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Determination of MK-0767 enantiomers in human plasma by normal phase LC–MS/MS

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Abstract

A sensitive and selective analytical method for the enantioselective determination of MK-0767, a dual peroxisome proliferator-activated receptor (PPAR) α/γ agonist, in human plasma has been developed and validated. The chromatography is based on normal-phase chiral separation on a Kromasil, 5 μm, CHI-DMB 250 mm × 4.6 mm column. The detection involves the direct introduction of the normal phase eluent into MS/MS without the addition of a post-column reagent. Atmospheric pressure chemical ionization (APcI) mode was selected as the ion source in this method. With proper sample handling and processing procedures, ex vivo interconversion of the enantiomers was kept to minimum during sample collection, preparation and short term storage of frozen human plasma samples. The method was successfully utilized to determine the concentrations of MK-0767 enantiomers in human plasma to support pharmacokinetic investigation in man.
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Keywords: Chiral separation; MK-0767 (KRP-297); LC–MS/MS; Liquid–liquid extraction; Normal phase; Dual PPAR; Diabetes; Thiazolidinediones

1. Introduction

MK-0767 (KRP-297), (±)-5-[2,4-dioxothiazolidin-5-yl) methyl]-2-methoxy-*N*-[[(4-trifluoromethyl)phenyl]methyl] benzamide, is a dual peroxisome proliferator-activated receptor (PPAR) α/γ agonist previous studied for the treatment of patients with diabetes and hyperlipidemia [1,2]. The thiazolidinediones-based ligand MK-0767, shown in Fig. 1, was developed as one of a series of 3-[(2,4-dioxothiazolidin-5-yl)methyl]benzamide derivatives. It is a racemate and has an asymmetric carbon in the 5-position of the thiazolidinediones (TZD) ring. Like other classic TZD compounds which undergo racemization at the C (5)-position under physiologic pH [3], MK-0767 underwent interconversion in non-clinical species producing both (*S*)- and (*R*)-enantiomers as well [4].

Both the US FDA and European Union have prepared guidance documents suggesting that the properties of enantiomers be evaluated in vitro in animals and that information on the disposition of enantiomers be determined in human [5,6].

In order to assess the pharmacokinetics of enantiomers of MK-0767 when given single enantiomers or racemate in man, a sensitive and selective LC–MS/MS method was developed to measure the concentrations of MK-0767 enantiomers in human plasma. To our knowledge, there are no published methods for the determination of MK-0767 enantiomers in human plasma. Suzuki et al. reported a HPLC/UV method to separate troglitazone stereoisomers, a TZD based compound with two chiral centers, in manufacturing process and stability testing [7]. Welch et al. also reported using Whelk-O column to separate a stereolabile 5-aryl-thiazolidinedione [8]. The present paper describes an analytical method which employs single step liquid–liquid extraction for the sample preparation, normal phase HPLC for enantiomer separation, and MS/MS for detection of the analytes. It allows sensitive quantitation of both enantiomers with a lower limit of quantitation (LLOQ) of 1 ng/mL in human plasma. There is no derivatization required for the chiral separation. With proper sample handing and processing procedures, in vitro interconversion of frozen plasma samples was controlled at less

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than 3% during sample collection, preparation and short term storage.

2. Experimental

2.1. Material

MK-0767 enantiomers were obtained from Kyorin Pharmaceutical Co. (Japan) and its internal standard (*I*) was obtained from Merck & Co. Inc. (Fig. 1). High performance liquid chromatography (HPLC) grade methanol was from Fisher (Fair Lawn, NJ, USA), HPLC grade ethyl acetate and isopropanol, ACS grade tetrahydrofuran (THF) were from EM Science (Darmstadt, Germany), HPLC grade Hexane (95% *n*-hexane) was from Acros Organics (Geel, Belgium). Formic acid (99%) was purchased from Sigma (St. Louis, MO, USA). Heparinized control human plasma was purchased from Biological Specialty Corp. (Colmar, PA, USA).

2.2. Standard solutions

Stock solutions of MK-0767 enantiomer (*R* or *S*) were prepared at concentration of 100 μg/mL in ethyl acetate, respectively. Stock solutions were further diluted to a series of working standard solutions in THF at concentrations of 20, 40, 100, 400, 2000 and 4000 ng/mL. All solutions were stored at −70 °C.

Internal standard (*I*) working solution was prepared at 1000 ng/mL in ethyl acetate. This solution was stored at −70 °C.

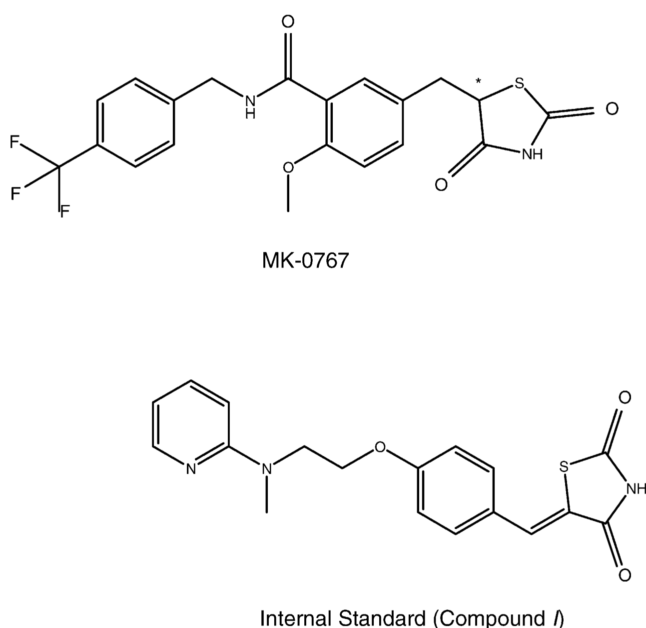

Fig. 1. Structures of MK-0767 and internal standard (compound *I*).

Enantiomer plasma standards were prepared separately. They were prepared by adding 50 μL of each working standard to 1 mL of human control plasma. The resulting plasma standard concentrations ranged from 1 to 200 ng/mL.

Quality control (QC) stock solutions for either (*R*)- or (*S*)-MK-0767 were prepared at a concentration of 100 μg/mL in ethyl acetate, respectively. Stock solutions were diluted with THF to generate working QC stock solutions. Plasma QC samples were prepared by adding the appropriate volume of working QC stock solution into control human plasma to yield final concentrations of 3.0, 30.0 and 160 ng/mL for *R* or *S*, respectively. All QC samples were stored at −70 °C.

2.3. LC–MS/MS

Chromatographic separation was performed using a Perkin-Elmer (Norwalk, CT, USA) Series 200 autosmapler and a quaternary pump. The separation was done on a Kromasil 5 μ CHI-DMB column (4.6 mm × 250 mm) (Eka Chemical AB Separation Products, Sweden). The mobile phase was a mixture of hexane and isopropanol with 0.1% formic acid (81:19, v/v). The flow rate was 1 mL/min and the column temperature was ambient. In order to keep the sample stable throughout the injection period, the autosampler temperature was kept at 4 °C.

A PE Sciex API 3000 (Thornhill, Canada) with an atmospheric pressure chemical ionization (APcI) ion source was used to monitor the analytes. The analytes were detected in positive ionization mode by monitoring their precursor-product combination in multiple-reaction monitoring (MRM) mode. After optimizing, the detecting channels were *m/z* 439.0 → *m/z* 264.0 for both *R* and *S* of MK-0767, and *m/z* 356.0 → *m/z* 135.0 for the internal standard. Fig. 2 shows the product scan mass spectra for the protonated molecule $[M+H]^+$ of the MK-0767 and its internal standard.

2.4. Sample preparation

As soon as frozen plasma samples thawed at room temperature, they were vortexed for 1 min using multi-tube vortexer and centrifuged for 5 min at 3000 rpm at 4 °C (centrifuge pre-cooled to 4 °C). The plasma sample tubes were placed into ice-water bath immediately. One milliliter of plasma was aliquoted into a 15 mL glass culture tube, followed by adding 50 μL of working internal standard. After vortexing, 5 mL of ethyl acetate was added to each tube and the tube was capped and rotated for 15 min at 60 rpm, and centrifuged at 4 °C for 5 min at 3000 rpm. After freezing the aqueous layer in a dry-ice/acetone bath, the organic layer was transferred into a 12 mL polypropylene conical tube and evaporated under nitrogen at 18 °C (without the ice-water bath) for 60 min in Turbo-Vap (Zymark, Hopkinton, MA, USA). The dried tubes were reconstituted with 100 μL ethyl acetate. Ten microliters of the final solution was injected onto the HPLC system. All sample preparations were in ice-

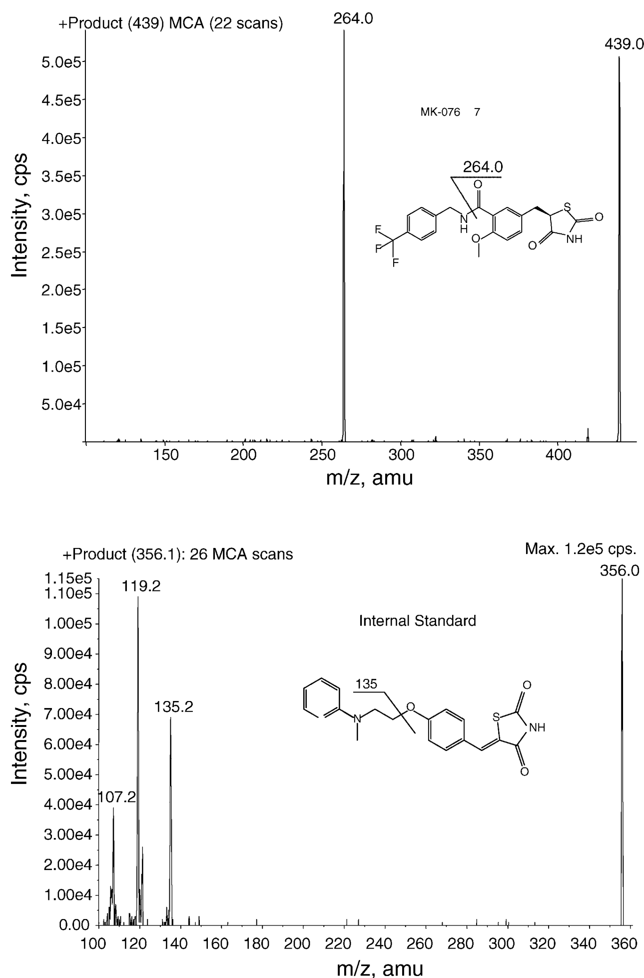

Fig. 2. Product scan spectra of MK-0767 and its internal standard.

water bath to minimize in vitro interconversion between two enantiomers.

2.5. Enantiomer interconversion calculation

The following formulae were used to assess the enantiomer interconversion during sample collection, preparation and storage. For (*S*)-MK-0767, the percentage of interconversion was expressed as $R_{\text{peak area}}/(R_{\text{peak area}} + S_{\text{peak area}}) \times 100\%$; For (*R*)-MK-0767, the percentage of interconversion was expressed as $S_{\text{peak area}}/(R_{\text{peak area}} + S_{\text{peak area}}) \times 100\%$.

2.6. Quantification

In each analytical run, the standard curves were constructed from peak area ratios of (*R*)- and (*S*)-MK-0767 to the internal standard versus the nominal concentration of the standards, respectively. Unknown sample concentrations were calculated from the equation ($y = mx + b$) as determined by the weighted ($1/x^2$) linear regression analysis of the standard curve.

3. Results and discussion

3.1. Chiral separation and normal phase LC–MS/MS sensitivity

Chiral compounds can be separated by derivatization of the enantiomers into diastereomers and subsequent separation by conventional HPLC, or by directly using chiral stationary phases in reverse- or normal-phase modes [9]. An important benefit of using derivatization technique is to increase sensitivity. Therefore, it is suitable for the analysis of trace amount of the enantiomers in complex matrices such as biological samples. However, it is essential that the chiral derivatization reaction proceeds completely for both enantiomers, and that racemization does not occur [10]. MK-0767 is optically unstable and readily racemized in basic condition. To minimize the possible ex vivo interconversion of the enantiomers, we chose to separate and quantitate the individual enantiomers without going through derivatization procedures. Chiral columns allow direct separation and quantitation of the individual enantiomers, and this approach is becoming increasingly popular in recent years [10]. Unfortunately, the relationship between the chemical structure and the type of chiral column to use is not obvious in chiral separation. Columns and solvents are usually found by trial and serror.

Reverse phase separation was tested first in our work since the aqueous mobile phase can couple with MS detector directly. The attempt of using Chirobiotic V (Astec, 4.6 mm × 250 mm) column in reversed phase mode to separate the two enantiomers of MK-0767 was not successful. Although the retention time was short, the peak shape was poor without adding modifier such as phosphoric acid, which was not suitable for MS detection.

In normal phase separation mode, we were unable to baseline separate these two enantiomers in polar organic condition using methonal or ethanol with acid or base modifiers as mobile phase.

Using hexane and isopropanol/ethanol as mobile phase, we tested many different columns with different stationary phases and chromatographic conditions. These columns included Chirex (R) NGLY DNB (Phenomenex, 4.6 mm × 50 mm), Chirex S-VAL and R-NEA (Phenomenex, 4.6 mm × 50 mm), Chiralpak AD (Chiral Technology, 4.6 mm × 250 mm), Chiral AGP (Regis, 4.6 mm × 250 mm) and Kromasil CHI-DMB (Eka Chemicals AB, 4.6 mm × 250 mm) column. Baseline separation of the two enantiomers was only achieved with Kromasil 5 μ CHI-DMB (250 mm × 4.6 mm) column using a mobile phase of hexane: isopropanol containing 0.1% formic acid (81:19, v/v). The retention times of the enantiomers of MK-0767 were approximately 23.5 and 26.0 min and the resolution R_S is 1.2. Fig. 3 shows the representative chromatograms of the separation of MK-0767 enantiomers in clinical plasma samples.

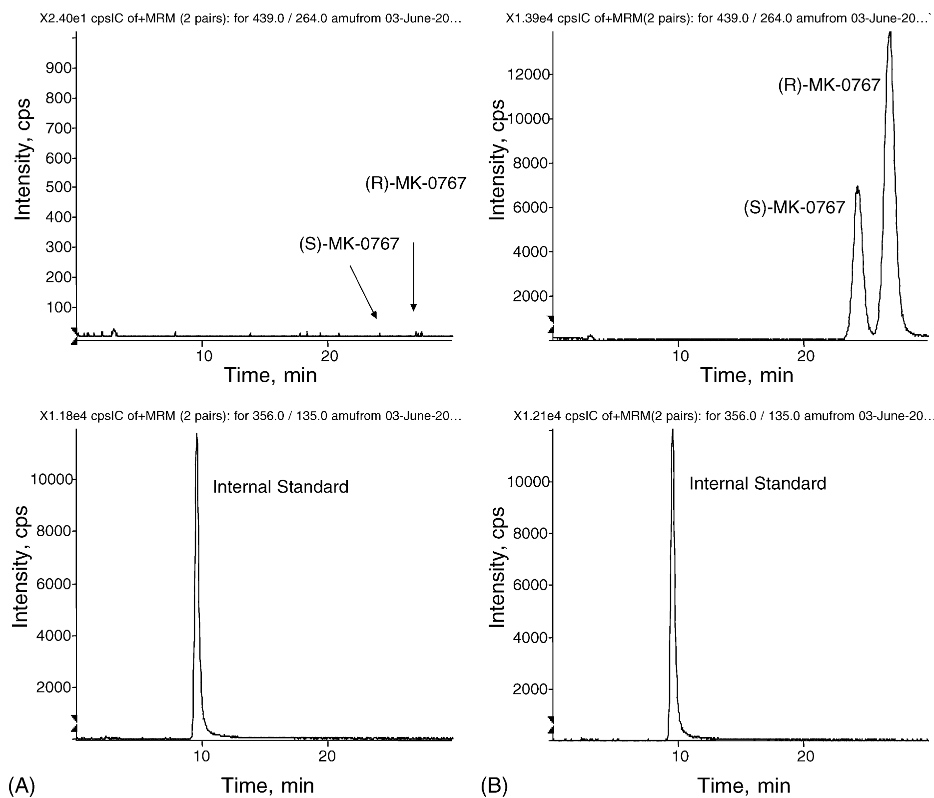

Fig. 3. Representative chromatograms of MK-0767 from clinical plasma samples (A) subject 004, predose (internal standard, 50 ng/mL), (B) subject 004, 10 h after receiving 12 mg of (*S*)-MK-0767. Analyzed concentration: (*R*)-MK-0767, 74.5 ng/mL; (*S*)-MK-0767, 31.9 ng/mL.

Both TurboIonSpray (TIS) and atmospheric pressure chemical ionization (APcI) were evaluated. Since the mobile phase is hexane-based, TIS mode generated very low response even with the addition of formic acid in mobile phase. It is possible to increase the sensitivity by adding an aqueous phase as the post-column reagent, but system setup would be more complicated. APcI produced reasonable sensitivity with the LLOQ at 1 ng/mL in the presence of formic acid. Therefore, APcI was selected as the source in our method.

The addition of formic acid into the mobile phase not only increased the sensitivity of MK-0767 in MS/MS detector, it also enhanced the enantiomer separation reproducibility from column to column.

3.2. Configurational stability of enantiomers

The chiral analytical method needs to serve as a tool to measure the extent of the stereoconversion in vivo, and should not introduce additional variability or bias by causing in vitro conversion [11]. Plasma sample stability and in vitro stereoconversion of enantiomers of MK-0767 were investigated during the course of our method development and validation.

3.2.1. Stability in sample preparation procedure

For chiral compound, configurational stability and lability are relative phenomena. Given proper conditions, no stereoisomer is configurationally stable [12]. Some molecules will racemize at physiological or acidic pH, while others will do so at increasing pH or temperature [13–15].

MK-0767 undergoes racemization under physiologic condition producing both (*S*)- and (*R*)-enantiomers [3]. It is necessary to test the stability of the MK-0767 enantiomeric ratio in sample preparation procedure in order to avoid interconversion during this period. The effect of pH and temperature on the MK-0767 enantiomers interconversion were investigated.

The (*R*)- or (*S*)-enantiomers solutions were prepared at 100 ng/mL separately in pH 2.5 formic acid solution, pH 4.2 and 6.5 ammonium acetate buffers. Interconversion was determined following preparation and 2 days later at room temperature by LC–MS/MS. The result showed that there was no interconversion observed for both enantiomers in the above buffers after immediate preparation. After 2 days, only 2% of the tested enantiomer was converted into its antipode in pH 2.5 solution, while there was ∼50% interconversion in pH 4.2 and 6.5 buffers. Therefore, pH plays a significant role in MK-0767 enantiomer interconversion.

Temperature is another factor that might affect the MK-0767 enantiomer interconversion in sample preparation procedure. Tests were done by spiking (*R*)- and (*S*)-enantiomers separately in human control plasma, and these samples were divided into two groups subsequently. One group of the samples was kept in room temperature while the other group of samples was kept in the ice/water bath. At different time

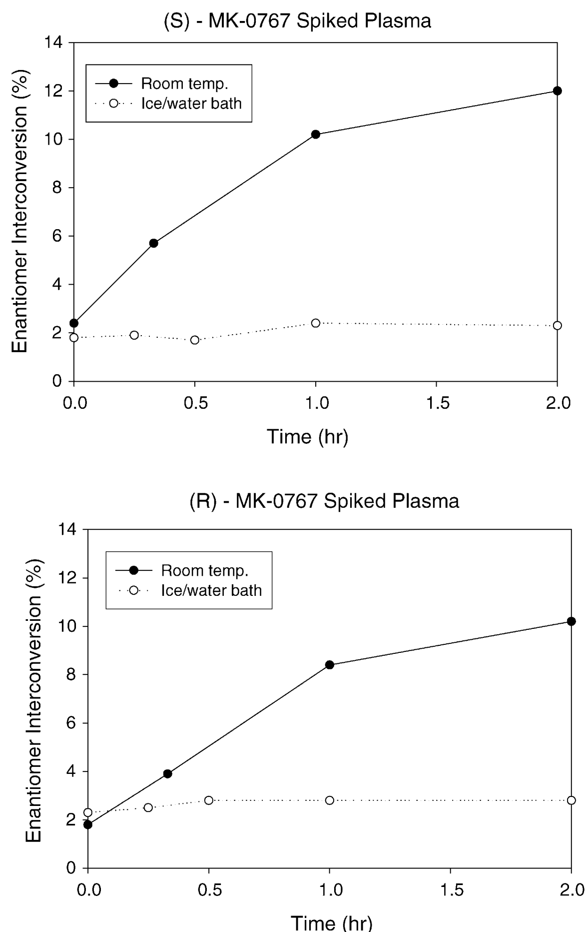

Fig. 4. Temperature effect on MK-0767 enantiomers in human plasma.

points within 2 h, the spiked samples were taken out and processed according to the sample preparing procedures described above. The extracted plasma samples were injected into LC–MS/MS system to measure the percentage of enantiomer interconversion. The result is illustrated in Fig. 4. For both enantiomers, interconversion was kept lower than 3% throughout the 2 h tested period at ice/water bath. However, for the enantiomer spiked plasma samples which were left at room temperature, there was around 10% interconversion in 2 h.

Since the in vitro interconversion of MK-0767 enantiomers is temperature dependent in plasma, we have adopted the procedure to submerge the plasma tubes in ice/water bath during sample preparation period and pre-cool the centrifuge to 4 °C before putting samples into centrifuge. Following this procedure, the in vitro interconversion can be controlled less than 3%.

3.2.2. Stability in sample collection procedure

From previous studies using achiral method, MK-0767 is stable in whole blood during sample collection [16]. To test the enantiomeric ratio stability in whole blood, MK-0767 enantiomers were spiked into three different lots of fresh human blood separately, and chilled the samples in ice/water bath immediately for 30 min. The samples were centrifuged at 4 °C for 15 min and stored at −70 °C immediately. The result showed that there was less than 2.5% of interconversion occurring in this period and was acceptable.

3.2.3. Stability in sample storage procedure

Enantiomers are not expected to possess the same affinities towards biological proteins, their presence can lead to specific drug–protein interaction and influence the degradation of each enantiomer in different fashion [11]. Short term storage stability of the enantiomers in frozen plasma at −70 °C was evaluated by spiking MK-0767 enantiomers into control plasma separately at concentration of 50 ng/mL. Over the time period of 50 days, samples were analyzed for enantiomeric ratio stability. The result showed there were no changes of the enantiomeric ratio up to 50 days at −70 °C.

3.3. Analytical performance

In order to validate this LC–MS/MS method for use as the routine analytical method, linearity of calibration curve, specificity, recovery, stability, precision, accuracy and ruggedness were investigated.

3.3.1. Internal standard selection

The internal standard used in previous racemate method [16] is an analog of MK-0767. It has a chiral center same as MK-0767. Since the enantiomers of this compound were also separated on the same column, it made the quantification of MK-0767 enantiomer complicated. The analysis of MK-0767 enantiomers was carried out using a different internal standard without any chiral centers. It produced good recovery and peak shape, but it is slightly photosensitive. It gave minor split peak if exposed in light for a long period. With precaution procedures such as keeping this internal standard in ambler vial and processing samples in the dark room, the analyzed result of MK-0767 enantiomers throughout the study showed excellent linearity and reproducibility.

3.3.2. Specificity

Human control plasma samples from five different sources were extracted and analyzed to assess the specificity of the method. The chromatographic conditions specified in this assay were found to be selective for both enantiomers and the internal standard. None of the control plasma samples had any detectable interference at the retention times of both enantiomers and internal standard.

Matrix effect was also evaluated. Five different lots blank plasma were extracted according to our extraction procedures, and then the working standard of the analyte was spiked into these matrixes. All these samples along with the neat solutions at the same concentration were analyzed by LC–MS/MS. The peak areas of the analytes from the neat standard and the extracts were similar. Thus, no significant matrix effect was observed for both enantiomers.

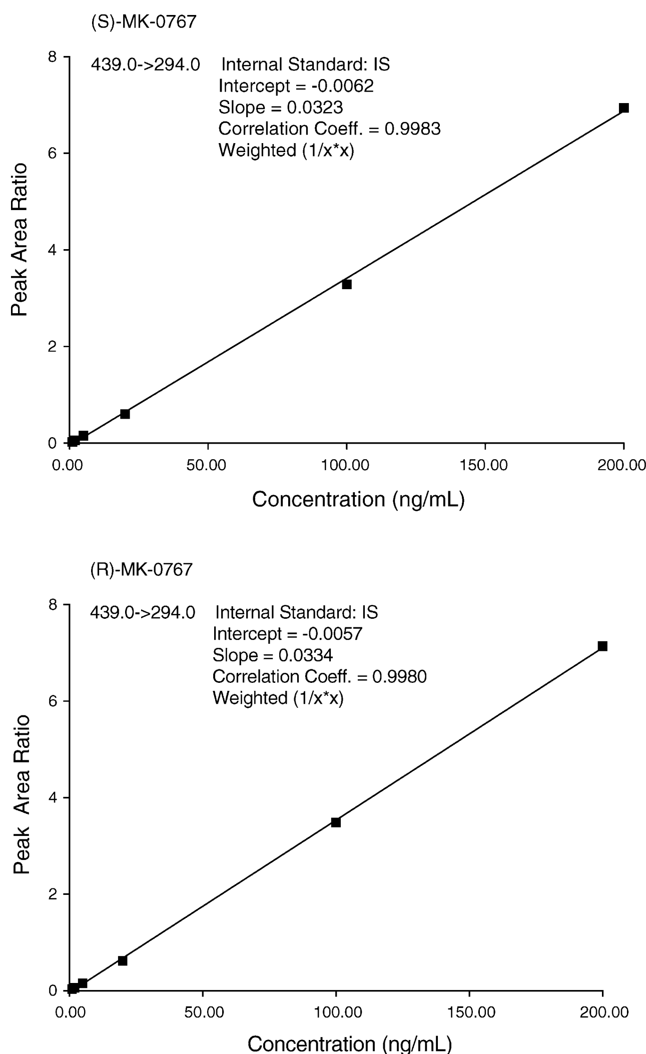

Fig. 5. Representative standard curves of MK-0767 enantiomers in human plasma.

3.3.3. Linearity, accuracy and precision

The linear dynamic range was from 1 to 200 ng/mL for both enantiomers. The correlation coefficient (r) using weighted ($1/x^2$) linear least-squares regression was greater than 0.990 for all the experimental runs. Fig. 5 is a representative standard curve.

The intra-day accuracy and precision were determined by analysis of five replicates of QCs at three different concentrations. The inter-day accuracy and precision were also determined by QCs from three different days. The precision and accuracy data of the QCs are summarizd in Table 1. The interconversion was less than 1.4% for all samples. Representative standard curve parameters from inter-day validation are shown in Table 2.

3.3.4. Recovery

Extraction recovery is determined for each enantiomer in five different lots of human control plasma at three different concentrations. The internal standard was evaluated only at the concentration used during extraction. In order to determine the recovery, both blank human plasma and analyte-spiked-plasma were extracted. The blank extracts were reconstituted with neat standard solution at the concentrations corresponding to the final concentration of the extracted plasma samples. The extraction recovery was calculated by comparing the mean chromatographic peak areas of spiked-before-extraction samples with the mean peak areas of the corresponding spike-after-extraction standards. The recoveries of (*R*)-MK-0767 were 102.6, 92.9 and 92.8% at the concentrations of 3, 30 and 120 ng/mL, respectively. The overall recovery was 96.1%. The recoveries of (*S*)-MK-0767 were 92.5, 88.3 and 87.2% at the concentrations of 3, 30 and 120 ng/mL, respectively. The overall recovery was 89.3%. Recovery of internal standard was 103.4%.

Table 1
Intra- and inter-day precision and accuracy of MK-0767 enantiomers in human plasma

	QC (ng/mL)	Found concentration (ng/mL)	Accuracy (%)[a]	CV (%)
Intra-day ($n=5$)				
(*R*)-MK-0767	3.0	2.9	96.7	4.2
	30.0	27.1	90.4	9.2
	160.0	160.9	104.3	6.4
(*S*)-MK-0767	3.0	2.8	93.3	3.9
	30.0	26.9	89.7	3.2
	160.0	160.9	100.6	5.4
Inter-day ($n=3$)				
(*R*)-MK-0767	3.0	2.8	93.3	5.2
	30.0	26.5	88.3	0.7
	160.0	159.2	99.5	1.4
(*S*)-MK-0767	3.0	2.9	96.7	4.6
	30.0	29.0	96.7	5.4
	160.0	165.7	103.6	3.5

[a] Accuracy (%) is expressed as mean found concentrations/nominal concentration × 100%.

Table 2
Representative standard curve parameters of MK-0767 enantiomers in human plasma

Run number	Slope	Intercept	Correlation coefficient
(*R*)-MK-0767			
1	0.0402	−0.00488	0.9965
2	0.0431	−0.00649	0.9971
3	0.0400	0.00451	0.9978
Mean	0.0411		
S.D.	0.0017		
CV (%)	4.2		
(*S*)-MK-0767			
1	0.0473	−0.00801	0.9995
2	0.0410	−0.00900	0.9994
3	0.0399	−0.01599	0.9988
Mean	0.0427		
S.D.	0.0040		
CV (%)	9.3		

Table 3
MK-0767 enantiomer plasma freeze–thaw and autosampler stability

Nominal concentration (ng/mL)	Fresh prepared sample ($n = 5$)		Three freeze–thaw sample ($n = 5$)		Re-injected sample after 24 h ($n = 5$)	
	Found concentration[a] (ng/mL)	Interconversion[b] (%)	Found concentration[a] (ng/mL)	Interconversion[b] (%)	Found concentration[a] (ng/mL)	Interconversion[b] (%)
(*S*)-MK-0767						
120.0	110.9 ± 9.2	1.1 ± 0.4	106.3 ± 6.9	2.9 ± 0.4	97.1 ± 3.3	1.1 ± 0.2
30.0	27.6 ± 1.3	0.4 ± 0.3	27.5 ± 1.0	2.9 ± 0.1	25.4 ± 0.7	0.4 ± 0.1
3.0	2.7 ± 0.2	1.1 ± 0.2	2.7 ± 0.2	2.9 ± 0.4	2.6 ± 0.1	N/A
(*R*)-MK-0767						
120.0	114.0 ± 4.1	1.6 ± 0.1	115.3 ± 5.2	2.7 ± 0.1	125.9 ± 4.7	1.6 ± 0.1
30.0	32.1 ± 2.1	1.3 ± 0.6	32.5 ± 1.9	2.9 ± 0.2	30.6 ± 0.8	1.9 ± 0.1
3.0	3.2 ± 0.2	N/A	3.5 ± 0.1	2.9 ± 0.8	3.0 ± 0.1	1.4 ± 0.1

[a] Found concentration is expressed as mean ± S.D.
[b] Interconversion (%) is expressed as $R_{\text{peak area}}/(R_{\text{peak area}} + S_{\text{peak area}}) \times 100\%$ for (*S*)-MK-0767, and $S_{\text{peak area}}/(R_{\text{peak area}} + S_{\text{peak area}}) \times 100\%$ for (*R*)-MK-0767.

Table 4
Precision and accuracy for MK-0767 plasma QC samples in clinical sample analysis ($n = 24$, over period of 10 months)

	(*S*)-MK-0767 (ng/mL)			(*R*)-MK-0767 (ng/mL)		
	High QC	Medium QC	Low QC	High QC	Medium QC	Low QC
Nominal	160.0	30.0	3.0	160.0	30.0	3.0
Mean	158.0	28.7	2.9	159.8	28.5	2.9
S.D.	8.66	1.75	0.19	9.87	2.39	0.22
CV (%)	5.5	6.1	6.6	6.2	8.4	7.7
Accuracy (%)	98.8	95.7	96.7	99.9	95.0	96.7

3.3.5. Freeze–thaw and autosampler stability

Freeze-thaw stability on enantiomeric ratio was evaluated for (*R*)- or (*S*)-MK-0767 separately. Stability samples were studied following three cycles of freeze–thaw procedure. In each cycle, the stability samples ($n = 5$) at each concentration level were thawed at room temperature and kept in ice-water bath for 2 h and then refrozen to storage temperature at −70 °C. These samples were processed along with the fresh thawed stability samples. The result showed that the interconversion of both enantiomers was kept below 2.9% and the overall stability of MK-0767 was acceptable following proper handling procedure (Table 3).

Autosampler stability was studied by comparing freshly injected samples with re-injected samples 24 h later. The result showed that MK-0767 enantiomer is stable and its enantiomeric ratio was not changed over 24 h in autosampler tray at 4 °C (Table 3).

3.3.6. Bioanalytical application

This method has been applied successfully to the analysis of plasma samples from clinical studies. QC samples were run in duplicate at each level with the clinical samples to monitor daily performance of the method. Table 4 shows the summarized QC information from one clinical study. For all QCs analyzed in this study over 10 months period, the precision (CV) for the QC samples were ≤8.4%. The accuracy ranged from 95.0 to 99.9% for both enantiomers. Results from the analysis of QC samples illustrate the robustness of the method.

4. Conclusions

An LC–MS/MS method for the determination of MK-0767 enantiomers in human plasma has been developed and validated. The validation results indicated that the method is sensitive, specific, accurate, and reproducible. The in vitro interconversion was controlled at a minimum following proper handling procedure. The method has been applied to the clinical sample analysis for the determination of the MK-0767 enantiomer concentrations in human plasma and provided satisfactory results.

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