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Simultaneous determination of Z-SU5416 and its interconvertible geometric E-isomer in rat plasma by LC/MS/MS

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Abstract

SU5416 is a selective inhibitor of vascular endothelial growth factor (VEGF) receptor, which plays a major role in vascular angiogenesis. SU5416 exists as the thermodynamically stable and pharmacologically active *cis* isomer (Z-isomer) in the solid state. In light-exposed solutions the unstable *trans* isomer (E-isomer) is formed. The E-isomer is unstable for synthesis and isolation and the analytical standard of the E-isomer is unavailable. A new, simple, fast and reliable LC/MS/MS method was developed to quantify both isomers simultaneously in rat plasma samples in order to support the study of disposition kinetics of Z- and E-SU5416. This method is sensitive (LOQ = 0.5 ng/ml), reproducible, and has a wide linear range (0.5–2500 ng/ml).

Keywords: Z-SU5416; LC/MS/MS; E- and Z-isomers

1. Introduction

SU5416, referred to as the Z-isomer in this manuscript, is an angiogenesis inhibitor with selectivity for the vascular endothelial growth factor (VEGF) receptor. 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. It acts by blocking the signaling pathway of VEGF and its receptor, fetal liver kinase-1/kinase insert domain-containing receptor (Flk-1/KDR), which is found on the surface of the endothelial cell lining in blood vessels. Flk-1/KDR is a primary driver of angiogenesis in most solid tumors, and its inhibition by SU5416 was extensively studied in our efforts in cancer research [1–4].

SU5416 exists in two stereoisomeric forms, the E (*trans*) or Z (*cis*) isomer around the double bond between 2-oxindole and the pyrrole ring as seen in Fig. 1. The solid substance exists only as the Z-isomer, which is the thermodynamically stable form. However, in solution it can spontaneously convert to its E-isomer when exposed to light. Specifically, the photoisomerization of the Z to E-isomer in methanol or acetonitrile at pH 10 (basified with NaOH) reaches equilibrium within 5–6 h. The Z-isomer is completely stable in acidified (pH 2) methanol or acetonitrile [5]. The E-isomer is not stable in solution, and readily

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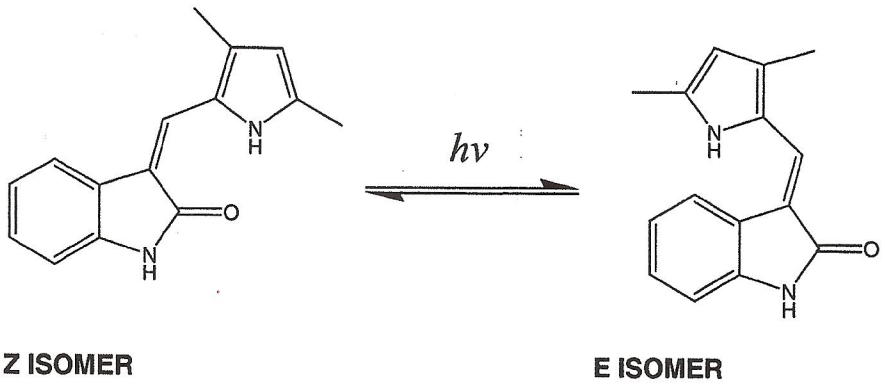

Fig. 1. The interconversion between the E- and Z-isomers of SU5416, 3-[(2,4-dimethylpyrrol-5-yl)methylidenyl]-2-indolinone.

converts back to the Z-isomer when the solution is protected from light. Thus the E- and Z-isomers are interconvertible in solution depending upon the conditions. The quantification of the E-isomer is difficult because an analytical standard is not available and could not be synthesized. The E-isomer converts to the Z-isomer during synthesis and only the Z-isomer could be isolated.

This paper describes a unique method developed to determine the two interconvertible geometric isomers simultaneously in rat plasma samples 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 simple and relatively short (25 min). The stability of the two isomers during the sample preparation and separation was also studied to obtain a suitable procedure to avoid the conversion between the Z- and E-isomers. The MS detection response of Z-isomer was identical to that of the E-isomer and thus the Z-isomer was successfully used as the reference standard to quantitate the E-isomer. This method was applied to a pharmacokinetic study of SU5416 in rats.

2. Experimental

2.1. Materials and reagents

SU5416, 3-[2,4-dimethylpyrrol-5-yl)methylidenyl]-2-indolinone reference standard (purity > 99.8%) and the internal standard SU9815 (the methyl ester derivative of 3-[2,4-dimethylpyrrol-5-yl)methylidenyl]-2-indolinone, purity > 99%), were obtained from Chemistry Department at SUGEN Inc. 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 3-[2,4-dimethylpyrrol-5-yl)methylidenyl]-2-indolinone and the internal standard solution used in this work were prepared in acetonitrile–water (50:50, v/v). The HPLC mobile phase contained acetonitrile–water–formic acid (45:55:0.03, v/v/v). Blank rat plasma was purchased from Pel-Freez Biologicals (Rogers, AK).

2.1.1. Experimental

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 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 was directed to the mass spectrometer. 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 239.0/224.1 (Q1/Q3) for the Z- and E-isomers, and 283.4/250.9 (Q1/Q3) for SU9815. The sample inlet was a heated nebulizer. A TurboVap LV evaporator and an IEC Centra MP4R centrifuge were used in this

study. The optimum parameters (a state file) of MS/MS system were obtained by perfusing 5 μg/ml solution of SU5416 at 10 μl/min and acetonitrile–water (90:10, v/v) 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.

2.1.2. Extraction procedure

The samples were prepared by spiking 20 μl of SU5416 standard solution and 50 μl of internal standard solution into 200 μl of blank rat plasma. Two milliliters of ethyl acetate was added after vortexing for 30 s. The aqueous phase was frozen by dry ice–methanol after vortexing for 2 min and centrifuging for 5 min at 4000 × g, 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 acetonitrile–water (50:50, v/v). 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 the results and discussions section.

3. Results and discussions

3.1. The responses of MS/MS detection for the Z- and E-isomers

In order to use the Z-isomer as the analytical reference standard for the E-isomer, the relationship between the responses of the Z- and E-isomers was studied. The spectra of the Z- and E-isomers are shown in Fig. 2B and C, respectively. Both compounds have the same molecular ions (MH+, m/z = 239), fragmentation ions, and ionization mechanism. The m/z of 224 amu is the major and stable fragmentation ion for both isomers. Since both compounds have identical ionization ratio ([MH^+]/[M]) and ion pairs (parent–daughter ions) under a certain condition, they should also have identical response to MS/MS detection. An experiment was designed to show that both isomers should have the same MS/MS response by using MRM scanning. Two sets of standard solutions of the Z-isomer were prepared under light protected conditions. Then, one was exposed to a normal laboratory light for 30 min and the other was protected from light. These two sets of standard solutions were analyzed by LC/MS/MS using the conditions described in the experimental section. If the response of both isomers with the MS/MS detection was to be similar, the mass spectral signal from the light protected solution (Z_p) should be equal to the signal sum of light exposed solution ($Z_p = Z_e + E_e$). The standard solution of the Z-isomer was prepared and handled under light-protected conditions. The photoisomerization of Z ↔ E follows first order kinetics in organic solvents and there is no evidence of photodegradation under laboratory fluorescence lighting (where predominant light intensities are >500 nm) or under yellow laboratory lighting [5]. Photodegradation during Z ↔ E isomerization is evident, however, when an organic solution of Z-SU5416 is exposed to sunlight (where predominant light intensities are ~350–500 nm) [5]. Table 1 shows that the decreased signal (intensity, cps) of the Z-isomer was equal to the increased signal (intensity, cps) of the E-isomer when the solution of the Z-isomer was exposed to light. The signal of Z-SU5416 in the light protected solution was equal to the sum of the signals of both isomers in the light exposed solution. This indicated that both isomers have the same mass spectral response, that there was no degradation of the E- and Z-isomers, and that the conversion between isomers occurs only in solution. This result also confirmed that a mass balance was maintained during the conversion between both isomers. Therefore, it is convincing that both isomers have identical response of MS/MS detection under the experimental conditions used in this study. The Z-isomer was therefore used as the analytical standard for quantitating the E-isomer of the compound in the rat pharmacokinetics study.

3.2. The chromatogram of the Z- and E-isomers

Fig. 2A shows a typical chromatogram of the Z- and E-isomers. The compounds were resolved by chromatography. Both compounds were scanned by using the same MS/MS channel. The retention time of the E- and Z-isomers were 1.15 and 1.89 min, respectively. The total separation time was less than 2.5 min. This rapid separation procedure significantly reduced the potential of the conversion between the isomers

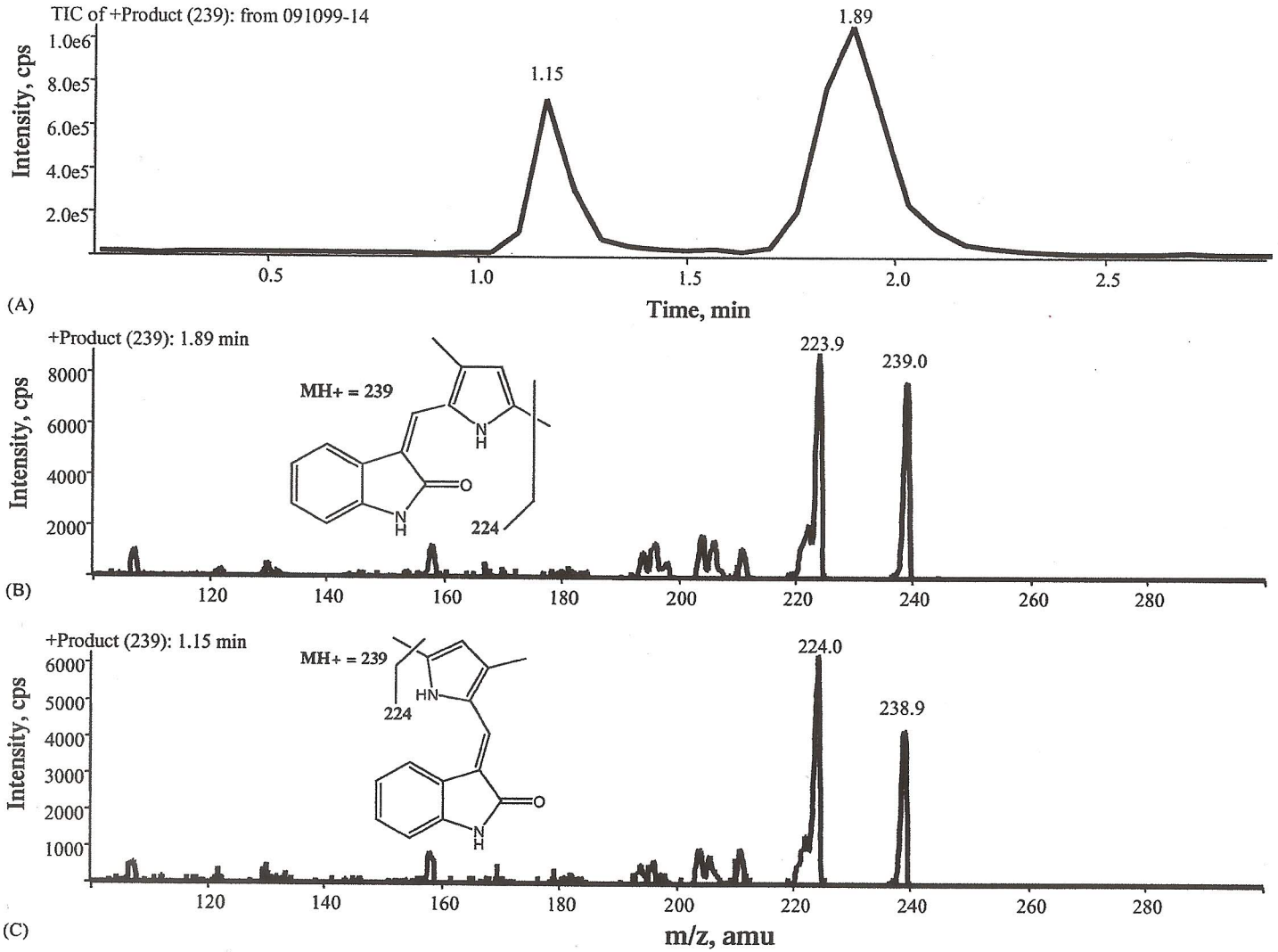

Fig. 2. A typical chromatogram (A) of the Z- and E-isomers, and their spectra (B and C). Fifty microliters of 250 ng/ml standard solution (in 50% acetonitrile) exposed to a light for 30 min was injected onto the LC/MS/MS system and the daughter ion of 239 scan was monitored.

Table 1
Experimental results of the response of the Z- and E-isomers

Concentration (ng/ml)	Z-SU5416 protected from light		Z-SU5416 exposed to light		Difference in peak area of Z-isomer, $\Delta Z = Z_p - Z_e$	Difference in peak area of E-isomer, $\Delta E = E_e - E_p$	Ratio $\Delta Z/\Delta E$
	(Z_p)	(E_p)	(Z_e)	(E_e)			
5	1991	0	1369	509	622	509	1.2
25	10480	0	6580	4068	3900	4068	1.0
50	19073	0	12495	7885	6578	7885	0.8
250	89629	0	43476	37725	46153	37725	1.2
500	180588	0	116404	67304	64184	67304	1.0
2500	803515	1142	582301	265873	221214	264731	0.8
5000	1511826	2547	1160936	407099	350890	404552	0.9

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 for 30 min; E_e: peak area of E-isomer in the standard solution exposed to light for 30 min.

during the injection and separation. In order to avoid the conversion of the Z-isomer to the E-isomer, a simple composition of mobile phase and a fast separation were critical. The mobile phase used in this procedure was acetonitrile–water–formic acid (45:55:0.03, v/v/v). The two compounds could be resolved well without formic acid. However, the sensitivity was poor without formic acid because for positive ionization acidic conditions are preferred. 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 high column efficiency with good resolution. A tee splitter was used to compensate for the high back-pressure and the poor sensitivity of the turbo ion spray source because of a high flow rate. The mobile phase was introduced at 300 μl/min to MS/MS system to obtain the optimal conditions.

3.3. Stability of the isomers during the sample preparation

The extent of photoisomerization of Z to E is dependent on solvent, concentrations, pH and light wavelength [5]. For example, the photoisomerization of Z to E reaches an equilibrium within 5–6 h under basic conditions (pH 10). Under our experimental conditions, acetonitrile–water (50:50, v/v), a continued isomerization of Z to E was evident for up to 9 h (Fig. 4). When protected from light, the unstable E-isomer converts back to the Z-isomer.

In order to assure that there was no conversion between the isomers during sample handling and preparation, the stability of both isomers was studied. A standard solution of 500 ng/ml in acetonitrile–water (50:50, v/v) was exposed to light for 30 min to generate the E-isomer, then kept under light protection for 9 h. The Z- and E-isomers were measured at 1-h intervals by using LC/MS/MS. The results are shown in Fig. 3A, which indicates that the Z-isomer was stable for 9 h and the concentration of E-isomer decreased slowly during the 9 h of light protection (<5%). In the first hour, the concentration of the E-isomer remained unchanged. Therefore, in a separate experiment, a standard solution of 500 ng/ml was exposed to light for 8 h, kept under light protection for 50 min, and then measured for both isomers at 10 min intervals. The results in Fig. 3B and the inset in Fig. 4 showed that both isomers were stable for 50 min under light protected condition. Therefore, a 40-min procedure for rat plasma sample preparation was deemed appropriate.

To study the stability of both isomers during the plasma extraction procedure, a standard solution of 500 ng/ml was exposed to light for 15 h to generate the E-isomer. Three groups of samples were prepared using this standard solution. In the first group, the analytes were spiked into blank rat plasma and extracted as detailed in the experimental section. In the second group, blank rat plasma was extracted in a similar way as group 1. Then the analyte was spiked into the reconstituted solution of the extracted plasma. In the third group, the procedure was the same as the second group

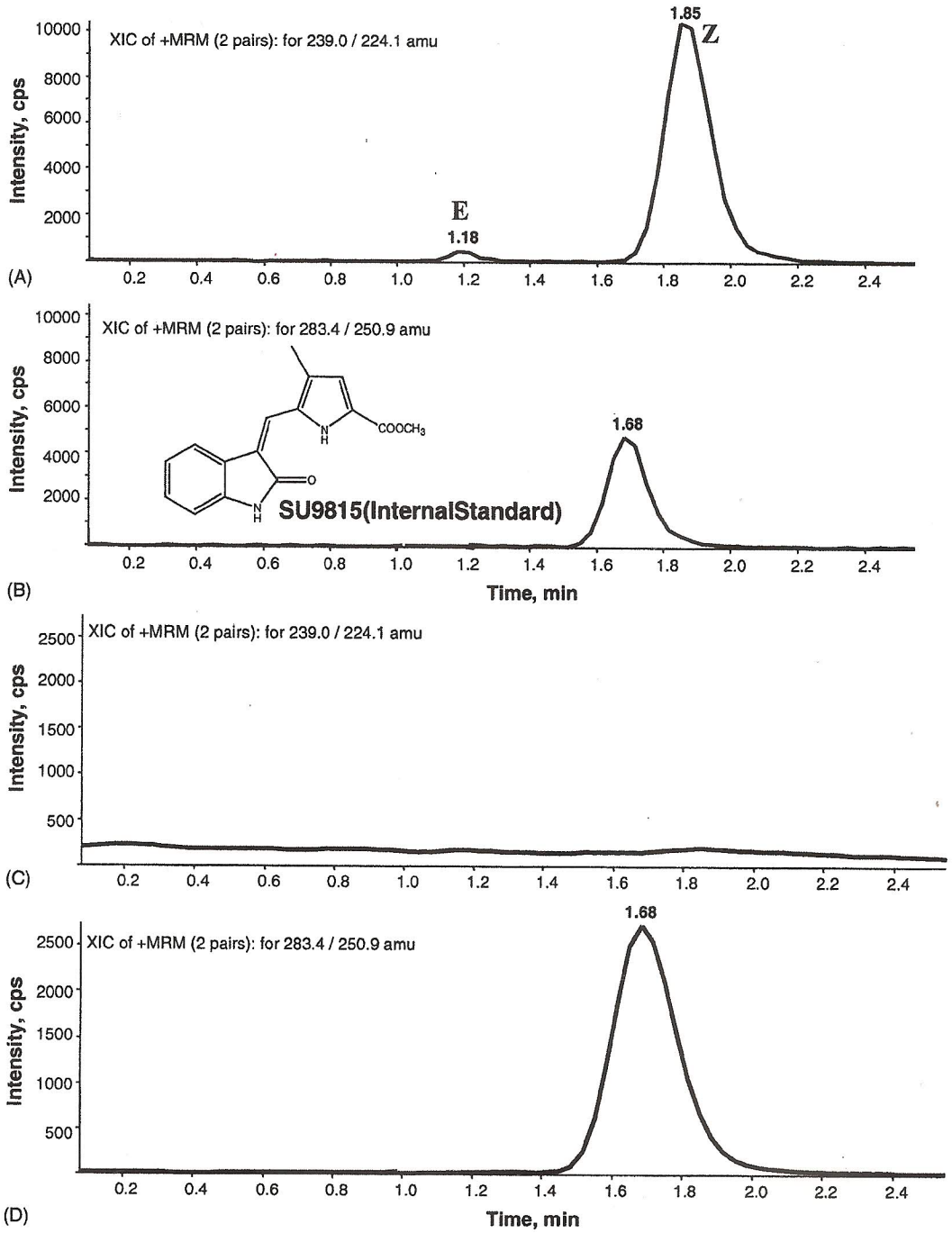

Fig. 3. A typical chromatogram of the Z- and E-isomers of SU5416 in rat plasma. (A) A rat plasma sample collected after dosing. (C) A rat plasma sample collected prior to dosing. (B) and (D) were the internal standard (SU9815) channel for both samples.

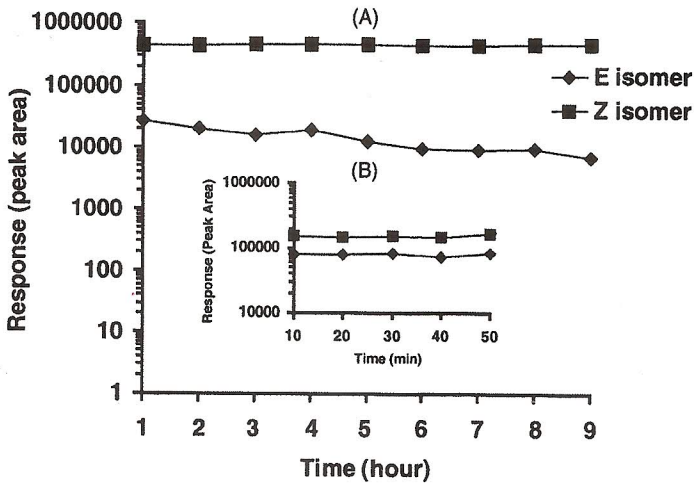

Fig. 4. The stability of the E- and Z-isomers in 50% acetonitrile solution: (A) a 9-h experiment and (B) a 50-min experiment.

except that 200 μl acetonitrile–water (50:50, v/v) was used instead of rat plasma. The composition of the individual isomers in these three groups was quite similar. The results shown in Table 2 demonstrate that there was no significant difference in the stabilities of the E- and Z-isomers for the three groups of samples. The minor difference in the response of the different groups of samples might have been due to matrix effect, extraction recovery, analytical error, and/or minor interconversion of isomers. This experiment showed that there was no significant conversion between the isomers during the sample preparation procedure. The entire sample preparation procedure took approximately 25 min, well within the necessary time limit of 40 min described above.

The stability of the isomers in the mobile phase and the column was studied under light protected and acidic 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 light for 15 h. It was then diluted 10-fold with acetonitrile–water (45:55, v/v) and the mobile phase, respectively. The two samples were then kept in the dark for 1.5 min (retention time of the E-isomer is only 1.15 min). The two samples were then analyzed by the LC/MS/MS system. The intensities of the E-isomers from the two samples were 8071 and 7871, and the ratios of the intensity of the E-isomer to the Z-isomer were 0.474 and 0.493, respectively. There was no isomerization in the dark and in acidic mobile phase. Therefore, the results suggested that there is no conversion between two isomers during the column elution procedure.

3.4. Performance of the assay

The performance of this assay 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 isomers). A correlation coefficient of 0.999 or

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

Sample	Spiking order of analytes	Response (peak area of isomer/peak area of internal standard)	
		Z-isomer	E-isomer
Group 1: analytes spiked in plasma	Before sample preparation	1.13 ± 0.09	0.51 ± 0.05
Group 2: analytes spiked in extract of plasma	After sample preparation	1.09 ± 0.05	0.60 ± 0.02
Group 3: analytes spiked in 50% ACN	After sample preparation	0.89 ± 0.01	0.55 ± 0.07

Table 3
Sensitivity, linear range, accuracy and precision of the assay for SU5416 isomers

Compound	LLOQ (ng/ml)	Linear range (ng/ml)	Correlation coefficient (r)	Sensitivity[a] slope (response vs. ng/ml)
Z-isomer	0.5	0.5–2500	0.9999	0.0076
E-isomer	0.5	0.5–2500	0.9995	0.0076
Compound	Standard concentration (ng/ml)	Concentration measured	CV (%)	RE (%)
Z-isomer	1.00	1.07 ± 0.04	3.6	7.0
	50.0	51.3 ± 3.9	7.5	2.6
	500	544 ± 29.6	5.4	8.8

The response was the ratio of peak area of the Z- or E-isomer to the internal standard.
[a] Standard solutions were prepared using Z-isomer of SU5416.

higher was 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 with 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. The coefficients of relative variation (CV) were lower than 7.5%.

The extraction recovery of the sample preparation was tested. Absolute recoveries were determined by comparing the total area of Z + E isomers in extracted samples to the area of a directly injected standard solution of Z-SU5416. Recovery (%) was defined as (extracted spiked sample/reference sample) × 100. 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. In summary, this method required 25 min for sample preparation and 2.5 min for the determination of both isomers by LC/MS/MS system.

3.5. *Application of the assay for pharmacokinetic and plasma stability studies*

The assay described in this manuscript was used to analyze rat plasma samples in support of the pharmacokinetic study of the Z- and E-isomers of SU5416 in rats. The Z-isomer was prepared in a Cremophor®-based formulation. The original concentration of the formulation was approximately 4.5 mg/ml, which 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 the 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 the 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 test tube, extracted, and assayed immediately. The Z-isomer reference standard was used as the

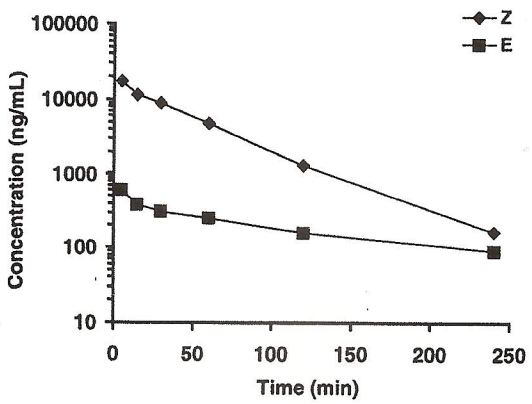

Fig. 5. The plasma concentration–time profiles of the Z- and E-isomers following intravenous administration of the Z-isomer (93.3%) containing 0.7% of E-isomer in rat.

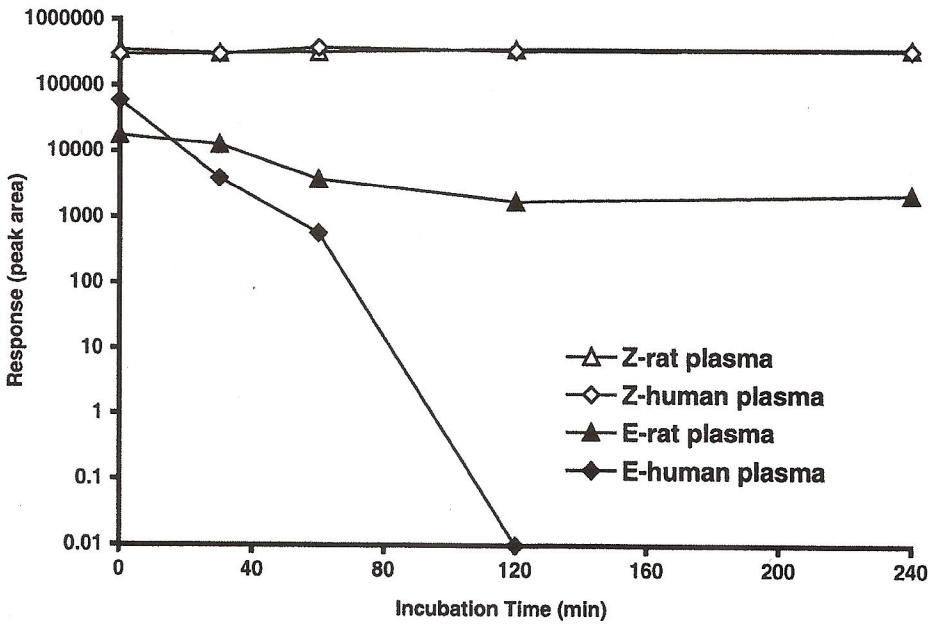

Fig. 6. The ex vivo stability of the Z- and E-isomers in human and rat plasma incubated at 37 °C.

analytical standard for both isomers. The calibration curve samples were prepared under light protected conditions (yellow laboratory lighting was modified with a wavelength cut-off of 580 nm). The experimental results denoting Z- and E-isomers stability are shown in Fig. 4. The concentration–time profile of the Z-isomer in the rat plasma is shown in Fig. 5. Fig. 2 shows the typical chromatogram of the isomers in rat plasma samples obtained from a pharmacokinetic study. No endogenous interfering peak was detected. The E-isomer of SU5416 was detected in the plasma although only a trace amount (0.7%) of the compound was administered to the rats.

This method was also used to study the ex vivo stability of both isomers in rat plasma and human plasma. The standard solution of Z-SU5416 in dimethyl sulfoxide (50 μg/ml) was exposed to light for 30 min. Two microliters of this solution were spiked into 200 μl rat and human plasma. These samples were incubated at 37 °C for 15, 30, 60, 120, and 240 min under light protected conditions, and then analyzed by using the method described in the experimental section. Fig. 6 shows the experimental results from ex vivo stability studies. Ex vivo, the Z-isomer was stable in both rat and human plasma. The E-isomer, on the other hand, was more stable in the rat than human plasma. This was particularly evident for the E-isomer, whereby the concentration of the E-isomer in human plasma was reduced by greater than 90% after a 60-min incubation. This experimental result also suggested that ex vivo in rat plasma, the thermodynamically unstable E-isomer might equilibrate to the thermodynamically stable Z-isomer.

4. Conclusion

A unique LC/MS/MS method was developed to quantify the interconvertible E- and Z-isomers of SU5416 in rat plasma to support the pharmacokinetic and the stability studies of the drug. This LC/MS/MS method is simple, fast, sensitive, and reproducible and has a broad linear range. This method was used to determine the concentration of the thermodynamically unstable E-isomer in the absence of an analytical reference standard and to ascertain that there was no conversion between the isomers during sample preparation and chromatography. This LC/MS/MS method was primarily developed to support pharmacokinetics of the geometric isomers of SU5416 in the rat.

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