Journal of Chromatography B, 878 (2010) 1885–1888

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Short communication

Quantitative analysis of 3α,6α,24-trihydroxy-24,24-di(trifluoromethyl)-5β-cholane, a potent synthetic steroidal liver X receptor agonist in plasma using liquid chromatography–tandem mass spectrometry

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ARTICLE INFO

Article history:
Received 28 January 2010
Accepted 5 May 2010
Available online 12 May 2010

Keywords:
Atherosclerosis
Cholanic acid
Pharmacokinetics
Quantitative analysis
Method validation
LC–MS–MS

ABSTRACT

The steroidal liver X receptor agonist, 3α,6α,24-trihydroxy-24,24-di(trifluoromethyl)-5β-cholane (ATI-829) is a potential therapeutic agent for the treatment of atherosclerosis. A sensitive and selective liquid chromatography–tandem mass spectrometry (LC–MS–MS) method for the quantification of ATI-829 in mouse plasma was developed and validated. Proteins in a 25 μL aliquot of mouse plasma were precipitated, and ATI-829 was extracted from the precipitate by the addition of 125 μL methanol. The overall extraction efficiency was greater than 99%. LC–MS–MS with negative ion electrospray and selected reaction monitoring was used for the quantitative analysis of ATI-829. The lower limit of quantitation of ATI-829 corresponded to 5.0 ng/mL (9.7 nM) plasma. Interference from matrix was negligible. The calibration curve was linear over the range 5–2000 ng/mL. The intra-day precision and inter-day precision of the analyses were <4.5% and <6%, respectively, and the accuracy ranged from 92% to 103%. ATI-829 in plasma was stable for at least 6 h at room temperature, 1 week at 4 °C, and 3 weeks at −20 °C. The validated method was then utilized for pharmacokinetic studies of ATI-829 administered to mice.

1. Introduction

Statins block cholesterol and lipid biosynthesis and decrease the influx of cholesterol into plaques but do not enhance cholesterol removal from arterial walls [1]. Liver X receptors (LXRs) are members of the nuclear receptor superfamily [2,3] and function as oxysterol receptors that mediate cholesterol efflux from foam cells to nascent and mature high density lipoprotein [4–7]. However, LXR activation in liver may lead to the accumulation of triglycerides at this site [8,9] as well as elevation of plasma triglycerides [10]. The steroidal LXR agonist ATI-829 (3α,6α,24-trihydroxy-24,24-di(trifluoromethyl)-5β-cholane; see structure in Fig. 1) has been reported to selectively activate LXR target gene expression in the intestine and macrophages but not in the liver [11]. To evaluate the pharmacokinetics of ATI-829 *in vivo*, a selective and sensitive analytical method is required to determine the concentration of ATI-829 in plasma.

ATI-829 is a synthetic derivative of cholanic acid that lacks a UV chromophore. As an alternative to UV absorbance detection, Torchia et al. [12] used HPLC with evaporative light scattering detection for the analysis of bile acids, but the linear range of quantitation of using this approach (0.08–10 nmol; dynamic range of 125) is too narrow for pharmacokinetic studies of ATI-829. Johnson et al. [13] used HPLC with tandem mass spectrometric detection (LC–MS–MS) for the analysis of bile acids after derivatization to form their dimethylaminoethyl esters. Scherer et al. [14] and Alnouti et al. [15] reported LC–MS–MS assays for the quantification of bile acids in serum that do not require derivatization with limit of detection (LOD) values of ∼10 nM and 2 μM, respectively. These and other mass spectrometry methods for the determination of bile acids in biological fluids have been reviewed by Griffiths and Sjövall [16].

In support of *in vivo* studies of the pharmacokinetics and anti-atherosclerotic effects of ATI-829, we developed and validated a selective and sensitive analytical method for its quantitative analysis in mouse plasma using LC–MS–MS. No derivatization is required,

Abbreviations: LXR, liver X receptor; SRM, selected reaction monitoring; ATI-829, 3α,6α,24-trihydroxy-24,24-di(trifluoromethyl)-5β-cholane; MTBE, methyl *tert*-butyl ether.

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doi:10.1016/j.jchromb.2010.05.009

the dynamic range is 5–2000 ng/mL, and the LOD is 0.3 nM in plasma. This method was then applied to the analysis of ATI-829 in mouse plasma in support of a pharmacokinetics study.

2. Experimental

2.1. Reagents and chemicals

HPLC-grade methanol, ethyl acetate, methyl *tert*-butyl ether (MTBE), and formic acid were purchased from Fisher Scientific (Fair Lawn, NJ, USA). 5β-Cholanic acid-3α,7α-diol-12-onediacetate (internal standard) was purchased from Steraloids (Newport, RI, USA). ATI-829 was synthesized as described previously [11]. Deionized water was produced using a Milli-Q purification system (Millipore, Milford, MA, USA). Blank pooled mouse plasma was purchased from Biochemed Pharmaceuticals (Winchester, VA, USA).

2.2. Preparation of standard solutions

Primary standard stock solutions of ATI-829 and internal standard were prepared separately in methanol at 1 mg/mL and were stored at 4 °C. The working solutions of ATI-829 and internal standard were prepared at 40 μg/mL by dilution from the primary stock solutions with 20% methanol (v/v). The standard working solutions at appropriate concentrations were obtained in 20% methanol by serial dilution (v/v) of working solutions. The calibration standards were prepared by spiking working solution into blank mouse plasma, giving final ATI-829 plasma concentrations of 5, 25, 50, 100, 200, 500, 1000, and 2000 ng/mL. Quality control (QC) samples were prepared in a manner similar to the calibration standards at three concentration levels (15, 250, and 1800 ng/mL).

2.3. Preparation of mouse plasma and pharmacokinetics analysis

Male C57BL/6 mice (5 animals per group) were administered ATI-829 in a 20% microemulsion by gavage at a single dose of 10 mg/kg. The control group received microemulsion (vehicle) only. After gavage, blood from the retro-orbital plexus was collected at different time points and no more than three bleedings were performed on each mouse. The blood samples were centrifuged at 1000 × *g* for 10 min to obtain plasma which was frozen at −80 °C until analysis. Pharmacokinetics parameters were calculated using Phoenix WinNonlin version 6.1 (Pharsight, Mountain View, CA) based on non-compartmental analysis.

2.4. Sample preparation

A working solution of internal standard was prepared in methanol at a final concentration of 200 ng/mL. A 25-μL aliquot of calibration standard, QC sample or plasma sample was vortex-mixed with 125 μL of internal standard solution for 30 s and centrifuged at 10,000 × *g* for 10 min. A 10 μL aliquot of each supernatant was injected into the LC–MS–MS system for analysis.

2.5. LC–MS–MS

The HPLC system consisted of Shimadzu (Columbia, MD, USA) LC-10ADvp pumps and an LC PAL autosampler (CTC Analytics AG, Switzerland) interfaced to an Applied Biosystems (Foster City, CA, USA) API 4000 triple quadrupole mass spectrometer. Chromatographic separations were carried out using a Zorbax SB-C_{18} analytical LC column (2.2 mm × 100 mm, 3.5 μm; Agilent Technologies, USA). The mobile phase consisted of a linear gradient from 70% to 95% methanol in water containing 0.05% formic acid at a flow rate of 0.2 mL/min. The injection volume was 10 μL. ATI-829 and the internal standard eluted at 7.1 and 4.8 min, respectively. The injector needle and sample loop were rinsed twice with methanol between injections to minimize sample carryover.

Negative ion electrospray tandem mass spectrometric analysis was carried out at unit resolution with collision-induced dissociation and selective reaction monitoring (SRM). The source temperature was 450 °C, the electrospray voltage was −4500 V, and the declustering potential was −110 V for ATI-829 and −90 V for the internal standard. Nitrogen was used as the collision gas at collision energies of −26 V and −32 V for ATI-829 and the internal standard, respectively. The ion transitions of *m*/*z* 513.4 to *m*/*z* 443.3 for ATI-829 and *m*/*z* 489.3 to *m*/*z* 447.2 for the internal standard were monitored with a dwell time of 400 ms per transition. Data were acquired and analyzed using Applied Biosystems Analyst 1.2 software.

2.6. Method validation

The specificity of the method was investigated by analyzing three batches of drug-free mouse plasma for any endogenous co-eluting interference at the retention times of ATI-829 and internal standard. Three lots of mouse plasma containing internal standard and ATI-829 at the LOD or LLOQ were also tested. Reproducibility of the LC–MS–MS response for these samples was used to evaluate the presence or absence of interference and the lot-to-lot variation.

Matrix interference caused by the suppression or enhancement of ionization during LC–MS–MS was evaluated by using post-column infusion measurements. A 10 μL aliquot of extract of blank mouse plasma was injected into the LC–MS–MS system. Simultaneously, a standard solution containing 100 ng/mL of ATI-829 or internal standard was infused post-column into the electrospray ion source at a flow rate of 10 μL/min. Matrix interference could be determined by changes in the MS–MS response at the retention times of ATI-829 or internal standard. Alternatively, the slopes of the standard curve were compared using three different lots of mouse plasma (matrix) according to the method of Matuszewski [17].

The calibration standards were analyzed in triplicate on 3 different days to determine the linearity of the LC–MS–MS signal response. Method validation was performed by evaluating intra-day and inter-day accuracy and precision of three quality control samples at low (15 ng/mL), medium (250 ng/mL) and high (1800 ng/mL) concentrations on 3 different study days. Each batch was processed on a separate day and had 6 replicates of QC samples. Quantification was based on the ratios of the peak areas of ATI-829 against that of the internal standard. Accuracy was defined as the percent bias of the measured concentration relative to the nominal concentration of each quality control sample. Precision was defined as percent relative standard deviation obtained from replicates of the QC samples.

The stability of ATI-829 in plasma was assessed using QC samples ($n = 5$) that were stored at room temperature for 6 h, at 4 °C in a refrigerator for 6 days and at −20 °C in a freezer for 3 weeks. All results were compared with those of freshly prepared QC samples. ATI-829 was considered to be stable in plasma when the measured concentration in the test samples ranged from 85% to 115% of the initial concentration.

The recoveries of ATI-829 from plasma using liquid–liquid extraction or protein precipitation were evaluated using quality control samples ($n = 3$) at low (15 ng/mL), medium (250 ng/mL) and high (1800 ng/mL) concentrations. The measured concentrations were compared with the spiked plasma concentrations. For liquid–liquid extraction, a 25 μL aliquot of plasma spiked with ATI-829 was extracted three times with 125 μL portions of MTBE, three times with 125 μL portions of ethyl acetate saturated with water, once with 500 μL MTBE, or once with 500 μL ethyl acetate saturated with water. After evaporation to dryness and reconstitution in

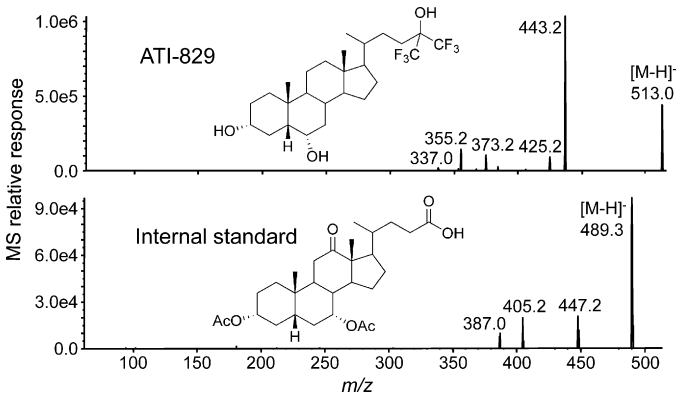

Fig. 1. Negative ion electrospray product ion tandem mass spectra of the deprotonated molecules of ATI-829 (*m*/*z* 513) and 5β-cholanic acid-3α,7α-diol-12-onediacetate (*m*/*z* 489). Nitrogen was used as the collision gas at collision energies of −26 V and −32 V for ATI-829 and the internal standard, respectively.

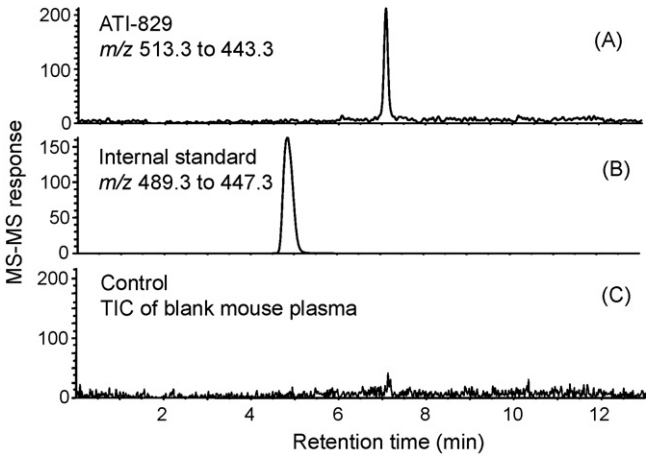

Fig. 2. LC–MS–MS selected reaction monitoring (SRM) chromatograms of ATI-829 and internal standard. (A) ATI-829 (0.5 ng/mL) in mouse plasma; (B) internal standard 5β-cholanic acid-3α,7α-diol-12-onediacetate (200 ng/mL); and (C) total ion chromatogram of extracted blank mouse plasma.

150 μL of 80% aqueous methanol, each extract was analyzed using LC–MS–MS. For evaluation of the sample preparation method using protein precipitation, a 25 μL aliquot of plasma was mixed with 125 μL methanol. After centrifugation, 10 μL of the supernatant was injected into the LC–MS–MS system.

3. Results and discussion

3.1. Recovery

Liquid–liquid extraction and protein precipitation, both common sample preparation approaches for the analysis of drug levels in plasma [18,19], were compared for the extraction of ATI-829 from plasma. Since ATI-829 is poorly soluble in most solvents, the non-polar solvent MTBE and a more polar solvent, ethyl acetate, were evaluated for the recovery of API-829 from plasma at three different concentrations. Extraction with MTBE gave poor recoveries of API-829 from plasma that ranged from 20.3% to 30.2% when a 20-volume excess of MTBE was used once. Extraction efficiency improved to nearly 50% when three extractions with a 5-volume excess of MTBE were used. Ethyl acetate provided better recovery of ATI-829 from plasma than did MTBE. The recovery of ATI-829 using a single extraction with ethyl acetate was between 45% and 65%, depending upon ATI-829 concentration, and improved to between 70% and 76% when using three ethyl acetate extractions.

The most efficient extraction of ATI-829 from plasma was obtained when using methanol. Treating plasma with 5-volumes of methanol served the dual purpose of precipitating proteins and extracting ATI-829. Using methanol, the recoveries of ATI-829 from plasma at initial concentrations of 15, 200 or 1800 ng/mL were 104%, 104%, and 99.1%, respectively, using the protein precipitation procedure. Furthermore, the background noise during LC–MS–MS analysis of plasma samples prepared using the protein precipitation method was low and as good as that observed when using liquid–liquid extraction. Protein precipitation and extraction of ATI-829 using methanol were used for the preparation of all subsequent plasma samples, because this procedure was simple, fast, provided quantitative recovery of ATI-829, and gave extracts with low background noise during LC–MS–MS.

3.2. Specificity and matrix effects

Deprotonated ATI-829 was detected at *m*/*z* 513 during negative ion electrospray mass spectrometry. ATI-829 did not form ions during positive ion electrospray mass spectrometry or when using atmospheric pressure chemical ionization. Collision-induced dissociation of the precursor ion of *m*/*z* 513 formed product ions of *m*/*z* 443, 425, 373, 355, and *m*/*z* 337, corresponding to $[M-H-HCF_3]^-$, $[M-H-HCF_3-H_2O]^-$, $[M-H-2HCF_3]^-$, $[M-H-2HCF_3-H_2O]^-$, and $[M-H-2HCF_3-2H_2O]^-$, respectively (Fig. 1). Since the ion of *m*/*z* 443 was the base peak of the product spectrum of the deprotonated molecule of *m*/*z* 513, the mass transition of *m*/*z* 513 to *m*/*z* 443 was used for selected reaction monitoring (SRM) and quantification of ATI-829 during LC–MS–MS. In addition, the ion transition of *m*/*z* 489 to *m*/*z* 447 was used for SRM to measure the internal standard 5β-cholanic acid-3α,7α-diol-12-one-diacetate. The total ion chromatogram of extracted blank mouse plasma and the SRM chromatogram of ATI-829 near the limit of quantification in an extracted calibration sample are shown in Fig. 2. No peaks corresponding to endogenous plasma compounds were detected at the retention times of ATI-829 or the internal standard during the analysis of blank mouse plasma samples.

Matrix effects are common during electrospray mass spectrometry of plasma when co-eluting matrix compounds attenuate or enhance the analyte signal. To determine if matrix interference affected the analysis of ATI-829, an extracted blank plasma sample was injected into the LC–MS–MS system while ATI-829 was introduced continuously post-column. No significant matrix interference was observed between 4.1 and 7.4 min which included the elution time of ATI-829 at 7.1 min and the internal standard eluting a 4.8 min (data not shown). Ion suppression caused by the matrix was observed at 1.5–4.0 and 7.5–11.0 min but did not affect the measurement of ATI-829. In a similar experiment (data not shown), no matrix interference was observed for the internal standard. Also, standard curves prepared using three difference lots of mouse plasma showed no significant variation in slope which indicated no matrix interference according to the method of Matuszewski [17].

3.3. Linearity and sensitivity

Fit by using the linear regression method with a $1/X$ weighting factor, the calibration curve for ATI-829 in plasma was linear over the concentration range of 5–2000 ng/mL. On 3 different days, the calibration curves for ATI-829 were described by the regression equations $y = 0.887x - 0.0022$, $y = 0.855x - 0.0029$, and $y = 0.895x - 0.0115$, respectively, all with coefficients of determination (R^2) greater than 0.996. The limit of detection (LOD) of ATI-829 in mouse plasma was 0.25 pg on-column (0.29 nM), based on a signal-to-noise ratio of 3:1. Using the lowest concentration of the calibration curve as the lower limit of quantification (LLOQ)

Table 1
Intra-assay and inter-assay accuracy and precision.

Concentration (ng/mL)	Intra-day (n = 6)		Inter-day (n = 18)	
	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)
15	92.7	3.7	96.6	5.8
250	99.2	2.3	102.9	5.9
1800	96.7	4.5	96.7	6.0

Table 2
Stability of ATI-829 in mouse plasma.

Concentration (ng/mL)	Relative difference (%; n = 5)[a]		
	22 °C for 6 h	4 °C for 6 days	−20 °C for 3 weeks
15	−2.8 ± 2.3[b]	−1.8 ± 1.8	−6.8 ± 1.9
250	5.5 ± 3.8	−0.2 ± 0.9	−6.6 ± 3.6
1800	−8.7 ± 2.2	−6.6 ± 0.8	−7.5 ± 0.4

[a] Stability values are expressed as relative differences between the measured concentrations of freshly prepared quality control samples and samples stored under various conditions.
[b] ±S.D.

according to FDA guidance [20], the LLOQ of this method is 5 ng/mL (9.7 nM).

3.4. Accuracy and precision

The precision of the assay was defined as the coefficient of variation (CV) calculated from multiple measurements of QCs at three different concentrations. The accuracy of the assay was defined as the percent of the mean of multiple measurements of three QCs compared to the true value. The resulting precision (CV) and accuracy are presented in Table 1. The intra-day precision (CV) and accuracy ranges were 2.3–4.5% and 93–97%, respectively. The inter-day precision ranges were ≤6.0% for all the QC samples, and accuracy was between 96% and 103%. At the LLOQ of 5 ng/mL (n = 6), the accuracy was 87.8%, and the precision was 7.7%. All intra-day and inter-day precision and accuracy values were ±15%, indicating that the assay is accurate, precise and reproducible for the quantification of ATI-829 over the concentration range of 5–2000 ng/mL in mouse plasma samples.

3.5. Stability

The stability of ATI-829 in mouse plasma during sample handling and storage was investigated by comparing the concentration remaining after storage at different temperatures to the concentration of ATI-829 in freshly prepared samples. These results are shown in Table 2. ATI-829 was determined to be stable in mouse plasma for at least 6 h at room temperature, for 6 days at 4 °C, and for at least 3 weeks at −20 °C.

3.6. Application

The method described above has been used successfully to investigate the plasma profile of ATI-829 after a signal oral dose of 10 mg/kg to C57BL/6 mice. A concentration–time curve of ATI-829 in mouse plasma is shown in Fig. 3. The pharmacokinetics parameters were calculated using these data and are as follows. The maximum concentration (C_{max}) in mouse plasma was 295 ± 49 ng/mL (0.574 ± 0.095 μM; ±S.D.). The time to maximum concentration (T_{max}) was 1.6 ± 1.3 h (S.D.) indicating that ATI-829 is absorbed rapidly. The area under the concentration–time curve,

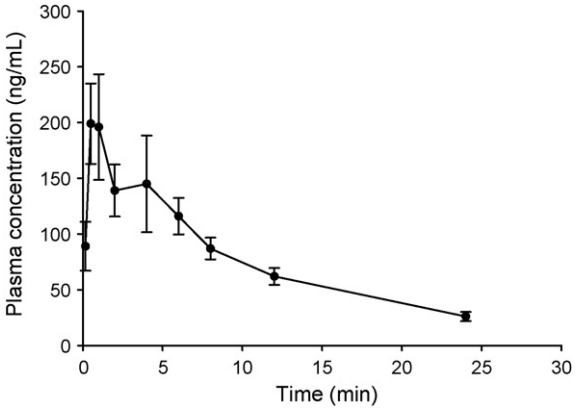

Fig. 3. Plasma concentration–time profile of ATI-829 following the administration of a single oral dose of 10 mg/kg AT-829 to male LDLR−/− mice.

AUC (0–24 h), was 2060 ± 340 h ng/mL (±S.D.). The elimination half-life ($T_{1/2}$) was 10.7 ± 3.7 h (±S.D.). These pharmacokinetics and half-life data suggest that ATI-829 exhibits favorable bioavailability and metabolic stability, which make it a promising lead compound for subsequent drug development.

4. Conclusions

In the present study, an assay based on LC–MS–MS was developed and validated for the quantitative analysis of ATI-829 in mouse plasma. The extraction procedure and the MS/MS conditions were optimized to give good recovery and excellent specificity and sensitivity. Given the low sample volume required (25 μL), this method is suitable for analyses of ATI-829 in support of pharmacokinetics studies especially when plasma quantities are small.

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