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Simultaneous determination of *Z*-3-[(2,4-dimethylpyrrol-5-yl) methylidenyl]-2-indolinone (SU5416) and its interconvertible geometric isomer (SU5886) in rat plasma by LC/MS/MS

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Abstract

Z-3-[(2,4-Dimethylpyrrol-5-yl)methylidenyl]-2-indolinone (SU5416) is a cytostatic substance in development as an anti-angiogenic agent. SU5416 exists as the thermodynamically stable *cis* or *Z*-isomer as a solid. Studies have shown that in light exposed solutions of SU5416, the unstable trans or *E*-isomer, namely SU5886, is formed. The *E*-isomer converts back to the *Z*-isomer when protected from light. The *E*-isomer is unstable for synthesis and isolation; therefore, the analytical standard of the *E*-isomer is not available. In this study, a simple, fast and reliable LC/MS/MS method has been developed to determinate both isomers simultaneously in rat plasma samples to support the study of disposition kinetics of SU5416. This method is sensitive (limit of quantitation (LOQ = 0.5 ng/mL)), reproducible and has a wide linear range (0.5–2500 ng/mL). There was no conversion between *E*- and *Z*-isomer during sample preparation procedure and sample determination with LC/MS/MS. Experimental results proved that SU5416 and SU5886 have identical detection response. Therefore, SU5416 (*Z*-isomer) was used successfully as analytical standard for SU5886 (*E*-isomer). This method has been applied to rat plasma samples obtained from a pharmacokinetic study. This study underscores the use of LC/MS/MS technique for bioanalytical methods where analytical standards are not available and analytes are interconvertible.

Keywords: *Z*-Isomer; *E*-Isomer; LC/MS/MS; SU5416

1. Introduction

Z-3-[(2,4-Dimethylpyrrol-5-yl)methylidenyl]-2-indolinone (SU5416) is an angiogenesis inhibitor in development [1]. It is a synthetic molecule designed to inhibit the growth of solid tumors by preventing the formation of new blood vessels (angiogenesis), which are required for nourishing the tumors. SU5416 acts by blocking the signaling pathway of the Flk-1 receptor, which is found on the surface of endothelial cell lining of the blood vessel walls. Flk-1 is a primary driver of angiogenesis in most solid tumors, suggesting a very important opportunity for the development of an effective inhibitor of this receptor [1–4]. SU5416 is currently in Phase II/III clinical trails as an anti-angiogenic agent for the treatment of cancer.

SU5416 can exist in two stereomeric forms, as *E*- and *Z*-isomers (see Fig. 1), around the double bond between 2-oxindol and the pyrrol ring of 3-[(2,4-dimethylpyrrol-5-yl)methylidenyl]-2-indolinone. The *E*-isomer of SU5416 is called SU5886, *E*-3-[(2,4-dimethylpyrrol-5-yl)methylidenyl]-2-indolinone. The solid substance exists only as *Z*-isomer. However, SU5416 can exist in two interconvertible forms in a solution. In order to support the in vivo pharmacokinetic and toxicological studies of SU5416, a sensitive, simple and reliable analytical method was developed. SU5416 is a light sensitive compound when it is in a solution. It can spontaneously convert to SU5886 when the solution of SU5416 is exposed to light, especially in dilute solutions. SU5886 is not stable in solution, and it readily converts back to SU5416 when the solution is protected from light. Addi-

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tionally, the quantification of SU5886 is also difficult because an analytical standard of SU5886 is not available. SU5886 could not be synthesized. SU5886 converts to SU5416 during its synthesis and only the *Z*-isomer could be isolated.

A HPLC method has been developed to determine these two isomers in support of preclinical pharmacokinetics studies. However, this method has a poor sensitivity (limit of quantitation (LOQ > 25 ng/mL)) and a long elution time (>10 min), which may cause the interconversion between *Z*- and *E*-isomers. This method was only used for assay of drug substance or formulation solutions. In this study, a unique method was developed to determine SU5416 (*Z*) and SU5886 (*E*) in rat plasma samples simultaneously by LC/MS/MS. This method was sensitive (LOQ = 0.5 ng/mL), fast (the separation time was 2 min), accurate, precise and with a good linear range (0.5–2500 ng/mL). The sample preparation procedure was very simple. The stability of SU5416 and SU5886 during the sample preparation and separation was studied to obtain a suitable procedure for avoiding the conversion between the *Z*- and *E*-isomers. The MS detection response of SU5416 and SU5886 was studied and SU5416 was successfully used as the analytical standard to quantitate SU5886. This method was applied to a pharmacokinetic study of SU5416 isomerization and disposition in rats.

2. Experimental

2.1. Instrumentation

A Perkin-Elmer Sciex Instruments (Foster City, CA) API-365 LC/MS/MS system with a Perkin-Elmer series 200 autosampler and two Shimadzu LC 10-AD pumps (Columbia, MD) was used in this study. The flow rate of the HPLC system was 0.8 mL/min. A post column tee splitter was used to reduce the flow rate to 0.3 mL/min mobile phase before the flow goes to the mass spectrometry. A BDS Hypersil silica column obtained from Keystone Scientific (Bellefonte, PA) was used to separate the *Z*- and *E*-isomers. The LC/MS/MS system was controlled by a power Macintosh 7300/200 computer with MassChrom software. Turbo IonSpray source with positive ion precursor/product monitoring was applied. The scan type was multiple reaction monitoring (MRM) and the masses measured were 239.0 (Q1) and 224.1 (Q3) for SU5416 and SU5886, and 283.4 (Q1) and 250.9 (Q3) for SU9815 (internal standard, see Fig. 1). A Zymark TurboVap LV evaporator (Hopkinton, MA) and a Thermo IEC Centra MP4R centrifuge (Waltham, MA) were used in this study.

2.2. Materials and reagents

SU5416 reference standard (purity > 99.8%) and an internal standard, SU9815 (purity > 99%) were obtained from Chemistry Department. HPLC grade acetonitrile, ethyl acetate and 90% formic acid were purchased from Fisher Scientific (Fair Lawn, NJ). All the stock and working standard solutions of SU5416 and the internal standard (SU9815) solution used in this work were prepared in 50% acetonitrile. The HPLC mobile phase contained 45% acetonitrile and 0.03% formic acid. Blank rat plasma was purchased from Pel-Freez Biologicals (Rogers, AK).

2.3. Experimental procedure

The optimum parameters (a state file) of MS/MS system were obtained by perfusing 5 μg/mL solution of SU5416

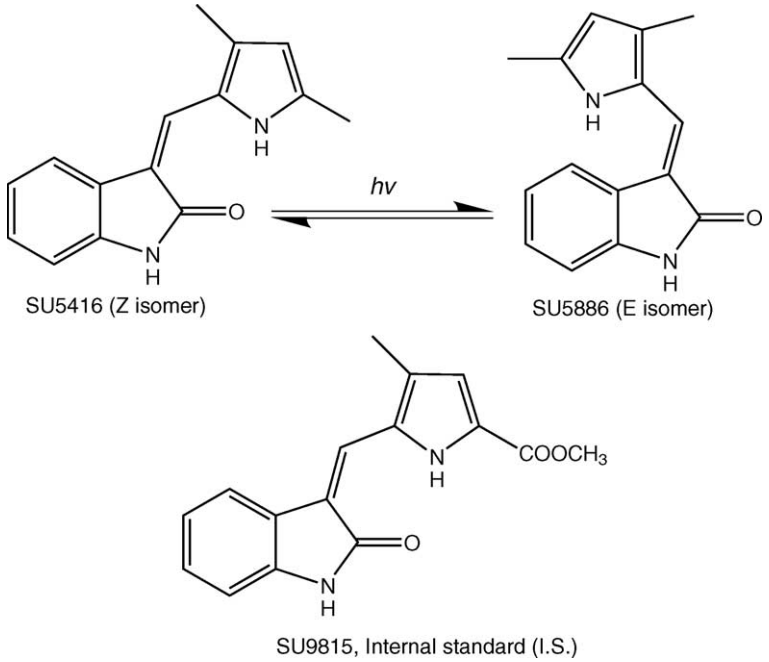

Fig. 1. The structures of test compounds and internal standard.

at 10 μL/min and 90% acetonatrile at 300 μL/min into the MS/MS system. The turbo ionspray gas flow of the system was 6000 mL/min and the source temperature was set at 350 °C. The molecular ion and fragmentation ions and state parameters were selected with an auto-tune procedure. The samples were prepared by spiking 20 μL of SU5416 standard solutions and 50 μL of internal standard solution (100 ng/mL SU9815) into 200 μL of blank rat plasma. Two milliliters of ethyl acetate was added after vortex mixing for 30 s. The aqueous phase was frozen by dry ice–methanol after vortex mixing for 2 min and centrifuging for 5 min at 4000 rpm, and the organic phase was transferred to a clean test tube. The organic solvent was dried by nitrogen gas. The residue was re-constituted with 150 μL of 50% acetonitrile. After vortexing for 30 s, the solution was transferred to an injection vial. Twenty microliters of the sample solution was injected onto the HPLC column. Other specific experimental procedures are described in detail in Section 3.

2.4. In vivo experimental procedure

A clinical formulation (A-005416-AB) of SU5416 was provided by the Formulation Research Department. This formulation at an approximate concentration of 4.5 mg/mL was diluted three-fold with 0.45% saline to obtain a final concentration of 1.5 mg/mL. The diluted formulation was exposed to light for 6 h to generate the *E*-isomer. The relative percentage of the *E*-isomer in the formulation was determined by the LC/MS/MS method as 0.7% *E*-isomer and 99.3% *Z*-isomer. The in vivo phase of this study was carried out under regular laboratory lights. The diluted formulation containing both isomers was administered intravenously to rats via jugular vein as a short infusion (over 1 min). The total dose of both isomers was 7.5 mg/kg. Blood samples were collected via carotid artery at 5, 15, 30, 60, 120 and 240 min after dosing. The blood samples were centrifuged immediately and the upper plasma layer was transferred to a tube, extracted and assayed immediately. SU5416 reference standard was used as the analytical standard for both SU5416 and SU5886. The calibration curve samples were prepared in the dark.

3. Results and discussion

3.1. Mass spectra of SU5416 (Z) and SU5886 (E)

The spectra of SU5416 and SU5886 are shown as Fig. 2 (B and C). SU5416 and SU5886 have identical spectra. They have identical molecular ion (MH^+, $m/z = 239$) and fragmentation ions. The *m*/*z* of major stable fragmentation ions of SU5416 and SU5886 are equal to 224 amu. The fragmentation ions of both compound were identical because the fragmentation did not involve the double bond between 2-oxindole and the pyrrole ring of 3-[(2,4-dimethylpyrrol-5-yl)methylidenyl]-2-indolinone. Therefore, it could be concluded that both SU5416 and SU5886 have the same ionization mechanism. These results indicate that both compounds cannot be resolved by MS/MS detection. However, these experiments show that it is possible to use SU5416 as the analytical standard of SU5886 because they have the same ion pairs for the MRM scan.

3.2. The chromatogram of SU5416 and SU5668

Fig. 2A shows the typical total ion chromatogram of SU5416 and SU5886. The two compounds were resolved by chromatography. Both compounds were scanned by using the same MS/MS channel. The retention time of SU5416 and SU5886 (*E*) were 1.15 and 1.89 min, respectively. The total separation time was shorter than 2.5 min. This fast separation procedure significantly reduced the potential of the conversion between SU5416 and SU5886 during the injection and separation. Fig. 3 shows the typical chromatogram of SU5416 and SU5886 in rat plasma samples obtained from an in vivo pharmacokinetic study. Fig. 4 shows the chromatogram obtained from a blank rat plasma sample. There was clean in the analyte channel. No endogenous interfering peak was detected. In order to avoid the conversion of SU5416 and SU5886, a simple composition of mobile phase, and a fast separation was critical. The mobile phase used in this work was 45% acetonitrile, 55% water and 0.05% formic acid. The two compounds could be resolved well without formic acid. However, the sensitivity was poor without formic acid because a positive ionization process prefers an acidic condition. The pH of the mobile phase was lower than 2 by adding formic acid. The mobile phase, therefore, provided abundant protons to the analytes to obtain a high percentage of positive ionization. Excessive acid, however, would cause the conversion and degradation of both compounds. A short column (50 mm × 4.6 mm) and a high flow rate (0.8 mL/min) were used to achieve a short separation time, and to achieve a high column efficiency and good resolution. A tee splitter was used to compensate for the high back-pressure and the poor sensitivity of the turbo ionspray source because of a high flow rate. The mobile phase at 300 μL/min was introduced to MS/MS system to obtain the optimal conditions.

3.3. The responses of MS/MS detection for SU5416 and SU5886

In order to use SU5416 (*Z*) as the analytical standard of SU5886, the relationship between the response of SU5416 and of SU5886 was studied. Fig. 2 indicates that both compounds have the same molecular and fragmentation ions and ionization mechanism. SU5416 and SU5886 are expected to have different ionization ratio ($[MH^+]/[M]$) in a neutral solution because they have different polarity. However, if the acidity of the solution is high enough, both compounds should have similarly high ionization ratios. Since both compounds have identical ionization ratio and ion pairs (parent–daughter ions) under a certain condition, they should have identical response to MS/MS detection. An experiment was de-

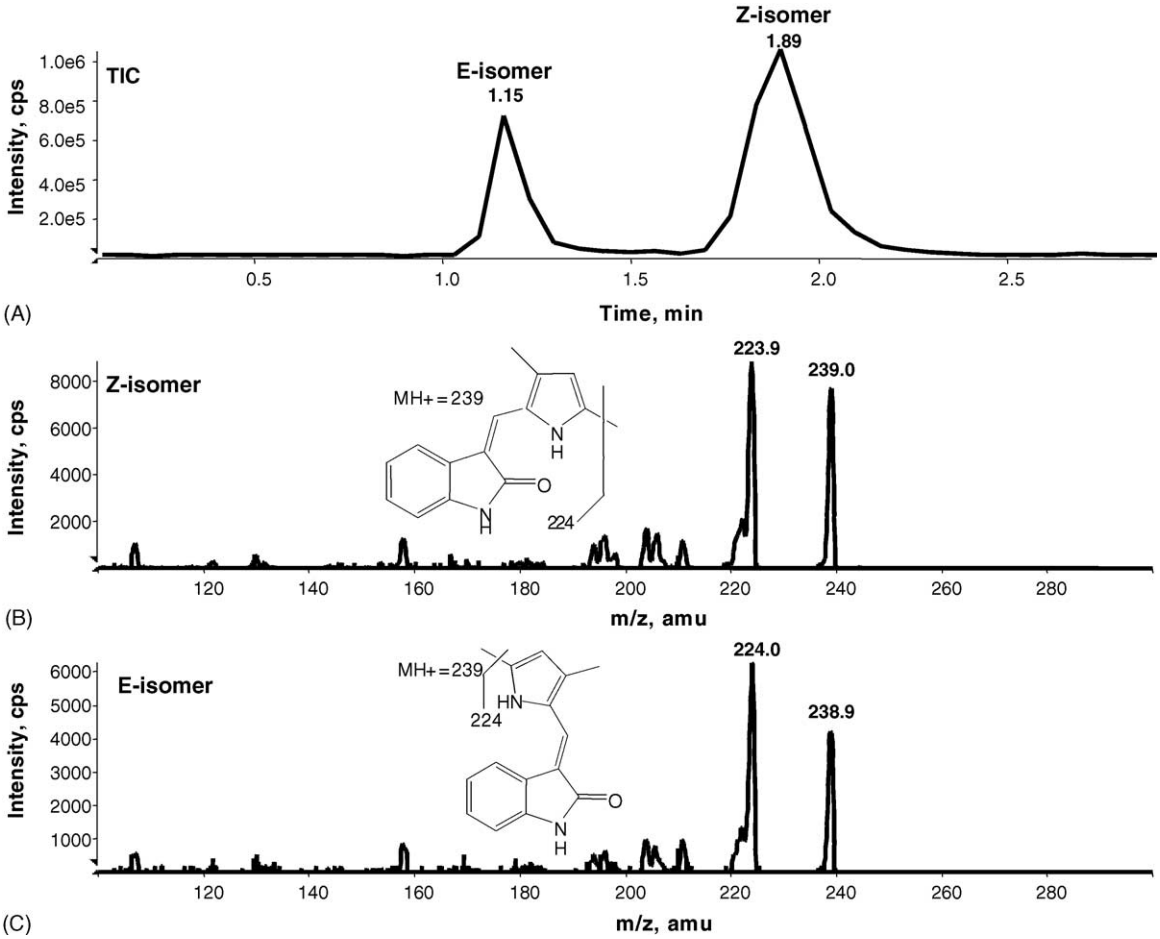

Fig. 2. A typical total ion chromatogram (A) of SU5416 (*Z*-isomer) and SU5886 (*E*-isomer), and the fragmentation mass spectra of SU5416 (B) and of SU5886 (C); 50 μL of 250 ng/mL standard solution (in 50% acetonitrile) exposed to a light for 30 min was injected onto the LC/MS/MS system and the daughter ion of 239 scan was carried out.

signed to show that both SU5416 and SU5886 should have the same MS/MS response by using MRM scanning. Two sets of standard solutions of SU5416 were prepared under a light protected condition. One was exposed to a normal laboratory light for 30 min and the other was protected from light. Then, the two sets of standard solutions were analyzed by LC/MS/MS using the conditions described in Section 2. Table 1 and Fig. 5 show the experimental results. Table 1 shows that the decreased signal (intensity, count per second (cps)) of SU5416 was equal to the increased signal (intensity, cps) of SU5886 when the solution of SU5416 was exposed to a light. In Fig. 5, *Z*-isomer protected (*Z*p) represents the signal–concentration profile of the light protected solution. *Z*-Isomer exposed and *E*-isomer exposed (*Z*e + *E*e) represents the signal–concentration profile of the light exposed solution. The concentration of SU5416 in light protected solution

Table 1
The experimental results of the response of SU5416 (*Z*) and SU5886 (*E*)

Concentration (ng/mL)	Standard solution (protected from light)		Standard solution (exposed to light)		Change of peak area *Z*p–*Z*e	Change of peak area *E*e–*E*p	Ratio (*Z*p–*Z*e)/(*E*e–*E*p)
	*Z*p	*E*p	*Z*e	*E*e			
5	1991	0	1369	509	622	509	1.22
25	10480	0	6580	4068	3900	4068	0.959
50	19073	0	12495	7885	6578	7885	0.834
250	89629	0	43476	37725	46153	37725	1.22
500	180588	0	116404	67304	64184	67304	0.954
2500	803515	1142	582301	265873	221214	264731	0.836
5000	1511826	2547	1160936	407099	350890	404552	0.867

*Z*p: Peak area of *Z*-isomer in the standard solution protected from light, *E*p: peak area of *E*-isomer in the standard solution protected from light, *Z*e: peak area of *Z*-isomer in the standard solution exposed to light, *E*e: peak area of *E*-isomer in the standard solution exposed to light.

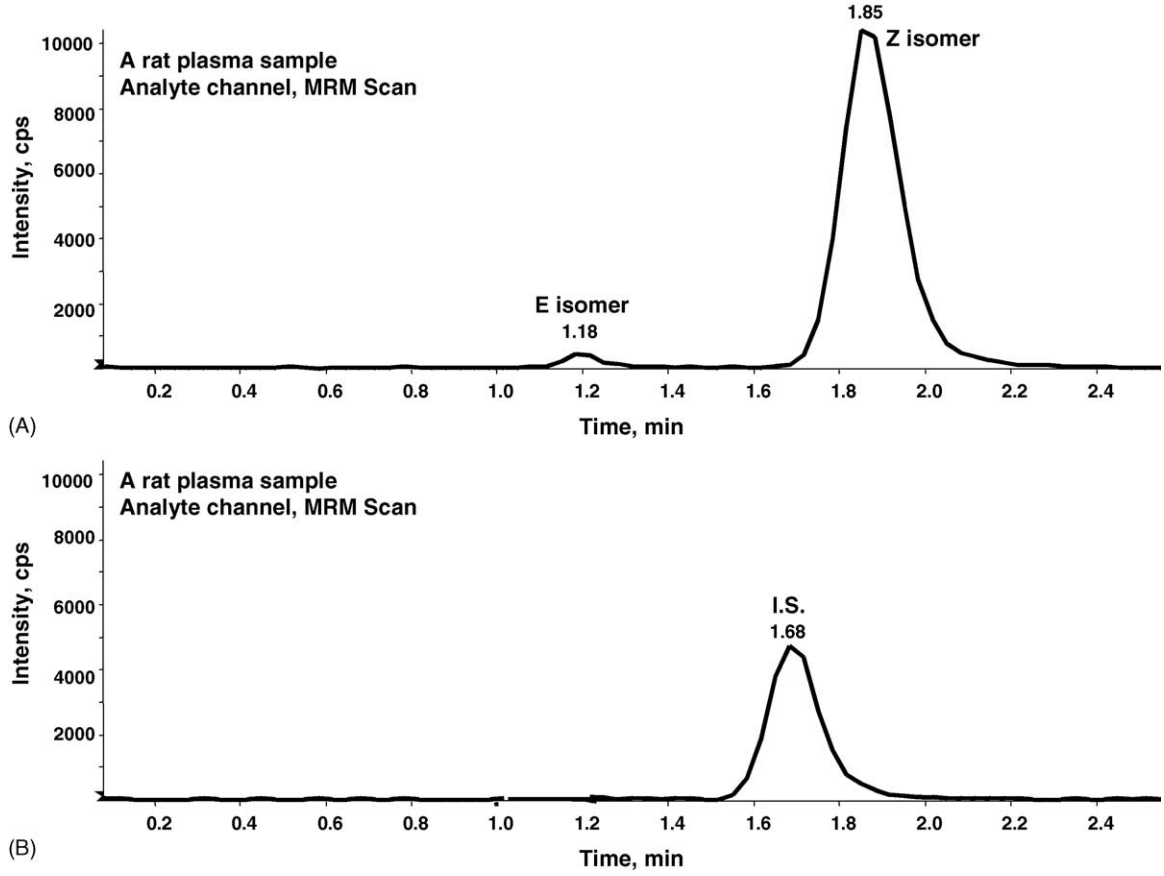

Fig. 3. A typical total ion chromatogram of SU5416 and SU5886 in rat plasma collected after dosing SU5416 and SU5886. (A) The analyte channel and (B) the internal standard (SU9815) channel.

should be equal to the sum of the concentrations of SU5416 and SU5886 in the light exposed solution. There was no degration of the *Z*- and *E*-isomers and only the conversion between both isomers occurred in the solution. If the response of both isomers with the MS/MS detection is the same, the signal of the light protected solutions should be equal to the signal sum of light exposed solutions ($Zp = Ze + Ee$). Table 1 and Fig. 5 indicate that the signal of SU5416 in the light protected solution of SU5416 was equal to sum of signals of SU5416 and SU5886 in the light exposed solution of SU5416. This result proved that both isomers have the same response. This result also indicated that a mass balance was maintained during the conversion between both isomers. Therefore, it is convincing that SU5416 and SU5886 have identical response of MS/MS detection under the experimental conditions used in this study. SU5416 was, therefore, used as the analytical standard for quantitating SU5886. The standard curve sample of SU5416 was prepared and handled under a light protected condition.

3.4. The stability of SU5416 and SU5886 during the sample preparation

In order to assure that there was no conversion between SU5416 and SU5886 during sample handling and preparation, the stability of both compounds was studied. A standard solution of 500 ng/mL in 50% acetonitrile was exposed to a light for 30 min, and then kept under light protection for 9 h. SU5416 and SU5886 were measured at 1-h intervals by using LC/MS/MS. The results are shown in Fig. 6A, which indicates that SU5416 was stable for 9 h and the concentration of SU5886 decreased slowly from the original concentration (<5%). In the first hour, the concentration of SU5886 remained unchanged. In a separate experiment, a standard solution of 500 ng/mL was exposed to light for 8 h, then kept under light protection for 50 min and measured both isomers at 10 min intervals. The results in Fig. 6B showed that both isomers were stable for 50 min under light protected condition. Therefore, the procedure of plasma sample preparation should be completed in 40 min.

The stability of both isomers in the sample extraction procedure was studied. A standard solution of 500 ng/mL was exposed to light for 15 h. Three groups of samples were prepared using this standard solution. Group 1: the analytes were spiked into blank plasma before sample extraction. The extraction procedure was described in Section 2 in detail. Group 2: the procedure was the same as the procedure described in Section 2 except where the standard solution was spiked into the re-constituted solution after sample extraction and before sample injection onto the LC/MS/MS. Group 3: the procedure

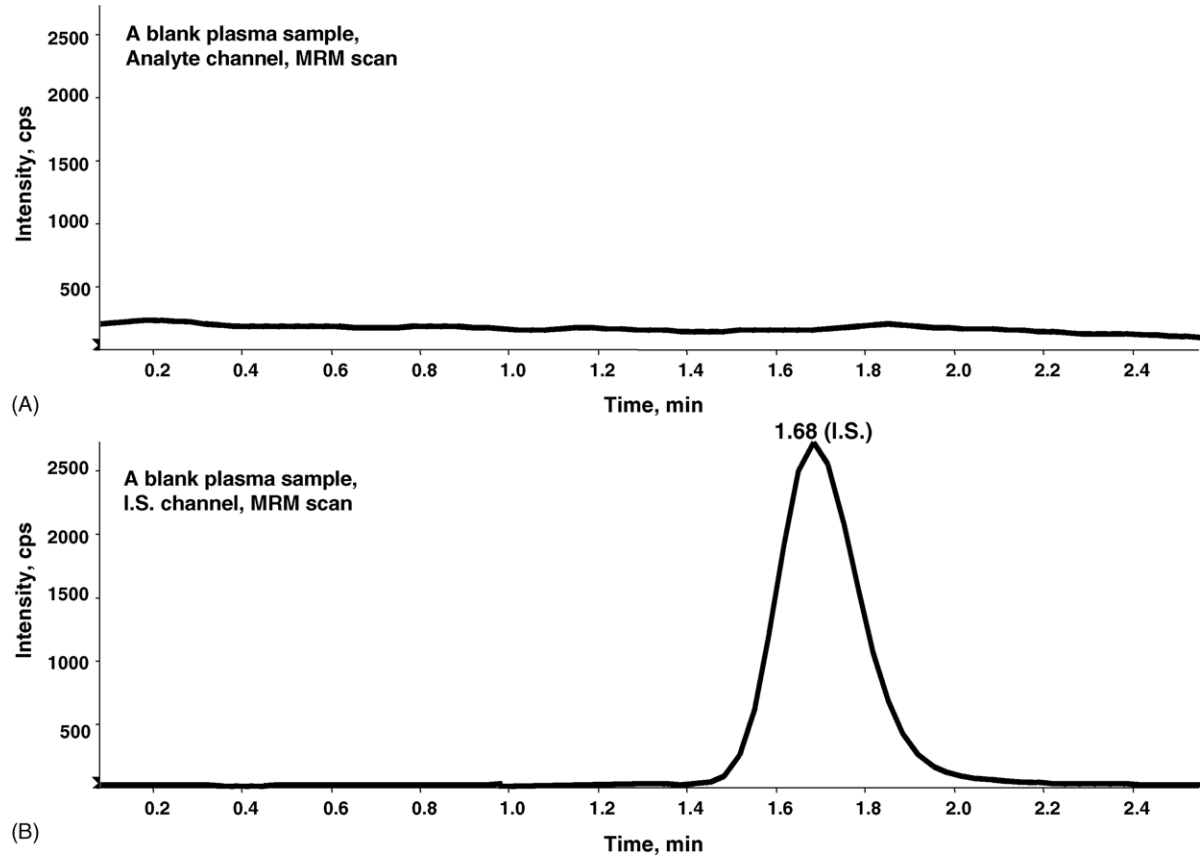

Fig. 4. A rat blank plasma sample collected before dosing SU5416 and SU5886. (A) The analyte channel and (B) the internal standard (SU9815) channel.

was same as Group 2 except that 200 μL of 50%ACN was used instead of rat plasma. The composition of SU5416 and SU5886 in these three groups was quite similar. The results shown in Table 2 demonstrated that there was no significant difference in the stability of *E*- and *Z*-isomers for the three groups of samples. The minor difference in the response for the different group of samples might be caused by the matrix effect, extraction recovery, analytical error and/or the minor conversion of both isomers. Therefore, no significant conversion between SU5416 and SU5886 was observed during the sample preparation procedure. The sample preparation procedure took about 25 min.

The stability of SU5416 and SU5886 in the mobile phase and the column was studied under light protected and acid conditions. The mobile phase used in this method contained 0.03% formic acid. In this experiment, a standard solution of 5 μg/mL SU5416 was exposed to lights for 15 h. Then it was diluted 10-fold with 45% acetonitrile and the mobile phase, respectively. The two samples were then kept in the dark for 1.5 min (retention time of SU5886 is only 1.15 min). Then, the two samples were analyzed by the LC/MS/MS system. The intensity of SU5886 (*E*) from the two samples was 8071 and 7871, and the ratios of the intensity of SU5886 to SU5416 were 0.474 and 0.493, respectively. There was no isomerization in the dark and in acidic mobile phase. Therefore, the results suggest that there is no conversion between two compounds during the column elution procedure of SU5886.

3.5. Performance of this method

The performance of this method for rat plasma samples is summarized in Table 3. The calibration curves were linear over the specified ranges (0.5–2500 ng/mL for both compounds). A correlation coefficient of 0.999 or higher was

Table 2
The stability of *Z*- and *E*-isomers of SU5416 during the sample preparation procedure

Sample	Spiking order of analytes	Response (peak area of isomer/peak area of I.S.)	
		Z-isomer	*E*-isomer
Group 1, analytes were spiked in plasma	Before sample preparation	1.13 ± 0.09	0.51 ± 0.05
Group 2, analytes were spiked in extract of plasma	After sample preparation	1.09 ± 0.05	0.60 ± 0.02
Group 3, analytes were spiked in 50% CAN	After sample preparation	0.89 ± 0.01	0.55 ± 0.07

Table 3
The sensitivity, linear range, accuracy and precision of the assay

Compound	LLOQ (ng/mL)	Linear rage (ng/mL)	Correlation coefficient (r)	Sensitivity[a] slop (response vs. ng/mL)	Nominal concentration (ng/mL)	Concentration measured (ng/mL)	CV (%)	RE (%)
SU5416	0.500	0.500–2500	0.9999	0.0076	1.00	1.07	3.61	7.00
					50.0	51.3	7.48	2.60
					500	456	5.42	8.80
SU5886	0.500	0.500–2501	0.9999	0.0076				

$N = 6$, CV: coefficient of relative variation, RE: relative error, LLOQ: lower limit of quantitation.
[a] The response was the ratio of peak area of SU5416 or SU5886 to SU9815 (I.S.).

obtained for the relationship between the peak area ratios (analyte/I.S.) and the corresponding calibration concentrations. The method shows good linearity over a broad concentration range and excellent sensitivity. A quantification limit of 0.5 ng/mL was achieved for both compounds in rat plasma. Table 3 also shows the accuracy and precision of this method.

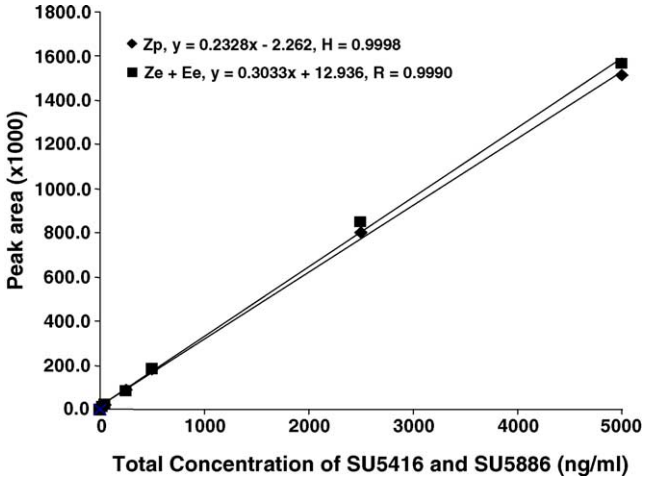

Fig. 5. The MS/MS response relationship between light protected solution of SU5416 and light exposed solution of SU5416 and SU5886 (*Z*p: *Z*-isomer in light protected solution; *Z*e and *E*e: *Z*- and *E*-isomers in light exposed solution).

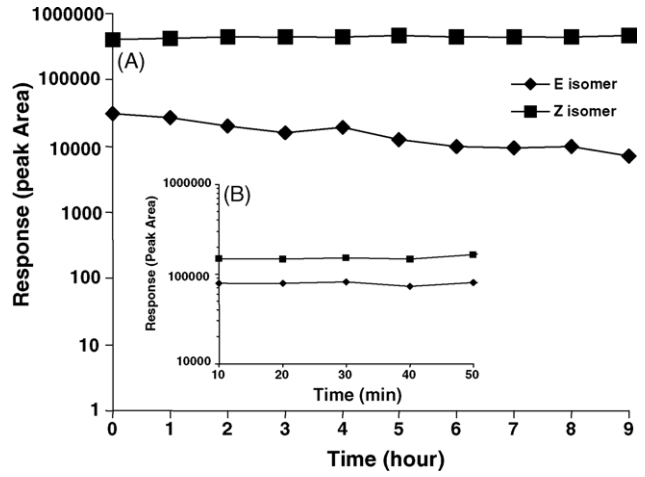

Fig. 6. The stability of SU5416 and SU5886 in 50% acetonitrile solution ((A) a 9 h experiment; (B) a 50 min experiment).

The relative errors (RE) were lower than 8.8% and the coefficients of relative variation (CV) were lower than 7.5%.

The extraction recovery of the sample preparation was tested. The liquid–liquid extraction method (with ethyl acetate) used in this study provided greater than 85% extraction recovery for both isomers. These results clearly indicate that the reproducibility, recovery and sensitivity are acceptable over the studied concentration range. This method requires 25 min for sample preparation and 2.5 min for the determination of both isomers by LC/MS/MS system.

3.6. The application of this method to pharmacokinetic and plasma stability studies

The method described in this paper was used to analyze rat plasma samples in support of the pharmacokinetic study of SU5416 and SU5886 in rats. The in vivo pharmacokinetic experimental procedure was described in Section 2. The experimental results are shown in Fig. 7. The *E*-isomer of SU5416 was detected in the plasma although only a trace amount of the compound was administered to the rats and the pharma-

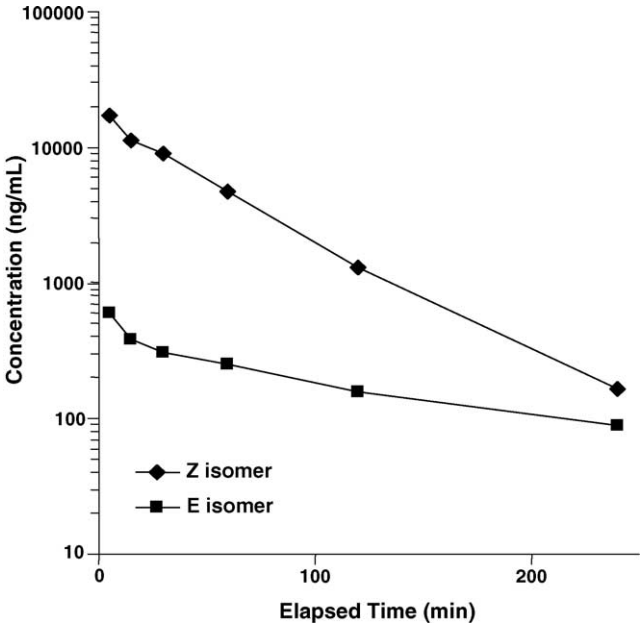

Fig. 7. The concentration–elapsed time profile of SU5416 (*Z*) and SU5886 (*E*) in rat plasma samples.

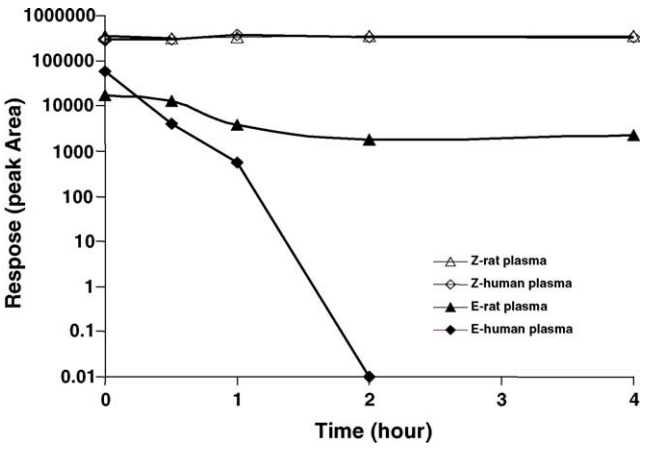

Fig. 8. The stability of SU5416 (*Z*) and SU5886 (*E*) in human and rat plasma incubated at 37 °C.

cokinetics of the *E*-isomer was different from SU5416. The two isomers have different disposition kinetics. This method was also used to study the in vitro stability of both isomers in rat plasma and human plasma. A 50 μg/mL SU5416 standard solution (in dimethyl sulfoxide) was exposed to light for 30 min. Two microliters of this solution was spiked into 200 μL rat and human plasma. These samples were incubated at 37 °C for 15, 30, 60, 120 and 240 min under a light protected condition, and then analyzed these samples by using the method described in Section 2. Fig. 8 shows the experimental results. In vitro, SU5416 was stable in both rat and human plasma. SU5886, on the other hand, was more stable in the rat than human plasma. Especially, the concentration of SU5886 in human plasma was reduced by greater than 90% after incubation for 60 min. This experimental result also suggests that in rat plasma, the thermodynamically unstable *E*-isomer may equilibrate to the thermodynamically stable *Z*-isomer, in vitro.

4. Conclusions

A unique LC/MS/MS method was developed to determine the two interconvertible isomers, namely SU5416 and SU5886, in rat plasma to support the pharmacokinetic and the stability studies of the drug. This LC/MS/MS method is simple, fast, sensitive, reproducible and with an broad linear range. This method was used to determine the concentration of SU5886 in the absence of an analytical standard and to ascertain that there was no conversion between the isomers in sample preparation, handling and separation. Ultimately, this LC/MS/MS method was used to study the pharmacokinetics of SU5416 and SU5886 in the rat.

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