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Quantitation of geometric isomers of a phosphodiesterase inhibitor in rat and monkey plasma using liquid chromatography–tandem mass spectrometry

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Abstract

Quantitation of geometric isomers of a phosphodiesterase inhibitor was required to determine the extent of interconversion following dosing of a single isomer in preclinical pharmacokinetic studies. Assays were developed for the simultaneous determination of Compound A (Fig. 1), 6-[1-methyl-1-(methylsulfonyl)ethyl-8(3-{(*E*)-2-(3-methyl-1,2,4-oxadiazol-5-yl)-2-[4-(methylsulfonyl)phenyl]ethenyl}phenyl)quinoline] and its geometric *Z*-isomer, Compound B, in plasma using liquid chromatography–tandem mass spectrometry. Sample clean-up was performed using a semi-automated liquid–liquid extraction procedure. Separation was achieved on a Phenomenex Synergi MAX-RP column. The method was validated in the linear range of 2–2000 ng/mL for Compound A and 0.5–500 ng/mL for Compound B in plasma and successfully applied to preclinical pharmacokinetic studies. Compound A was dosed in rats and Compound B in monkeys and the degree of conversion was determined by comparing the area under the curve. The relative amount of conversion was less than 1 and 10% in rats and monkeys, respectively. Because of the small amount of conversion and minor peak tailing of the dosed geometric isomer, the order of elution of the two analytes was important in order to achieve best quantitative results. The minor component needs to elute first; thus, a second assay was developed in which the order of elution was reversed. This was achieved by changing the mobile phase modifier.

Keywords: LC–MS/MS; Geometric isomers; Reverse phase chromatography; Tandem mass spectrometry; Bioanalytical assay

1. Introduction

Compound A, a potent type IV phosphodiesterase (PDE4) inhibitor, was tested as a potential treatment of asthma [1]. In early preclinical studies, it was found the metabolism of Compound A in rats gave rise to a minor metabolite, Compound B, which is the *Z*-isomer of Compound A. In humans, Compound B concentrations in plasma widely varied between 1.4 and 21.5% of Compound A based on plasma area under the curve [2]. A metabolic mechanism for the formation of Compound B from Compound A was proposed which first required the addition of glutathione (GSH) to the electrophilic double bond [3]. This is followed by loss of the GSH moiety and reformation of the double bond, with partial conversion to Compound B. The mechanism was supported by the observation that the relative concentration of Compound B in human subjects depended upon their GST genotype [4]. A similar mechanism could lead to conversion of Compound B to A.

LC–MS/MS has become a very popular and powerful tool for the quantification of drugs and their metabolites in biological fluids [5]. Tandem mass spectrometry makes it possible to use short columns and fast gradients to meet increasing demand in drug discovery and development. In general, less chromatographic separation is required because tandem mass spectrometry is very specific when used in selective reaction monitoring (SRM) mode. However, in some cases, increased chromatographic separation is required in order to avoid problems with matrix effects, metabolite and endogenous compound interferences in quantitative drug determinations [6–9]. Chromatographic separation is also needed for quantification of geometric isomers.

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To test the theory that metabolic conversion of each isomer could occur in preclinical species, two studies were conducted. In the first, Compound A (*E*-isomer) was dosed in rats and in the second, Compound B (*Z*-isomer) was dosed in monkeys. Preliminary work indicated the relative amount of conversion resulting from GSH conjugation/deconjugation of the dosed compound was relatively small compared to humans. Thus, a sensitive method had to be developed to measure the metabolite concentration. LC–MS/MS was found to provide the required sensitivity and selectivity for the assay. Because geometric isomers have the same molecular weight and fragmentation pattern, chromatographic separation using HPLC was necessary. The order of elution of geometric isomers was important because: (1) there was a relatively low amount of metabolic conversion occurring and (2) the HPLC peaks exhibited a small amount of peak tailing. Details of the assays are described and their application to pharmacokinetic studies is presented.

2. Experimental

2.1. Materials and reagents

Compounds A and B and d6-Compound A (Fig. 1) were synthesized by Merck Research Laboratories. Methanol (HPLC grade), methyl *t*-butyl ether (MTBE, HPLC grade) and ammonium acetate (certified grade) were purchased from Fisher Scientific. Acetonitrile (HPLC grade) was purchased from Acros. Formic acid (Analytical Reagent grade) was from Riedel-DeHaen. Deionized water was produced using a Milli-Q water system (Millipore Corporation). The 1 mM ammonium acetate buffer used for the mobile phase was prepared by diluting 1 M ammonium acetate with deionized water and then adjusting the pH to 3 using formic acid.

Control rat and monkey plasma were provided by the Laboratory Animal Resource Group of Merck at West Point. Rhesus monkeys were administered a single oral 3 mg/kg dose of Compound B. Sprague–Dawley rats were administered a single oral dose at 6, 12 and 24 mg/kg of Compound A, separately. All animal experimental procedures were conducted at Merck Research Laboratories, West Point, which is an accredited facility approved by the Association for Assessment and Accreditation of Laboratory Animal Care.

2.2. Instrument conditions

Liquid chromatography (LC) was performed using Perkin-Elmer Series 200 micro pumps coupled with a PE Sciex 3000 mass spectrometer (Applied Biosystem), equipped with a TurboIonSpray^{TM} (TIS) interface. The data were processed using Analyst 1.1 software and the pharmacokinetic parameters and profiles were generated using Watson LIMS system.

2.2.1. HPLC method 1

Separation using method 1 was done under isocratic conditions using acetonitrile as the organic modifier. The mobile phase was prepared by premixing 450 mL of acetonitrile with 550 mL of 1 mM ammonium acetate buffer (adjusted to pH 3 with formic acid). The flow rate was 250 μL/min and the run time was 20 min. The column was a Synergi MAX-RP C12 (250 mm × 2 mm, 4 μm, Phenomenex).

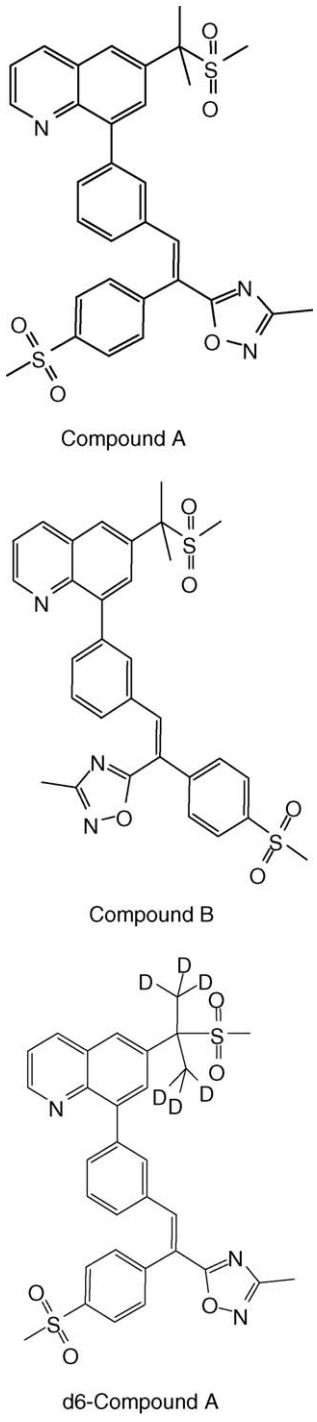

Fig. 1. Chemical structures of Compounds A and B and d6-Compound A (internal standard).

2.2.2. HPLC method 2

Separation using method 2 was done using a binary gradient with: (A) 1 mM ammonium acetate buffer (adjusted to pH 3 with formic acid) and (B) methanol. Starting conditions were 20% B, holding for 1 min after the injection. This was followed

by a linear increase to 60% B over 4 min which was held for 10 min to elute the analytes. Finally, B was decreased back to its starting condition for a 6.5 min equilibration time. The flow rate was 250 μL/min and the total run time was about 22 min. The column was a Synergi MAX-RP C12 (150 mm × 2 mm, 4 μm, Phenomenex).

2.2.3. MS/MS conditions

A PE Sciex 3000 mass spectrometer was interfaced via TurboIonSpray™ to the HPLC system. Positive ionization mode was employed and the spray temperature was maintained at 450 °C. The ionization potential was set to 4000 V. Selected reaction monitoring mode was used for the mass spectrometer with Q1 selecting the protonated molecule $(M+H)^+$. The collision energy was 39 eV and all other parameters were also optimized. The precursor and product ions monitored during assay were: *m/z* 588.3 → 509.3 for Compound A, *m/z* 588.3 → 509.3 for Compound B and *m/z* 594.3 → 515.3 for d6-Compound A.

2.3. Standard and quality control samples

Stock solutions of Compounds A and B at 1 mg/mL were prepared in HPLC grade acetonitrile and stored at −20 °C. A series of dilutions were made to the stock solutions with 20% acetonitrile in water to yield working standards. In method 1, the nominal plasma concentrations of standards were from 0.5 to 500 ng/mL for Compounds A and B. In method 2, the nominal plasma concentrations of standards were from 2 to 2000 ng/mL for Compound A and 0.5 to 500 ng/mL for Compound B. The QCs were prepared at four different concentrations covering a low to high concentration range.

2.4. Internal standard solution

The internal working standard was a solution of 20 ng/mL of d6-Compound A in 20% acetonitrile in water. It was prepared from a stock solution of 1 mg/mL in acetonitrile stored at −20 °C.

2.5. Plasma sample preparation

One hundred microliters of control plasma was added to a 2-mL 96-well plate. Fifty microliters of working standard and 50 μL of internal standard were added to standard and QC samples. Fifty microliters of 20% acetonitrile in water and 50 μL of internal standard were added to the study samples. One hundred microliters of 50 mM ammonium acetate was added to all samples and they were vortexed briefly. Extraction was performed by adding 500 μL of MTBE to the samples and the plate was sealed and vortexed vigorously in a multi-tube vortexer for 10 min. The plate was centrifuged at 2500 rpm at room temperature for 10 min and the supernatant was transferred to a 1 mL 96-well plate. Extracts were evaporated using a gentle stream of nitrogen at 35 °C. The samples were reconstituted with 100 μL of mobile phase and the plate was sealed, vortexed briefly and centrifuged at 2500 rpm. Fifty microliters of supernatant was injected onto LC–MS/MS system. The study samples were stored at −70 °C until analysis. All the study samples were thawed in a water bath at room temperature. The samples with concentrations above the limit of quantification were diluted with control plasma and reassayed.

2.6. Precision, accuracy, linearity and recovery

The intra-day precision and accuracy of method 2 were assessed by analyzing four replicates of plasma quality control samples at four different concentrations analyzed as a single batch. This was performed three times on separate days. The inter-day precision and accuracy were determined using the same set of data, combined from all three days. Standard curves were generated using a $1/x^2$ weighted linear regression of analyte/internal standard peak area ratio versus analyte concentration. The precision was calculated as relative standard deviation (R.S.D.). Accuracy was expressed by %Bias (100 − (observed concentration/nominal concentration) × 100). The recovery was determined by comparing the peak area ratio of an extracted sample to a non-extracted sample. The data were processed using Analyst 1.1 and Watson data system.

3. Results and discussion

3.1. HPLC–MS/MS assay development and its application to the rat pharmacokinetic studies

The product ion mass spectra of Compounds A and B are shown in Fig. 2. Compounds A and B fragment to yield ions with identical mass to charge ratios. The mass transitions with a *m/z* 588.3–509.3 were used for the quantification of Compounds A and B and 594.3–515.3 for d6-Compound A.

Method 1 was previously developed for the determination of Compound A and its metabolite, Compound B, in plasma after dosing of Compound A in humans [4]. In this method, Compound A elutes first from the column. Data from a human ADME study showed the relative amount of Compound B in plasma ranged from 1.4 to 21.5% of Compound A after 10 mg dosage of Compound A. The relative amount of Compound B was dependent upon individual GST genotype. This method was applied to a rat study after a single oral dose of Compound A (6 mg/kg). Preliminary analysis of samples from the rat study indicated significantly less conversion than in human (Compound B was 0.3% of Compound A based on plasma mean $AUC_{0-24\,h}$, $n=4$). Measured concentrations of quality control samples prepared at a similar ratio of Compound A to B indicated the analyte to internal standard peak ratio of Compound B was affected by the presence of the large amount of Compound A (Fig. 3). Attempts were made to modify the method and increase separation and minimize tailing but these efforts were unsuccessful. Reversing the order of elution of analytes so that the minor component eluted first [10] provided an alternative for analysis since the first part of the chromatographic peaks exhibited a sharp rise from baseline (Fig. 4).

It has been reported that the elution order of enantiomers can be reversed by changes in the column or mobile phase [10,11]. The reversal of the elution order of enantiomers on

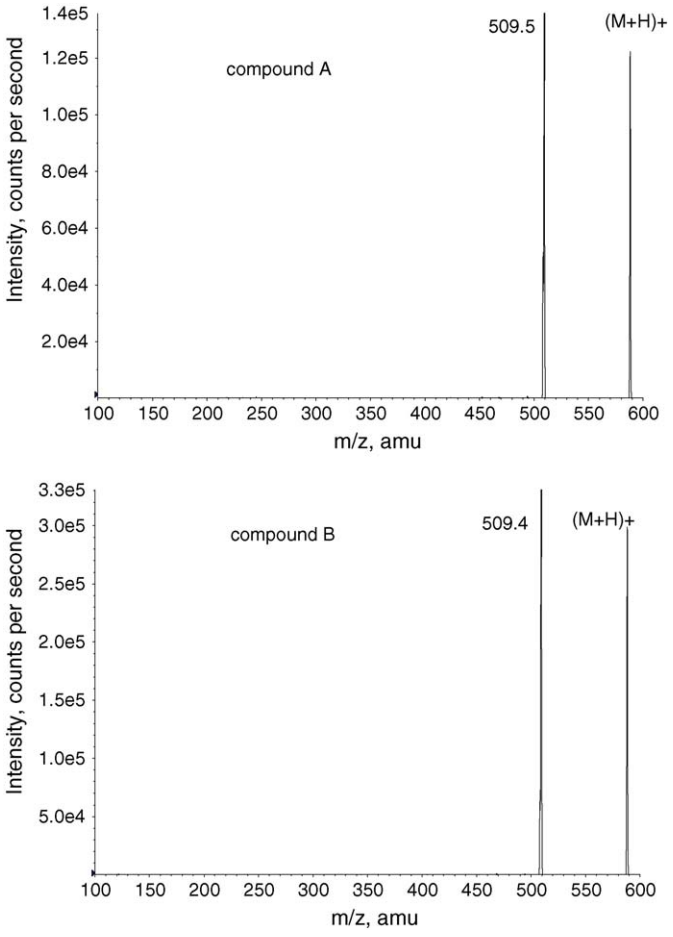

Fig. 2. Production ion mass spectrum of $(M+H)^+$ for Compounds A and B.

cellulose-based chiral separation phases has been reported by a number of research groups, achieved primarily by changing the alcohol modifiers in the mobile phase [12–14]. In this study, during the method development, it was found when the mobile phase was changed from acetonitrile to methanol, the elution order of the *E*/*Z*-isomer pair on the same column (Phenomenex Synergi MAX-RP, 4 μm, 150 mm × 2 mm and 250 mm × 2 mm) was reversed.

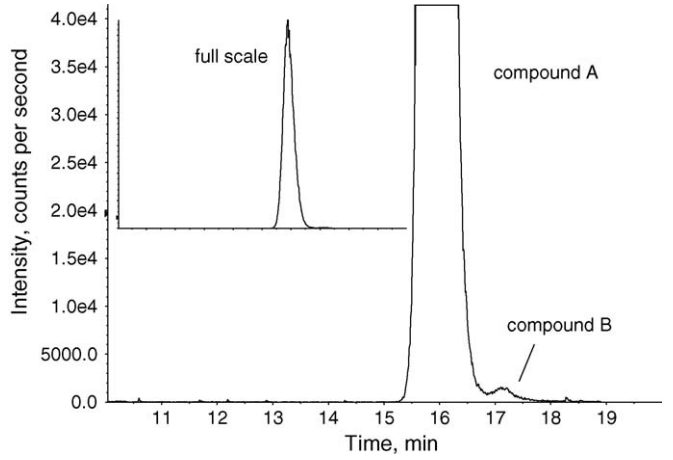

Fig. 3. LC–MS/MS chromatogram using LC method 1 resulting from injection of an extract from rat plasma obtained at 2 h after a 6 mg/kg dose of Compound A. Inset shows same chromatogram at full scale.

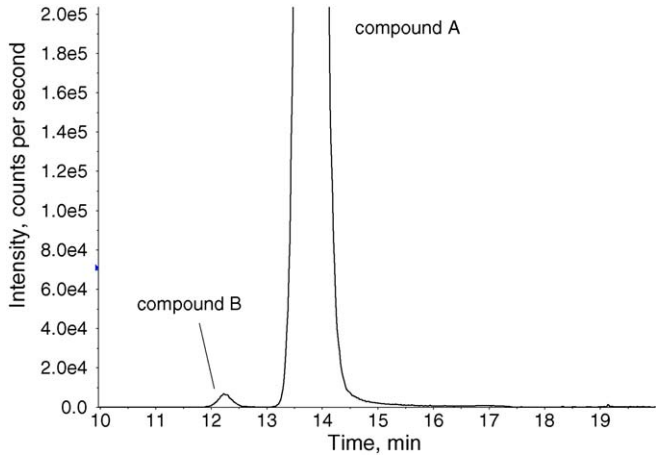

Fig. 4. LC–MS/MS chromatogram using LC method 2 resulting from injection of an extract from rat plasma obtained at 2 h after a 6 mg/kg dose of Compound A.

By substituting methanol for acetonitrile as the organic solvent in the mobile phase, the order of analytes was reversed and Compound B eluted first at 12.2 min followed by Compound A at 13.5 min (Fig. 4).

3.1.1. Specificity, linearity, precision, accuracy and recovery of method 2 assay

Method 2 was validated in rat plasma. Control plasma from five different lots were tested and no interferences were observed (Fig. 5). The linear range of standard curves was 0.5–500 ng/mL for Compound B and 2–2000 ng/mL for Compound A. The correlation coefficients of the standard curves were greater than 0.997 for all five curves. The inter- and intra-assay precision and accuracy are shown in Tables 1 and 2. The intra- and inter-assay accuracy was generally within ±12% (expressed as bias) and the precision was less than 11%, expressed as coefficient

Table 1
Summary of Compound A precision and accuracy from replicate quality control samples on three separate days in rat plasma using method 2

	Day	Nominal concentration (ng/mL)			
		2.0	4.0	20	1000
Intra-run mean ($n=4$)	1	2.0	4	20.5	984.6
Intra-run %CV		3.6	3.0	1.1	3.0
Intra-run %Bias		−1.5	1.0	2.5	1.5
Intra-run mean ($n=4$)	2	2.2	4.4	20.1	998.6
Intra-run %CV		2.7	6.0	2.2	3.7
Intra-run %Bias		11.0	8.8	0.7	−0.1
Intra-run mean ($n=4$)	3	2.1	4.1	21.3	1099.4
Intra-run %CV		2.9	5.6	6.1	5.5
Intra-run %Bias		3.5	3.3	6.3	9.9
Inter-run mean ($n=12$)		2.1	4.2	20.6	1027.5
Inter-run %CV		3.1	4.9	3.1	4.1
Inter-run %Bias		4.3	4.4	3.2	3.8

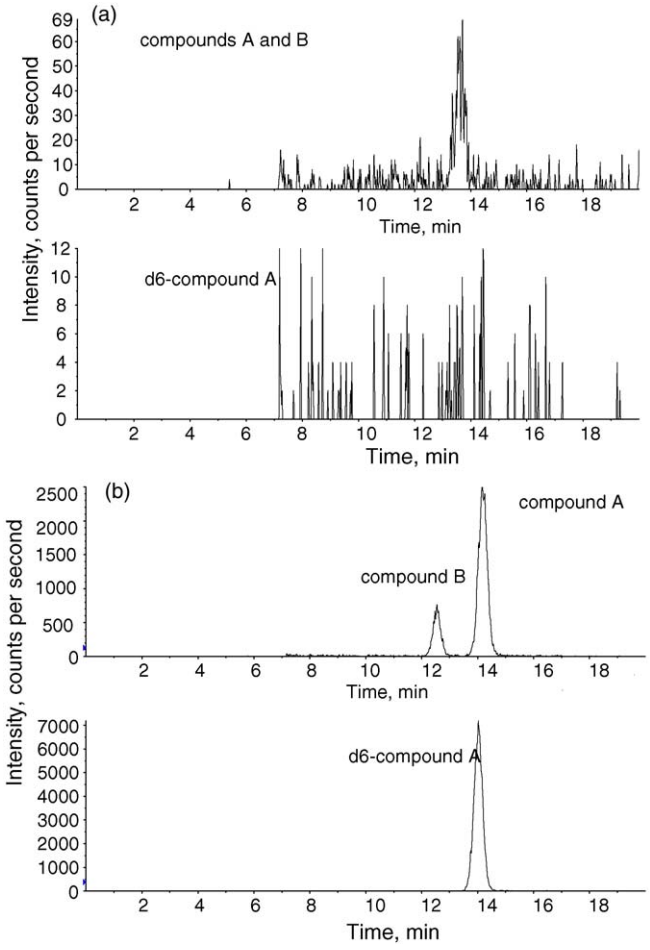

Fig. 5. LC–MS/MS chromatograms resulting from injection of an extract from rat plasma ((a) double blank and (b) 0.5 ng/mL for Compound B and 2 ng/mL for Compound A).

of variation, for both analytes. The recovery of Compound A in rat plasma was 90% at 2 ng/mL and 86% at 2000 ng/mL. The recovery of Compound B in rat plasma was 84% at 0.5 ng/mL and 83% at 500 ng/mL.

Table 2
Summary of Compound B precision and accuracy from replicate quality control samples on three separate days in rat plasma using method 2

	Day	Nominal concentration (ng/mL)			
		0.5	1	5	250
Intra-run mean ($n=4$)	1	0.5	0.9	4.6	252.1
Intra-run %CV		8.7	9.7	8.0	8.2
Intra-run %Bias		−8.0	−7.0	−7.2	0.8
Intra-run mean ($n=4$)	2	0.6	1.1	4.9	246.2
Intra-run %CV		8.9	5.6	10.8	2.4
Intra-run %Bias		12	8	−2.0	−1.5
Intra-run mean ($n=4$)	3	0.5	1.0	5.1	273.6
Intra-run %CV		3.8	3.9	5.7	2.9
Intra-run %Bias		6	3	2.2	9.4
Inter-run mean ($n=12$)		0.5	1.0	4.9	257.3
Inter-run %CV		7.1	6.4	8.2	4.5
Inter-run %Bias		3.3	1.3	−2.3	2.9

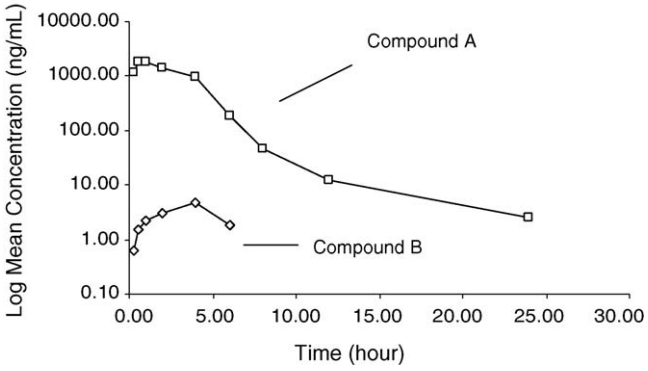

Fig. 6. Compounds A and B plasma concentrations (mean of four animals) following a single 6 mg/kg oral dose of Compound A in rats.

3.1.2. Application to pharmacokinetic study of Compound A dosed in rats

The assay (method 2) was successfully applied to the pharmacokinetic study of Compound A in rats at 6, 12 and 24 mg/kg following oral administration of Compound A. A representative chromatogram from rat study sample is shown in Fig. 4 and a typical concentration–time profile of Compounds A and B in rat plasma at 6 mg/kg is shown in Fig. 6.

3.2. Pharmacokinetic study of Compound B dosed in monkey

It is possible that *Z* to *E* conversion could also occur in any species. A study was carried out to test this by administering Compound B to monkeys. Preliminary examination of plasma from monkeys dosed orally at 3 mg/kg indicated that Compound B concentrations were much higher than Compound A. The samples were assayed using method 1 so the lesser abundant compound, Compound A, eluted first and eliminated the peak tailing effect on accuracy. Method 1 was successfully cross-validated from human to monkey (data not shown) and used to support this study. The concentrations of Compounds A and B in monkey plasma are shown in Fig. 7. The study confirmed conversion of Compound B to A could occur in monkeys, but was less than 15% based upon area under the curve.

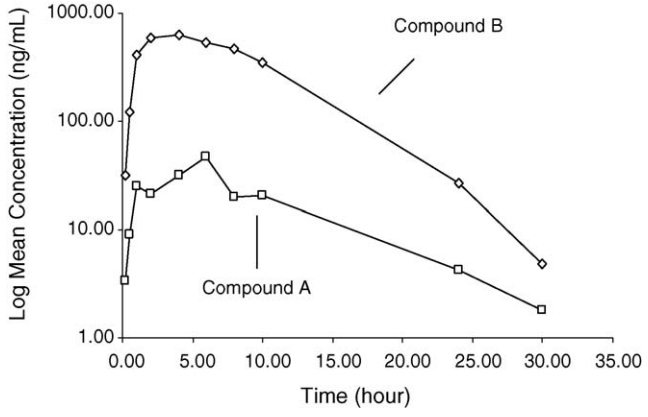

Fig. 7. Compounds A and B mean plasma concentrations ($n=4$) following a single 3 mg/kg oral dose of Compound B in Rhesus monkey.

4. Conclusions

Methods were developed to provide pharmacokinetic data for the geometric isomers in rat and monkey studies. The methods were applied to determine the relative amount of conversion of each isomer following dosing of either Compound A or B in separate studies. The order of elution of analytes was important because of minor peak tailing and the large difference in concentrations of each geometric isomer. It was necessary to develop two methods with the order of analytes reversed relative to each other. This was achieved through modification of the mobile phase.

Future work could include focusing on improving separation efficiency and reduction of peak width and tailing. Elevated mobile phase temperatures or Ultra Performance Chromatography (UPC) need to be tested to determine their utility. The dependence of alkene geometric isomer conversion upon preclinical species GSH genotype and glutathione concentration could also be studied.

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