolites is a demanding task, especially at concentrations <10 ng/mL. Typical chromatograms of blank and isotretinoin spiked plasma and brain samples are shown in Fig. 2A–D. Isotretinoin and internal standard (benz[α]anthrancene-7,12-

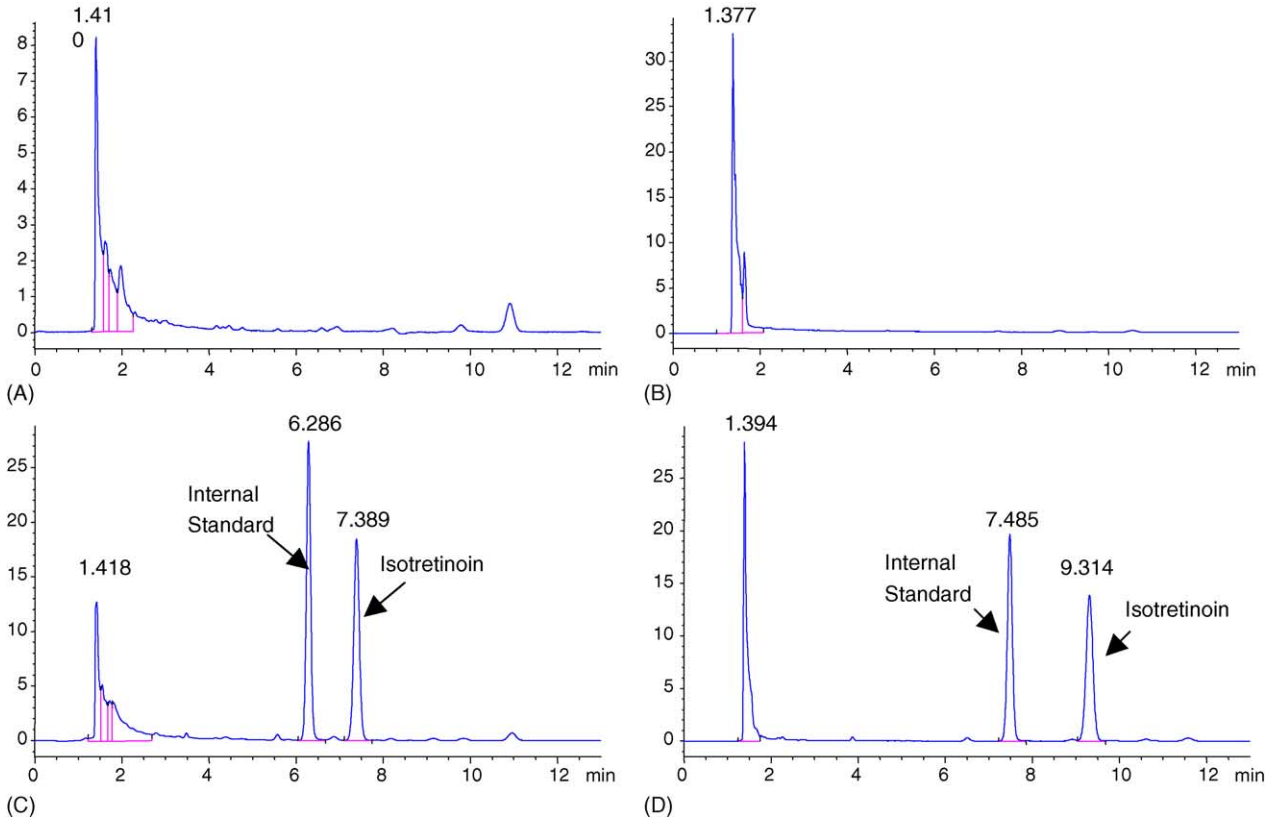

Fig. 2. Chromatograms of a blank plasma sample (A), a blank brain sample (B), a plasma spiked with isotretinoin and internal standard benz[α]anthrancene-7,12-dione (C), and a brain spiked with isotretinoin and internal standard benz[α]anthrancene-7,12-dione (D). Separation is accomplished on the 250 mm × 4.6 mm Hydro-RP 80A column with mobile phase of acetonitrile–aqueous 0.5% acetic acid (85:15, v/v) delivered isocratically at a rate of 1.5 mL/min. Detection was performed at 360 nm.

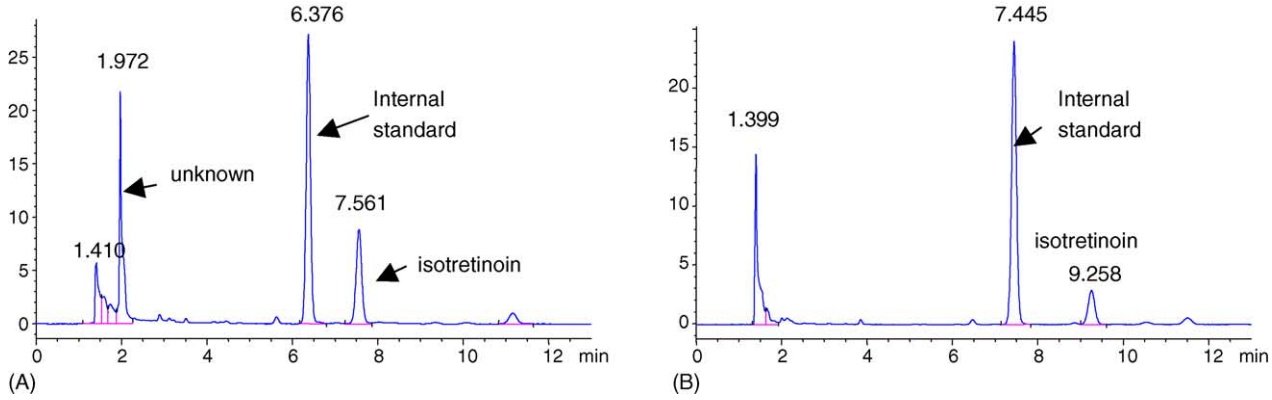

Fig. 3. Typical chromatograms of a plasma sample (A) and a brain sample (B) from mice treated with oral dose of accutane showing separation of unknown polar material, internal standard and isotretinoin.

Table 1
Parameters of calibration curve for murine plasma samples ($n = 3$)

	Validation 1	Validation 2	Validation 3	In study
Linear range (ng/mL)	30–600	30–600	30–600	30–600
Calibrators	8	8	8	8
R^2 value	0.9973	0.9891	0.9935	0.9980
Slope	0.0023	0.0020	0.0021	0.0021

dione) peaks in these chromatogram are well resolved from each other and from its major metabolites. A typical chromatogram of plasma and brain from mice dosed orally with accutane is shown in Fig. 3A and B.

3.1.3. Linearity and limit of quantification

The calibration curve was linear over the validated range 30–600 ng/mL for spiked plasma standards and 30–600 ng/0.1 g for spiked brain standards. Correlation coefficient was greater than 0.99 on 4 different days (Tables 1 and 2). The limit of quantification was 30 ng/mL for plasma samples and 30 ng/0.1 g for brain samples.

3.1.4. Precision and accuracy

Intra- and inter-assay precision and accuracy results are listed in Tables 3–5. The mean relative standard deviations were 1.5–7.2% for plasma and 0.4–1.4% for brain, respectively. The mean accuracies were 94–104% for plasma and 96% to 101% for brain, respectively. The mean inter-day precisions were 5.7–15.3% for plasma and 5.3–8.2% for brain, respectively. The acceptance ranges of accuracies are 80–120% for LLOQ, and 85–115% for the low, intermediate and high QCs. The allowable limits of %R.S.D. are 20% for LLOQ, and 15% for the low, intermediate and high QCs. Both accuracy and precision were found to be acceptable.

Table 2
Parameters of calibration curve for murine brain samples ($n = 3$)

	Validation 1	Validation 2	Validation 3	In study
Linear range (ng/0.1 g)	30–600	30–600	30–600	30–600
Calibrators	8	8	8	8
R^2 value	0.9942	0.9986	0.9989	0.9985
Slope	0.0023	0.0021	0.0024	0.0023

3.1.5. Recovery

The recovery from plasma and brain was determined following replicate analysis, by comparison of peak areas of spiked samples, processed as described above, with the peak areas obtained from the true concentration of the pure authentic standard. Recovery experiments were performed by comparing the analytical results for extracted samples at three concentrations (low, medium, and high) with unextracted standards that represent 100% recovery. The recoveries of the analytes were greater than 95% for plasma and 82% for brain, respectively.

3.2. Pharmacokinetics

The mean plasma and brain concentration–time profiles of isotretinoin are presented in Fig. 4. The pharmacokinetics of isotretinoin following an oral dose can be adequately described by a single compartment model. The absorption was rapid following the oral suspension dose. The concentration of isotretinoin in plasma increased to a maximum of 2.36 μg/mL within 1 h after dosing, then declined exponentially with a $t_{1/2}$ value of 46 min (Fig. 4). The mean area under curves in plasma were 6.13 μg h/mL. Maximum concentrations of 0.34 μg/g in brain was observed at 1 h after dosing (Fig. 4). There exist some interesting differences when the pharmacokinetic parameters presented herein are compared to those previously published by Kalin et al. [9]. The previous study was conducted by orally single dosing isotretinoin suspension in mice using an aqueous-based formulation and a smaller dose (10 mg/kg). The elimination of isotretinoin from serum and tissues also proceeded exponentially after initial accumulation phases. Serum levels of isotretinoin reached their maxima within 15–30 min with a concentration of 8.33 μg/mL and $t_{1/2}$ values of 19 min. Brain

Table 3
Intra-day precision and accuracy for murine plasma samples ($n = 5$)

Standards (ng/mL)	Precision (%R.S.D.)			Accuracy (%)				
	Validation 1	Validation 2	Validation 3	In study	Validation 1	Validation 2	Validation 3	In study
30	10.8	5.2	5.1	NA	80	107	108	NA
90	2.6	1.9	7.0	1.5	92	103	113	102
250	1.1	6.4	4.6	2.0	88	95	95	94
500	4.5	4.7	5.6	7.2	93	101	95	104

NA: not applicable.

Table 4
Intra-day precision and accuracy for murine brain samples ($n = 5$)

Standards (ng/0.1 g)	Precision (%R.S.D.)			Accuracy (%)				
	Validation 1	Validation 2	Validation 3	In study	Validation 1	Validation 2	Validation 3	In study
30	3.0	3.1	4.8	NA	102	85	94	NA
90	1.9	4.8	3.0	0.5	103	100	98	96
250	3.7	4.2	3.9	0.4	96	100	93	96
500	6.4	5.0	4.2	1.4	91	94	96	101

NA: not applicable.

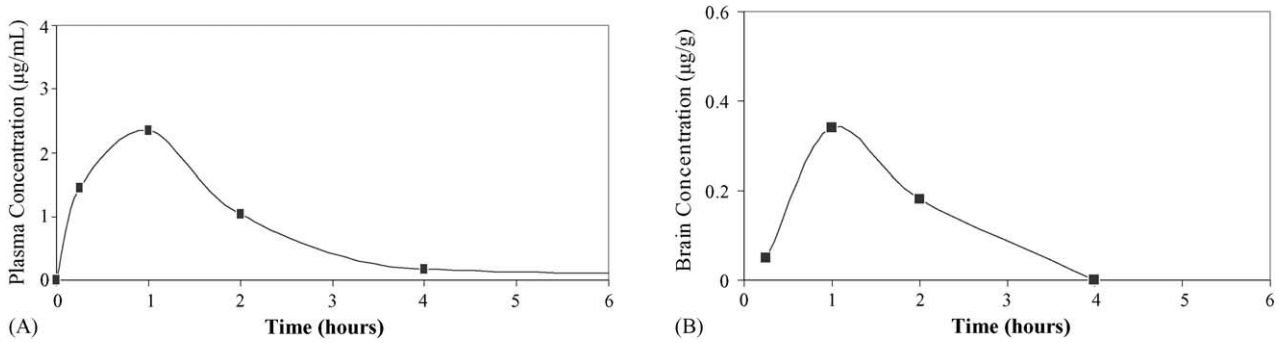

Fig. 4. The time-course of the mean plasma (A) and brain (B) concentrations profile of isotretinoin following a single oral dose of accutane.

levels of isotretinoin reached their maxima within 15–30 min with a concentration of 0.97 μg/mL and $t_{1/2}$ values of 16 min. The rapid absorption, higher C_{max} and rapid elimination of isotretinoin with short half-lives observed from the previous study were discrepant with those presented herein. The difference in composition of formulations may be the major reason to cause this discrepancy. It is reported that soybean oil based formulations and chain length of fatty acids can significantly delay gastric emptying and thereby reduce absorption [20]. Additionally hydrogenated vegetable oil flakes present in this formulation [21] and increases in formulation viscosity [22] can also delay gastric emptying. The pH of the previous study's formulation was alkaline which significantly enhances gastric emptying [23]. Since the mean gastric emptying time in mice is 30 min [24] and the previous study detected peak isotretinoin levels within 15 to 30 min, it is likely the significant formulation differences contributed to the discrepancy.

Table 5
Inter-day precision for murine plasma and brain samples ($n = 15$–20)

Standards (ng/mL)	Precision of plasma (%R.S.D.)	Standards (ng/0.1 g)	Precision of brain (%R.S.D.)
30	15.3	30	8.2
90	9.6	90	4.0
250	5.8	250	4.6
500	5.7	500	5.3

In the human, 4-oxo-13-*cis*-retinoic acid is by far the most predominant retinoid present during isotretinoin therapy [25,26]; also, 13-*cis*-retinoyl-β-glucuronide was identified as a metabolite [27]. The 4-oxo-13-*cis*-retinoic acid has been shown to be active in the mouse papilloma model as well as the rat sebum model. The main metabolites of isotretinoin in the monkey were the 4-oxo-13-*cis*-retinoic acid and the 13-*cis*-retinoyl-β-glucuronide, while the predominant metabolite of isotretinoin in mice was found to be the 13-*cis*-retinoyl-β-glucuronide [27,28]. As in the Fig. 3A and B, a relatively small peak of parent compound was detected in both plasma and brain samples from mice orally administered isotretinoin. The oxidized metabolites, 4-oxo-13-*cis*-retinoic acid or 4-oxo-all-*trans*-retinoic acid, were below the limit of quantification. In contrast, a large peak of unknown material with

retention time of only 2 min was eluted out immediately following a solvent peak. It suggests this is a very polar material. Interestingly, Kalin et al. [9] found similar polar materials present in bile, feces, and urine in mice following oral dosing isotretinoin. However, they were unable to identify this polar compound or determine the route of elimination. We suspect this might be a possible glucuronidated conjugate metabolite, since glucuronidation increases hydrophilicity. Mass spectrometry was performed on this polar material. Mass spectrometry data revealed a molecular ion peak of 475 consistent with an isotretinoin-glucuronide species. The resulting fragmentation of the 475 peak generated a 299 peak and fragmentation consistent with a 13-*cis*-retinoic acid standard (data not shown). Therefore, the polar material is very likely to be 13-*cis*-retinoyl-β-glucuronide. When the reference standards for 13-*cis*-retinoyl-β-glucuronide or other conjugate compounds become available we will further identify and quantify this material in mice.

4. Conclusions

An isocratic RP-HPLC method for the determination of isotretinoin in murine plasma and brain tissue has been validated according to FDA's "Guidance for Industry"—Bioanalytical Method Validation. The method was accurate, sensitive, selective and reproducible. The simple sample preparation, easily handling procedures and efficient isocratic reverse-phase HPLC are the major advantages of the method. The usefulness of this method is demonstrated by its successful application for pharmacokinetic studies of isotretinoin in murine plasma and brain. The method has been shown to be stable and reproducible for application to large-scale pre-clinical studies.

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