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Journal of Pharmaceutical and Biomedical Analysis
25 (2001) 821–832

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

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Simultaneous determination of SU5416 and its phase I and phase II metabolites in rat and dog plasma by LC/MS/MS

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Received 2 August 2000; received in revised form 7 December 2000; accepted 18 December 2000

Abstract

SU5416, *Z*-3-[(2,4-dimethylpyrrol-5-yl)methylidenyl]-2-indolinone, is a cytostatic substance in development as an anti-angiogenic agent. SU5416 has several phase I and phase II metabolites including SU9838, SU6595, SU6689, 5′-hydroxy glucuronide of SU5416 and 5′-acyl glucuronide of SU5416. In order to support the preclinical studies, a liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) method for simultaneous determination of SU5416 and its metabolites in rat and dog plasma was developed. This method is fast, simple, sensitive (LOQ = 2.0 ng/ml), reproducible and has a wide linear range (2.0–5000 ng/ml for SU5416, 2.0–2000 ng/ml for SU6689 and 2.0–1000 ng/ml for SU9838 and SU6595). This method was applied to rat and dog plasma samples obtained from pharmacokinetic and toxicokinetic studies.

Keywords: SU5416; Drug analysis; Liquid chromatography; Mass spectrometry; LC/MS/MS

1. Introduction

SU5416, *Z*-3-[(2,4-dimethylpyrrol-5-yl)methylidenyl]-2-indolinone, 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 one of the primary drivers 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. The objective of this paper is to illustrate a sensitive and validated liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) method developed to analyze SU5416 and its metabolites.

Biotransformation studies on SU5416 [6] have indicated that SU5416 has several metabolites including SU9838, SU6595, SU6689, 5′-hydroxy glucuronide of SU5416 and 5′-acyl glucuronide of SU5416 (Fig. 1). In order to support the pharmacokinetic and toxicological studies of SU5416 and

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0731-7085/01/$ - see front matter
PII: S0731-7085(01)00341-7

its major metabolites, a unique method was developed to simultaneously determine SU5416 and its phase I and II metabolites in rat and dog plasma samples by LC/MS/MS. This method was sensitive (LOQ = 2.0 ng/ml), fast (the separation time was 4 min), accurate, precise and with good linear range (2.0–5000 ng/ml for SU5416, 2.0–2000 ng/ml for SU6689 and 2.0–1000 ng/ml for SU9838 and SU6595). The sample preparation procedure was simple. This method was applied to pharmacokinetic, toxicokinetic and formulation evaluation studies of SU5416 in dogs and rats.

2. Experimental

2.1. Apparatus

A Perkin–Elmer Sciex API-365 LC/MS/MS system with a Perkin–Elmer series 200 autosampler and two Shimadzu LC 10-AD pumps were used in this study. A BDS Hypersil silica column (150 × 4.6 mm, 5 μm) from Keystone Scientific was used to separate the analytes. The flow rate of the high performance liquid chromatography (HPLC) system was 0.8 ml/min. A post column tee splitter was used to reduce the flow rate to 0.3 ml/min for the mobile phase before the flow was introduced into the mass spectrometer. The LC/MS/MS system was controlled by a Power Macintosh 7300/200 computer with MassChrom 1.1. The turbo IonSpray source was operated at 350°C with positive ion precursor/product monitoring. The scan type was multiple reaction monitoring (MRM) and the masses measured at $Q1/Q3$ were 239.5/224.2 for SU5416; 255.3/240.2 for SU6689; 255.0/225.3 for SU9838; 269.0/225.3 for SU6595; 283.4/250.9 for SU9815 (internal standard). The sprayer of the Turbo Ionspray source was aligned at a suitable position. When the probe of the ion

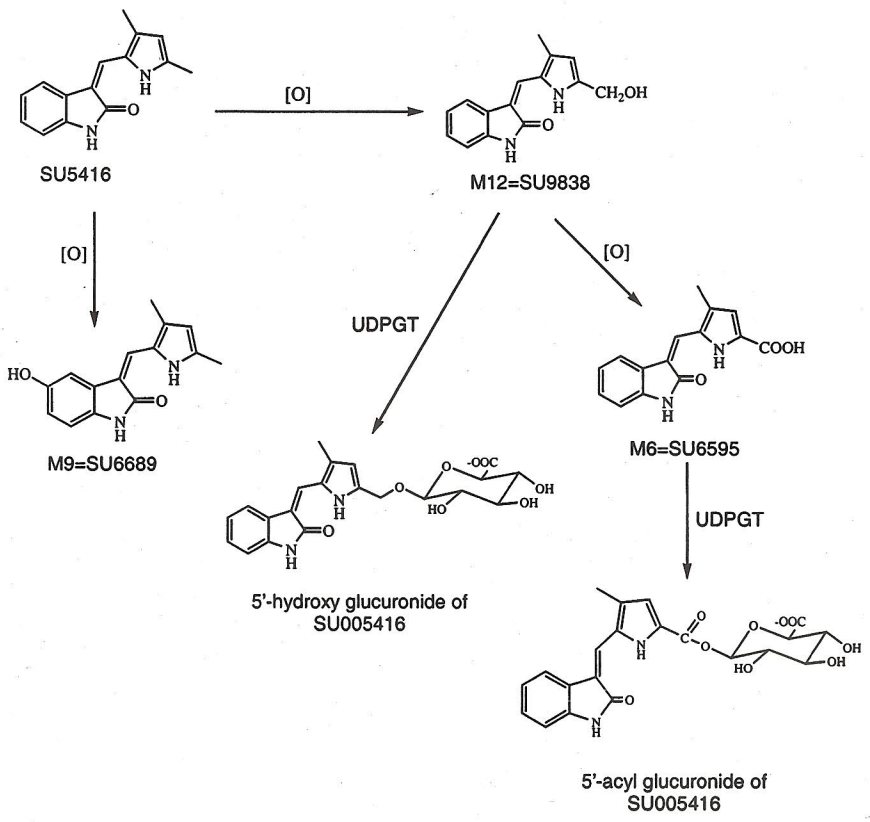

Fig. 1. Biotransformation of SU5416.

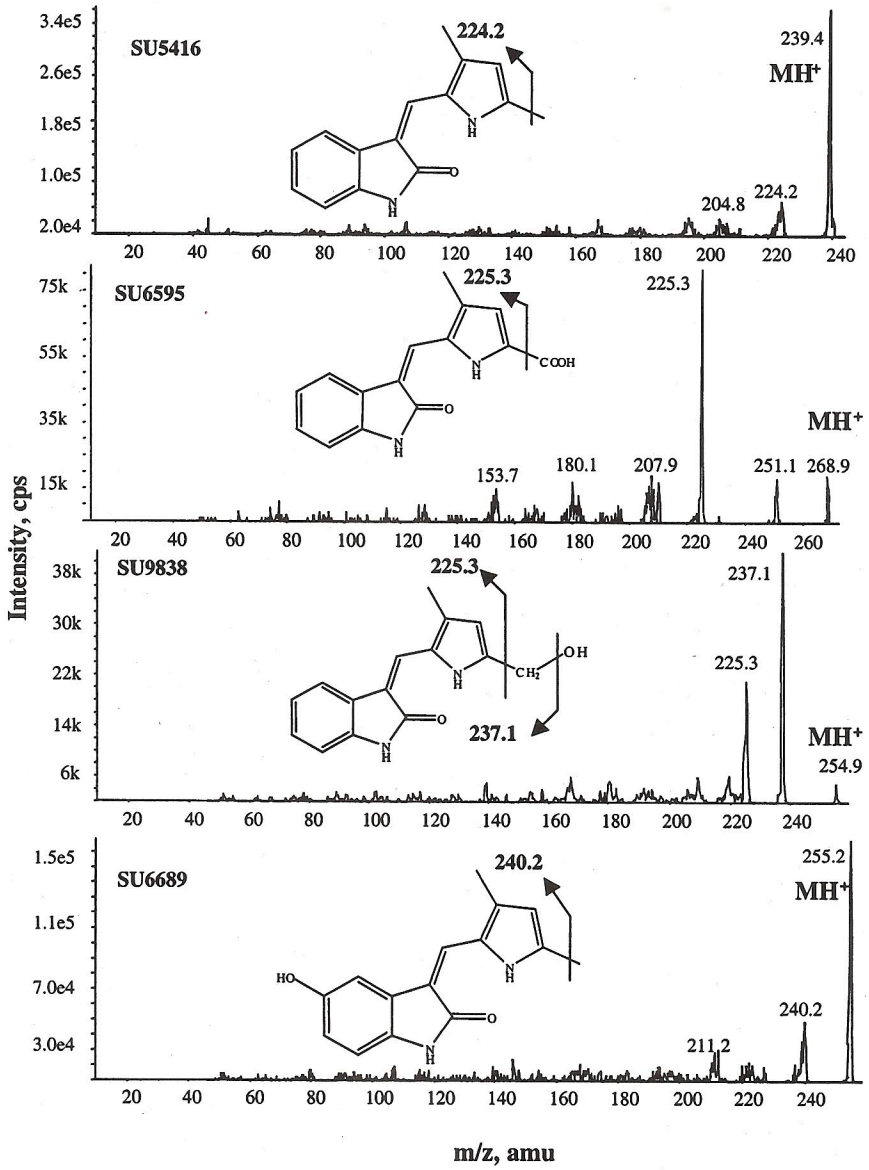

Fig. 2. Spectra of SU5416 and its phase I metabolites.

spray source is too close to the orifice of the curtain plate, the signal stability was reduced because of matrix effects. The ESI electrode voltage was set at 4000 V, nebulizer gas pressure at 9 psi, turbo gas flow at 7.5 l/min, and curtain gas pressure at 8 psi. Collisionally activated dissociation (CAD) was performed using nitrogen with a collision cell gas pressure of 6 mTorr. The collision energy was set at 29 eV for SU5416, 13 eV for SU9838, 30 eV for SU6689, 16 eV for SU6595 and 29 eV for SU9815. The CEM was operated at 2300 V, and the dwell time for each transition was 300 ms. A TurboVap LV evaporator and an IEC Centra MP4R centrifuge were used during sample preparation in this study.

2.2. Materials and reagents

SU5416, SU6595, SU6689 and SU9838 reference standards (purity > 99.8%) and an internal standard, SU9815 (purity > 99%), were obtained from the Chemistry Department at SUGEN. HPLC grade acetonitrile, ethyl acetate, and 90% formic acid were purchased from Fisher Scientific (Fair Lawn, NJ). All working standard solutions of SU5416, SU6689, SU6595, SU9838 and the internal standard (SU9815) solution used in this work were prepared with HPLC grade acetonitrile. The HPLC mobile phase A contained 10% acetonitrile, 90% water and 0.7% formic acid and the mobile phase B contained 90% acetonitrile, 10% water and 0.7% formic acid. Blank rat and dog plasma was purchased from Pel-Freez Biologicals (Rogers, AK).

2.3. Experimental procedure

2.3.1. Optimization of MS/MS system

The optimal parameters (a state file) of MS/MS system were obtained by infusing 5 μg/ml solution of SU5416 and its metabolites individually at a flow rate of 10 μl/min along with a flow of mobile phase B 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.

2.3.2. Preparation of standard solutions

To make four individual stock solutions of 5.0 mg/ml, 10.0 mg each of SU5416, SU6689, SU6595 and SU9838 (I.S.) were dissolved in 2.0 ml dimethylsulfoxide (DMSO). Twenty microliters from each stock solution were mixed together. This mixture solution (80 μl) was diluted with 1.92 ml of 50% acetonitrile to obtain a standard solution containing the four compounds each at 50 μg/ml. This solution was diluted with 50% acetonitrile to obtain standard solutions of 20 and 10 μg/ml. Standard working solutions were prepared by diluting the three standard solutions (50, 20 and 10 μg/ml) with 50% acetonitrile. The final

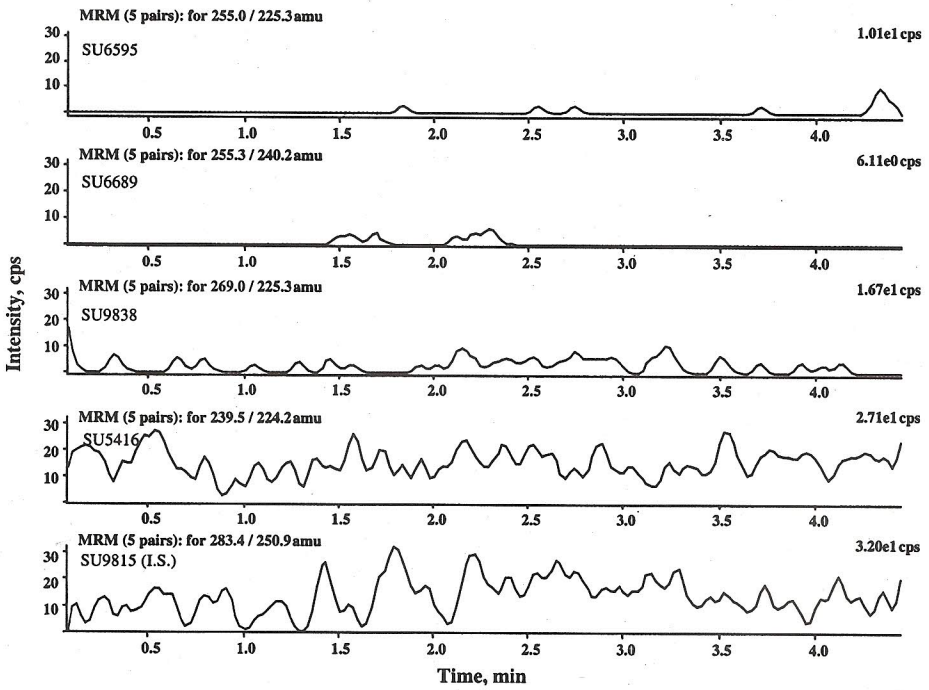

Fig. 3. The chromatogram of blank dog plasma.

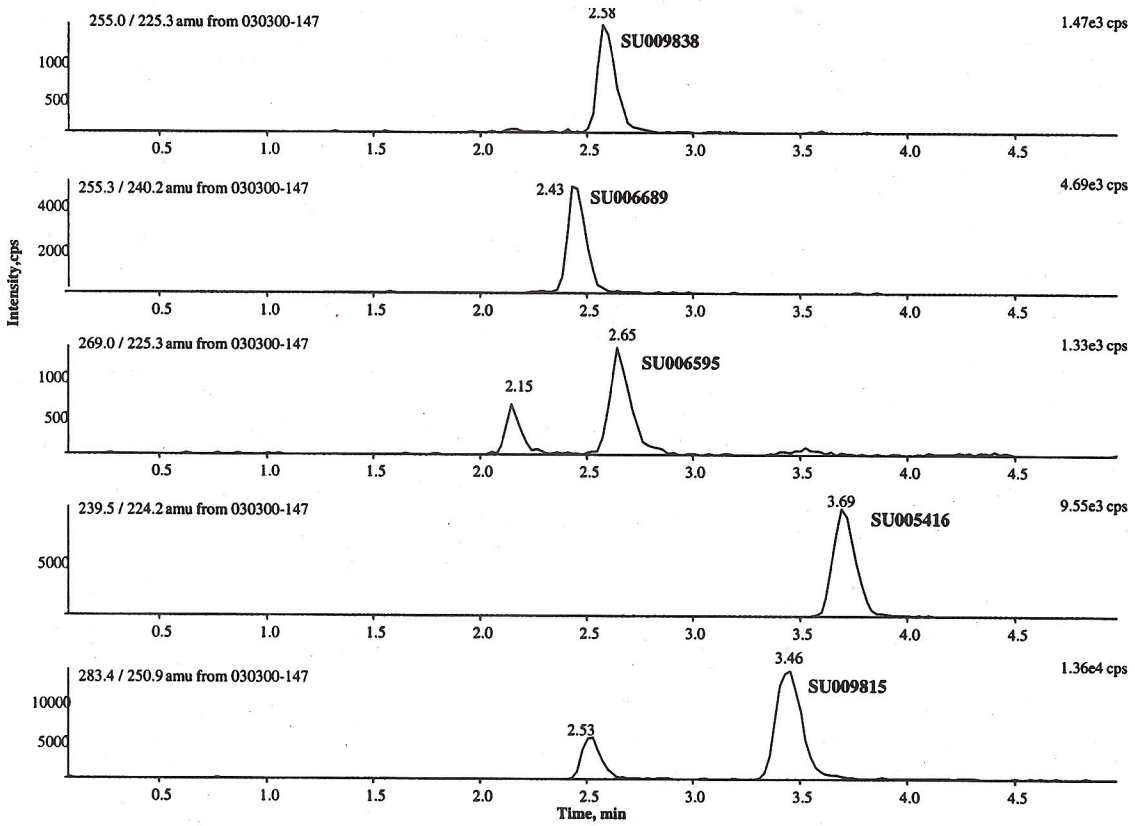

Fig. 4. A typical chromatogram of dog plasma sample spiked with analytes (100 ng/ml).

Table 1
Gradient elution program[a]

Step	Time (min)	Duration (min)	Flow (μl/min)	Mobile phase A (%)	Mobile phase B (%)
0	0.00	0.00	800	15.0	85.0
1	0.00	2.00	800	15.0	85.0
2	2.00	0.10	800	0.00	100
3	2.10	2.00	800	0.00	100
4	4.10	0.40	800	15.0	85.0

[a] Mobile phase A, 10% ACN; 90% water; and 0.7% formic acid. Mobile phase B, 90% ACN; 10% water; and 0.7% formic acid.

concentrations of these standard working solutions for the four analytes were 0.020, 0.050, 0.10, 0.20, 0.50, 1.0, 2.0, 5.0, 10, 20 and 50 μg/ml. An internal standard solution was prepared separately. SU9815 (5 mg) was dissolved in 10 ml of acetonitrile and further diluted with acetonitrile to obtain an internal standard solution of 500 ng/ml.

2.3.3. Sample preparation

The samples were prepared by spiking 20 μl of the standard working solutions of SU5416 and its metabolites and 100 μl of internal standard solution (500 ng/ml SU9815 in 100% acetonitrile) into 200 μl of blank rat and dog plasma, followed by vortexing for 30 s. The final plasma concentra-

tions of standard samples were 2.0, 5.0, 10.0, 20.0, 50.0, 100, 200, 500, 1000, 2000 and 5000 ng/ml. Ethyl acetate (3 ml) was added to each sample, followed by vortexing for 3 min. The organic phase was separated from the aqueous phase by centrifugation at 4000 rpm for 5 min in an IEC Centra MP4R centrifuge. The aqueous phase was frozen by putting the test tubes in dry ice–acetone. The organic phase was transferred to a clean test tube. The organic solvent was evaporated under a stream of nitrogen gas. The residue was re-constituted with 100 μl of the mobile phase B. After vortexing for 30 s, the solution was transferred to an HPLC vial. Sample solution (50 μl) was injected onto the HPLC column. Plasma samples from in vivo studies were prepared using the above procedure.

In order to avoid the isomerization of SU5416 and other compounds, the standard solution and sample preparations were carried out under light-protected condition. Plasma standard and in vivo study samples were processed immediately after thawing on ice.

Table 2
Inter-run variability of quality control samples

	Nominal concentration (ng/ml)			
	1000	400	100	5.00
SU5416				
n	12	12	12	12
Mean	985	395	100	5.15
S.D.	107	24.5	3.88	0.50
CV (%)	10.8	6.2	3.80	9.80
Error	−14.8	−5.08	−0.27	0.15
R.E. (%)	−1.48	−1.27	−0.27	3.00
SU9838				
n	12	12	12	10
Mean	952	425	112	5.01
S.D.	92.7	49.9	13.2	0.38
CV (%)	9.73	11.7	11.8	7.62
Error	−48.0	25.3	12.2	0.01
R.E. (%)	−4.80	6.33	12.2	0.26
SU6689				
n	12	12	12	12
Mean	1021	442	109	4.94
S.D.	97	62.2	11.8	0.51
CV (%)	9.5	14.1	10.8	10.3
Error	20.8	41.7	8.97	−0.06
R.E. (%)	2.08	10.4	8.97	−1.23
SU6595				
n	12	12	12	11
Mean	950	416	104	5.20
S.D.	109	47.1	11.6	0.64
CV (%)	11.5	11.3	11.2	12.3
Error	−50.4	15.5	3.87	0.20
R.E. (%)	−5.04	3.88	3.87	3.93

2.3.4. Hydrolysis of SU5416 glucuronides

β-Glucuronidase (EC3.2.1.31; from *Escherichia coli*, in 50% glycerol solution; 72 U/μl) was purchased from SIGMA (G-8162). β-Glucuronidase (15 μl) solution was transferred to an eppendorf tube, 85 μl of a Tris buffer was added to make 10.7 U/μl enzyme working solution for this experiment. The glucuronidase treatment procedure was as follows: 200 μl of sample plasma which were obtained from an in vivo study was added into a test tube. Fifty microliter of 5 mM Tris buffer which contained 2 mM $MgCl_2$, pH at 7.4 and 10 μl of enzyme working solution of 10.7 U/μl were added into the sample test tube. After vortexing for 15 s, the sample tube was incubated at 37°C for 30 min in a shaking water bath. The sample was then processed immediately using the method described in the Section 2.3 after spiking 100 μl of 500 ng/ml SU9815 internal standard solution into the sample.

2.3.5. Analytical method validation

This method was validated in terms of accuracy, precision, sensitivity, specificity, linearity, and reproducibility according to the FDA guideline for bioanalytical methods validation for human studies [5]. Briefly, blank rat and dog plasma samples from various sources were analyzed. Six standard curves in rat and dog plasma were prepared and analyzed. Quality control (QC) samples containing 5, 100, 400, and 1000 ng/ml of SU5416, SU9838, SU6595, and SU6689 each were analyzed on six different days.

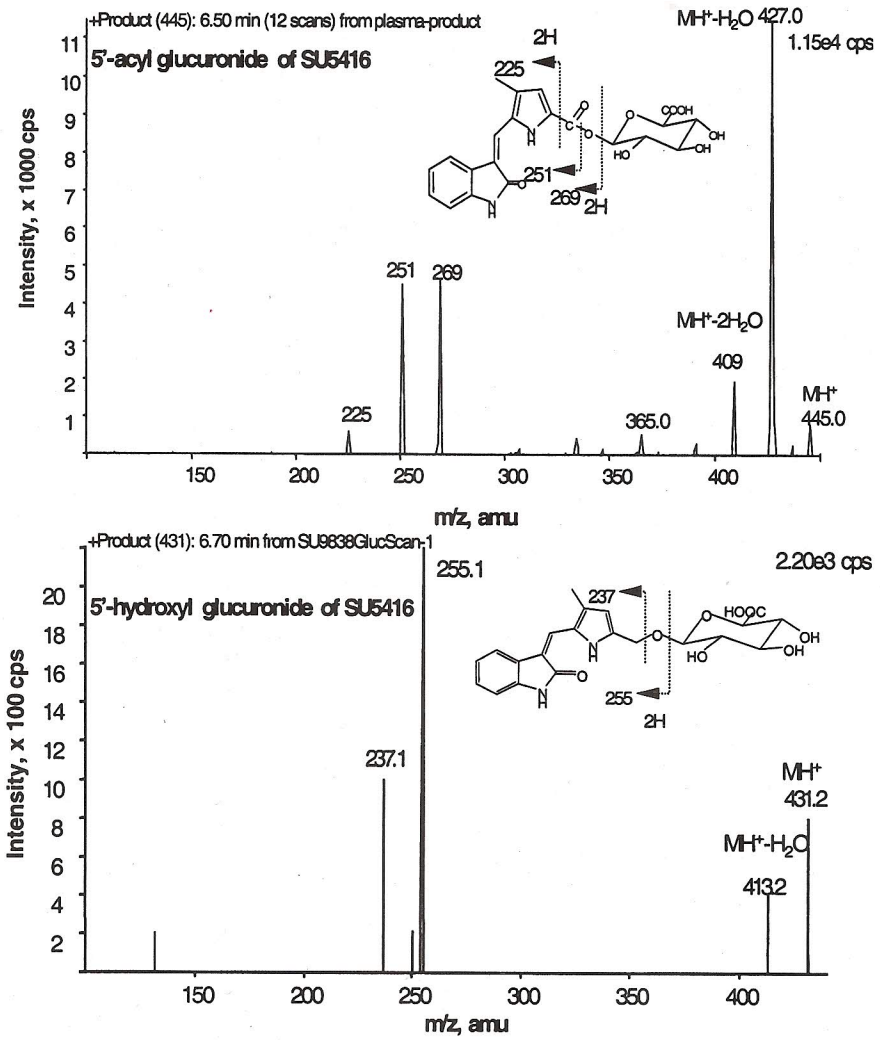

Fig. 5. Spectra of 5′-acyl glucuronide of SU5416 and 5′-hydroxyl glucuronide of SU5416.

3. Results and discussion

3.1. Mass spectra of SU5416 and its metabolites

The spectra of SU5416, SU6689, SU6595 and SU9838 are shown as Fig. 2. The ion pairs selected for quantitation of SU5416, SU6595 and SU6689 were the molecular ions and the most sensitive and stable fragmentation ions. In order to avoid the cross talk between SU6689 and SU9838 and to reduce the background noise, a second fragmentation ion ($m/z = 225.3$) was selected for quantitation of SU9838. No significant cross talks between the four compounds (channels) were observed.

3.2. The chromatograms of SU5416 and its metabolites

Fig. 3 shows a chromatogram of a blank dog plasma sample. No endogenous interfering peak was observed. Fig. 4 shows a typical chromatogram of SU5416 and its metabolites in dog plasma samples. A gradient elution procedure was

applied (see Table 1). The compounds were resolved from sample matrix by the chromatography procedure. The retention times of SU5416, SU6689, SU6595, SU9838 and SU9815 were 3.69, 2.43, 2.65, 2.58 and 3.46 min, respectively. The total separation time was less than 4.5 min. In the SU6595 and SU9815 channels, two peaks in front of the analyte peak were observed. These peaks were subsequently shown to have the same fragmentation pattern as the corresponding analyte, and were therefore attributed to the geometric isomers of SU6595 and SU9815. Experiments also indicated that the cross-talk between internal standard and analytes were not observed.

3.3. Assay performance

The performance of this method is summarized in Tables 2 and 3. This method is sensitive with a wide linear range. The limits of quantification are 2.0 ng/ml for four compounds. The linear range was from 2.0 to 5000 ng/ml for SU5416, from 2.0 to 2000 ng/ml for SU6689, and from 2.0 to 1000 ng/ml for SU6595 and SU9838. This method was validated in dog plasma for these concentration ranges with six inter-day experiments. The correlation coefficients of the standard curves were >0.99 for these analytes. The relative errors (R.E.) of quality control samples was $<9\%$ and coefficient of relative variation (CV) was $<12\%$ (Table 2), which indicated that this method was accurate and precise. The R.E. and the CV of standard samples and LOQ samples were $<15\%$ (Table 3). This validation data met the FDA criteria for an acceptable bioanalytical method [5]. The method was demonstrated to be precise, accurate, and sufficiently reproducible for analysis of in vivo plasma samples to support preclinical pharmacokinetic and toxicological studies.

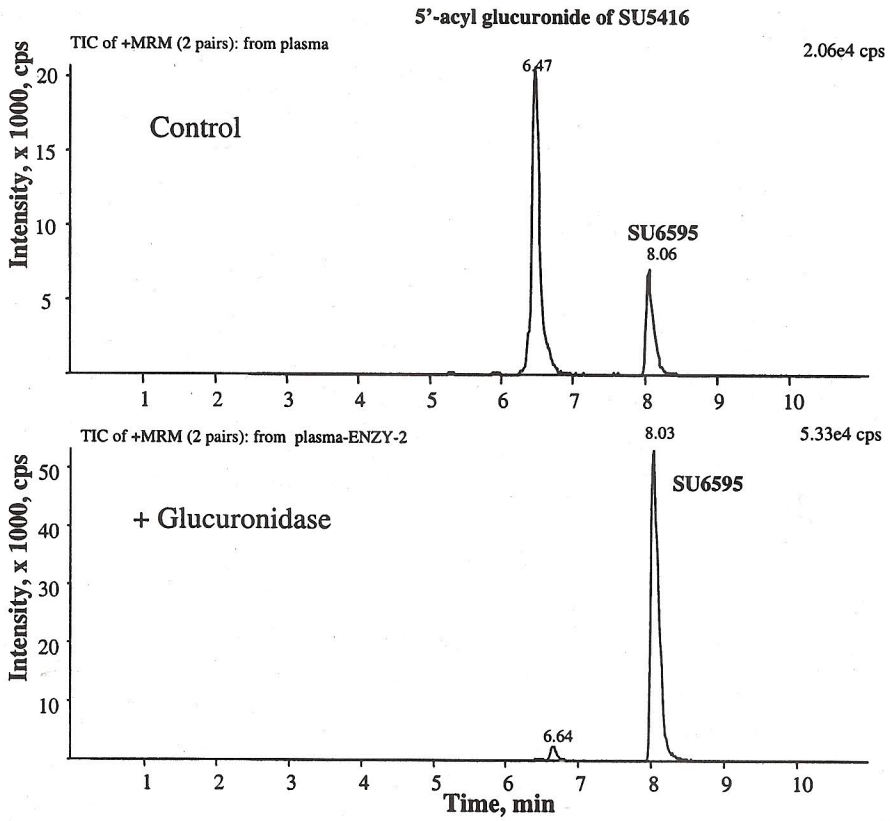

Fig. 6. Effect of glucuronidase treatment on 5'-acylglucuronide of SU5416 and SU6595.