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Short communication

High performance liquid chromatography with ultraviolet detection for the determination of SYUIQ-5, a novel telomerase inhibitor for cancer therapy: Application to an enzyme kinetic study in rat liver microsomes

Qi-Biao Su [a], Fan He [a], Su Guan [a], Yu-Jing Lu [b], Lian-Quan Gu [b], Zhi-Shu Huang [b], Xiao Chen [c], Min Huang [a,**], Chun-Guang Li [d], Balram Chowbay [e], Shu-Feng Zhou [f,*]

[a] *Institute of Clinical Pharmacology, School of Pharmaceutical Sciences, Sun Yat-Sen University, 74 Zhongshan Road, Section 2, Guangzhou, China*
[b] *School of Chemistry and Chemical Engineering, Sun Yat-Sen University, Guangzhou, China*
[c] *Department of Pharmacy, First Affiliated Hospital, Sun Yat-Sen University, Guangzhou, China*
[d] *The Chinese Medicine Research Group, Division of Chinese Medicine, RMIT University, Melbourne, Australia*
[e] *Clinical Pharmacology Lab, Division of Medical Sciences, National Cancer Centre, Singapore 169610, Singapore*
[f] *Division of Pharmacy, School of Life Sciences, Queensland University of Technology, 2 George Street, , G.P.O. Box 2434, Brisbane, Queensland 4001, Australia*

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Abstract

A sensitive assay for the determination of SYUIQ-5, a novel telomerase inhibitor and anti-tumor drug, in rat liver microsomes was developed by using high-performance liquid chromatography with ultraviolet detection. SYUIQ-5 was incubated in vitro with liver microsomes from rats pretreated with control vehicle, β-naphthofIavone, phenobarbital, 20% ethanol or dexamethasone. The analytes were extracted with diethyl ether and separated a C_{18} 5-μm analytical column. Elution was conducted with 30 mM dipotassium hydrogen phosphate (pH 8.0)–methanol–triethylamine (30:70:0.05, v/v/v) at a flow-rate of 1.0 ml/min and the detection of UV absorbance was conducted at 278 nm. Intra-day and inter-day precision and accuracy of the method were within 10%. The mean analytical recoveries of SYUIQ-5 ranged from 78.8 to 95.3%. The linearity of the calibration curve was in the range of 1.0–80.0 μM. The lower limit of quantification (LOQ) was 1.0 μM. Kinetic analysis showed that β-naphthofIavone and dexamethasone significantly induced SYUIQ-5 metabolism, suggesting that cytochrome P450 1A and 3A are the major contributor to SYUIQ-5 metabolism in rat liver microsomes.

Keywords: SYUIQ-5; HPLC; Rat liver microsomes; Metabolism

1. Introduction

SYUIQ-5 (*N*′-(10H-Indolo [3,2-b] quinolin-11-yl)-*N*,*N*-dimethyl-propane-1,3-diamine) (Fig. 1), a novel quindoline derivative synthesized by the School of Pharmaceutical Sciences, Sun Yat-sen University, has been verified to interfere with telomere replication by blocking the elongation step catalyzed by telomerase or telomerase-independent mechanism and could therefore act as a novel potent telomerase inhibitor and promising anti-tumor agent [1,2]. Treatment with quindoline

Abbreviations: CL_{int}, intrinsic clearance; CV, coefficient of variation; CYP, cytochrome P450; K_m, Michaelis–Menten constant; LOQ, lower limit of quantification; NADPH, reduced β-nicotinamide adenine dinucleotide phosphate; V_{max}, maximum velocity of reaction

* Corresponding author. Tel.: +61 7 31381340; fax: +61 7 31381534.
** Co-corresponding author. Tel.: +86 20 873 34521; fax: +86 20 873 34718.
E-mail addresses: huangmin@mail.sysu.edu.cn (M. Huang), s4.zhou@qut.edu.au (S.-F. Zhou).

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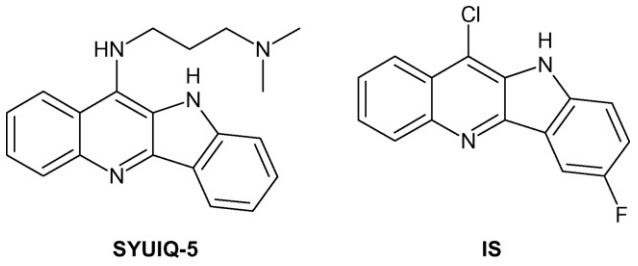

Fig. 1. The chemical structure of SYUIQ-5 (*N*′-(10H-Indolo [3,2-b] quinolin-11-yl)-*N*,*N*-dimethyl-propane-1, 3-diamine) and I.S.

derivatives including SYUIQ-5 inhibited telomerase activity in human leukemia K562 cells and colon cancer SW620 cells. SYUIQ-5 increased time of population doublings of K562 and SW620 cells, induced a marked cessation in cell growth and cellular senescence phenotype, which was accompanied by a shortening of telomere length, and induction of p16, p21 and p27 protein expression [2].

To efficiently inhibit telomerase activity in cancer cells *in vitro*, critical concentrations of SYUIQ-5 must be given. Pharmacokinetic studies are now needed to develop a dosage schedule that can produce the necessary concentrations *in vivo*. The pharmacokinetics and metabolism of SYUIQ-5 need to be examined in details before it can enter Phase I study in cancer patients. In order to study the kinetic profile of the SYUIQ-5 metabolism in rat liver microsomes, we developed and validated a sensitive high performance liquid chromatography (HPLC) method for the detection of SYUIQ-5 in rat liver microsomes. Enzyme kinetics was studied by measuring depletion of the substrate.

2. Experimental

2.1. Chemicals and reagents

SYUIQ-5 and the internal standard (Fig. 1) were synthesized as described previously [1]. Dexamethasone, phenobarbital, β-naphthofIavone, and NADPH were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO). All other chemicals and solvents were of analytical reagent or HPLC grade and obtained from common commercial sources. The water used was of Milli-Q grade purified by a Milli-Q UV Purification System (Millipore, Bedford, MA).

2.2. Animals

Male Sprague-Dawley rats (weighing about 185–220 g) obtained from the Experimental Animal Center of Sun Yat-Sen University (Guangzhou, China) were used throughout this study. Rats housed in cages were kept in a room under controlled temperature (23–24 °C) and 12-h day-night cycle. Animals had free access to tap water and regular diet. All procedures were approved by the Animal Ethics Committee of the Sun Yat-sen University, Guangzhou, China, in accordance with National Institute of Health and Nutrition Guidelines for the Care and Use of Laboratory Animals.

2.3. Administration of enzyme inducers

Healthy male Sprague-Dawley rats ($n = 8$ per group) were randomized to be treated with control vehicle, or several known enzyme inducers, including β-naphthofIavone (80 mg/kg/day, i.p., daily for 3 days), phenobarbital (80 mg/kg, i.p, daily for 3 days), 20% ethanol (5 ml/kg per day, intragastric gavage, daily for 4 days), or dexamethasone (100 mg/kg/day, i.p, daily for 4 days) [3].

2.4. Preparation of rat liver microsomes

On scheduled day, the rats were euthanized with diethyl ether and the livers collected, washed with ice-cold 0.9% NaCl solution and stored at −80 °C. The liver microsomes were prepared by differential centrifugation as described by Zhou et al. [4]. Liver samples were thawed and weighed, and 3 volumes of ice-cold homogenisation medium (50 mmol/l Tris-HCL buffer at pH 7.4 containing 0.25 M sucrose) were then added. The tissue was chopped using scissors, and homogenized with an automatic homogenizer at 500 rpm (IKA Labortechnik, Staufen, Germany). The resultant homogenates were transferred to centrifuge tubes, and centrifuged at $9000 \times g$ for 20 min at 4 °C using a Beckman centrifuge (Beckman Coulter, Inc., Fullerton, CA). The supernatant (S_9) was collected and centrifuged at $105{,}000 \times g$ for 1 h at 4 °C using a L8-70 Beckman ultracentrifuge ((Beckman Coulter, Inc., Fullerton, CA). The microsomal pellet was resuspended with homogenisation medium. Hepatic microsomal suspensions were aliquotted (0.5 ml) into a 1.5-ml test tube and stored at −80 °C until used. The cytochrome P450 (CYP) content was determined by the Omura and Sato method [5]. Protein concentrations were determined by the Lowry et al. [6] method.

2.5. Microsomal incubation

Typical incubation was performed with a 0.5 ml mixture consisting of the substrate, rat liver microsomes (0.25 mg protein/ml), 100 mM potassium phosphate buffer (pH 7.4) and NADPH (1 mM). The linearity of metabolism was examined with respect to incubation time and microsomal concentration. The mixture containing microsomes and substrate was pre-incubated for 5 min at 37 °C, 50 μl NADPH solution was added to initiate the reaction. After incubation at 37 °C for 3.5 min, 2.0 ml ice-cold diethyl ether were added to the reaction tube, followed by vortex-mixing to stop the reaction. Then 10 μl of I.S. at 100 mg/ml and 200 μl ammonium chloride buffer (pH 10.0) were added, vortex-mixed for 2 min and centrifuged at $2000 \times g$ for 10 min. The organic phase was evaporated under nitrogen gas, and the residues were immediately reconstituted in 200 μl of mobile phase and an aliquot (10 μl) was injected into HPLC system for analysis.

2.6. Apparatus and chromatographic conditions

The chromatographic system (Waters, Avondale, CA) consisted of a pump (Waters 515), an automatic injector (Waters

717 plus), a UV detector (Waters 2487), and a Workstation (Millennium32). Hypersil BDS C_{18} column (5 μm, 4.6 mm × 150 mm, Dalian Elite Analytical Instruments Co., Ltd., Dalian, China) was used and preceded with a C8 cartridge guard column (5 μm, 4 mm × 50 mm, Dalian Elite Analytical Instruments Co., Ltd., Dalian, China). The mobile phase consisted of 30 mmol/l dipotassium hydrogen phosphate (pH 8.0)–methanol–triethylamine (30:70:0.05, v/v/v). The analytes were separated by the analytical column set at ambiguous room temperature. Detection was performed at wavelength of 278 nm. The flow-rate was 1 ml/min.

2.7. Preparation of standards and quality control

Stock solutions of SYUIQ-5 were prepared by dissolving it in a volumetric flask, 7.98 mg of SYUIQ-5 precisely weighed, then dissolved in 25 ml water to yield a concentration of 2 mM. Similarly, a stock solution of the I.S. was prepared by dissolving a precisely weighed amount of the substance in a volumetric flask to yield a concentration of 100 mg/ml. The stock solutions were stored in glass tubes at 4 °C.

For the preparation of calibration standards, a series of working solutions of analytes were produced by diluting appropriate volumes of stock solutions with water to yield concentrations of 50–800 μM SYUIQ-5. Calibration standards of 1, 2, 5, 10, 20, 40, and 80 μM of SYUIQ-5 were obtained by double dilution. Appropriate volumes of working solutions were added to 0.5 ml liver microsomes mixtures (0.25 mg protein/ml) containing 100 mM potassium phosphate buffer (pH 7.4) at the final concentration indicated.

A second set of stock solutions, weighed separately, were used for preparation of quality control samples to yield concentrations of 2, 20, and 80 μM of SYUIQ-5. Quality control samples were used to determine intra- and inter-day variability of the HPLC method.

2.8. Validation of method

2.8.1. Specificity

The interference of endogenous compounds was investigated by analysis of a drug-free rat liver microsomal sample.

2.8.2. Calibration curve and calculation procedures

The calibration curve for the assay was constructed by analyzing a serial of blank microsomes spiked with SYUIQ-5 in the concentration range from 1 to 80 μM. Peak area ratios (Y) of SYUIQ-5 versus the internal standard were measured and plotted against the concentration (X) of SYUIQ-5.

2.8.3. Precision and accuracy of the assay

In order to evaluate the intra-day precision and accuracy, samples were analyzed for each concentration on the same day. The inter-day precision and accuracy were evaluated on consecutive 4 days. The assays for both intra- and inter-day precision and accuracy evaluation were assessed using rat liver microsomes spiked with SYUIQ-5 at concentrations of 2, 20, and 80 μM. The accuracy was evaluated as percentage error [(found concentration-spiked concentration)/spiked concentration] × 100%, and the precision was determined by the coefficient of variation (C.V. [(S.D./mean) × 100]%). The acceptance criteria are not more than 15% deviation from the nominal value for accuracy and not more than 15% CV for precision.

2.8.4. Recovery and limits of quantification

Extraction efficiency from microsomal samples, at the concentrations of 2, 20, and 80 μM of SYUIQ-5 and 10 μg/ml of I.S. was examined by comparing with the peak area equivalent to pure compounds dissolved in water. The limit of quantification (LOQ) was defined as the lowest concentration for which the coefficient of variation and accuracy were lower than 15%.

2.9. Statistical analysis

Data are expressed as mean ± S.D. Several models to describe the kinetics of SYUIQ-5 metabolism (single and two binding sites, substrate inhibition, and the sigmoid models) (Eqs. (1)–(4)) were fitted and compared using the Prism 3.0 program.

$$v = \frac{V_{\max} \times [S]}{K_{\mathrm{m}} + [S]} \tag{1}$$

$$v = \frac{V_{\max 1} \times [S]}{K_{\mathrm{m1}} + [S]} + \frac{V_{\max 2} \times [S]}{K_{m2} + [S]} \tag{2}$$

$$v = \frac{V_{\max} \times [S]}{K + [S] + [S]^2/K_{\mathrm{is}}} \tag{3}$$

$$v = \frac{V_{\max} \times [S]^{h'}}{K^{h'} + [S]^{h'}} \tag{4}$$

where v is the rate of metabolism; $V_{\max}$, is the maximum velocity; K_{m}, the Michaelis–Menten constant; [S], the substrate concentration; K_{is}, the substrate inhibition constant; h', the Hill coefficient for cooperative substrate binding; and subscripts 1 and 2 represent the first and the second type of enzyme binding sites. The choice of model was confirmed by F-test and comparison of Akaike's information criterion values [7]. The intrinsic clearance (CL_{int}) was determined by $V_{\max}$ divided by K_{m}. This parameter may be used to predict in vivo clearance when a proper scaling procedure was followed. The statistical analysis to evaluate the differences in the mean kinetic values among different treatment groups was carried out by a one-way analysis of variance (ANOVA) followed with a post-hoc test (Dunnett's multiple comparison test).

3. Results and discussion

3.1. Specificity

Typical chromatograms of extracts of SYUIQ-5 and the I. S. from rat liver microsomal samples are shown in Fig. 2. Following the microsomal incubations, chromatographic peaks were detected with retention times corresponding to those of SYUIQ-5 (5.2 min) and I.S. (6.5 min). The total run time was about

Table 1
Intra and inter-day assay accuracy and precision for the determination of SYUIQ-5 in rat liver microsomes ($n = 4$)

Nominal concentration (μM)	Intra-day ($n = 4$)			Inter-day ($n = 4$)		
	Measured concentration (μM)	CV (%)	Accuracy (%)	Measured concentration (μM)	CV (%)	Accuracy (%)
2	2.02 ± 0.20	9.9	1	1.96 ± 0.20	9.9	−2
20	20.4 ± 0.78	3.8	2	19.6 ± 0.98	4.9	−2
80	72.01 ± 1.87	2.6	−10	73.60 ± 1.25	1.7	−8

Data are the mean ± S.D.

8.0 min. The peak of SYUIQ-5 and its metabolites was largely symmetric or slightly skewed to the right. We evaluated peak skew using the asymmetry coefficient $A_s = b/a$, where b is the distance after the peak maximum and a is the distance before the peak maximum, both a and b being measured at 10% of the total peak height. The asymmetry coefficients for SYUIQ-5 were between 1.03 and 1.07. The measurement of absorbance of SYUIQ-5 by the UVE detector was conducted as an optimal wavelength of 278 nm with a reference wavelength of 350 nm, which gave a maximum signal response.

Matrix-specific interfering peaks that required modification of the mobile phase composition were not observed in any cases, including in the presence of either SYUIQ-5 or I.S.

3.2. Calibration curves

Peak area ratios (Y) of SYUIQ-5 versus I.S. were measured and plotted against the concentration (X) of SYUIQ-5. The linearity of the calibration curve was in the range of 1.0–80.0 μM. The regression equation of the calibration curves was $Y = 0.2235X + 0.031$ ($r^2 = 0.9989$, $n = 5$). For each point on the calibration curves for SYUIQ-5, the concentrations back-calculated from the equation of the regression analysis were within acceptable limits for accuracy and precision of ±20%. A linear regression of the back-calculated concentrations versus the nominal values provided a unit slope and an intercept not significantly different from zero. The distribution of the residuals showed random variation, was normally distributed and centered on zero. The bias was not statistically different from zero, and the 95% confidence intervals included zero (data not shown).

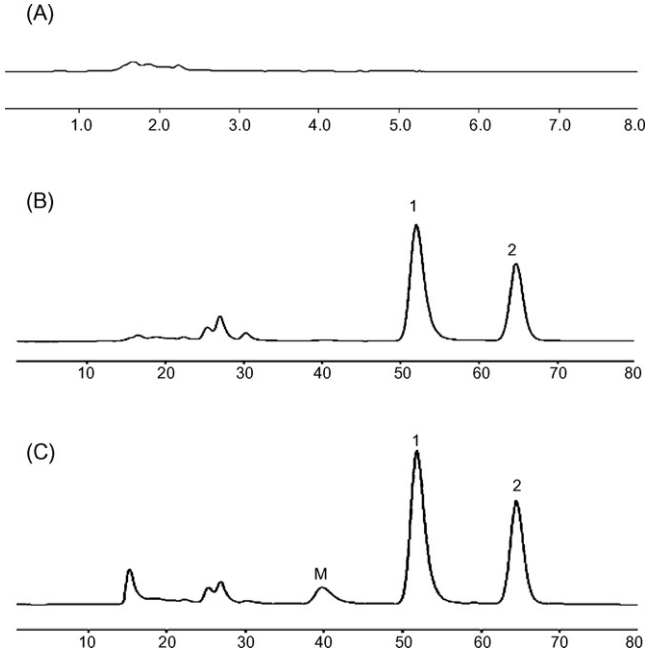

Fig. 2. Representative chromatograms of SYUIQ-5 and I.S in rat liver microsomes. (A) Liver microsomes from rats without any drug treatment; (B) rat liver microsomes spiked with SYUIQ-5 and I.S.; and (C) rat liver microsomes incubated with SYUIQ-5 at 20 μM and I.S. at 2 mg/ml. Peak 1: SYUIQ-5; peak 2: I.S.; and peak M: metabolite of SYUIQ-5.

3.3. Precision and accuracy of the assay

Intra-day and inter-day precision and accuracy of the method were determined for SYUIQ-5. Table 1 shows that the CVs were 1.7–10% for SYUIQ-5 at different concentrations. In addition, the accuracy was estimated to be within 10%.

3.4. Recovery

The recovery following the extraction procedure was determined by comparing peak areas of stock solutions of analytes directly injected into the system, with those of extracted samples from rat liver microsomes. The I.S. recovery was determined at the concentration (2 mg/ml) used in the assay procedure. The mean analytical recoveries of SYUIQ-5 at 2, 20, and 80 μM (Table 2) ranged from 78.8 to 95.3% and the recovery of the I.S. was 71.9 ± 5.6%. Each recovery was acceptable for the determination.

Diethyl ether was used in this study to extract SYUIQ-5, its metabolites and I.S. from the rat liver microsomal mixtures. We have also tested the recoveries of SYUIQ-5 and I.S. using other organic solvents including acetone, alcohol, acetonitrile and methanol. All of these organic solvents except acetonitrile gave a 67.5–82.3% recovery of SYUIQ-5 which was lower than by diethyl ether. Acetonitrile resulted in a high recovery (90.2%), but the recovery of I.S. was moderate (56.3%). Thus, diethyl

Table 2
Analytical recoveries of SYUIQ-5 and the internal standard (IS) from rat liver microsomes ($n = 5$)

Compound	Concentration added	Recovery (%)
SYUIQ-5	2 μM	95.3 ± 9.8
	20 μM	81.6 ± 3.1
	80 μM	78.8 ± 3.1
IS	1 mg/ml	71.9 ± 5.6

Data are the mean ± S.D.

Table 3
Kinetic parameters of SYUIQ-5 metabolism in rat liver microsomes treated with control vehicle and various inducers ($n = 8$ per group)

Treatment	K_m (μM)	V_{max} (nmol/min/mg protein)	CL_{int} (ml/min/mg)
Control vehicle	12.26 ± 1.6	2.01 ± 0.19	0.16 ± 0.00
β-NaphthofIavone	8.12 ± 0.21*	3.20 ± 0.34*	0.39 ± 0.01*
Phenobarbital	11.56 ± 0.57	2.30 ± 0.41	0.20 ± 0.01
Ethanol	13.55 ± 1.7	1.97 ± 0.09	0.15 ± 0.00
Dexamethasone	7.05 ± 0.64*	3.10 ± 0.45*	0.44 ± 0.01*

Data are the mean ± S.D. The apparent K_m and V_{max} were derived by data fit to a one binding-site equation (best model).
* $P < 0.05$, compared with rats treated with control vehicle (by an ANOVA followed with a Dunnett's multiple comparison test).

ether was chosen for extraction of the analytes from the liver microsomes.

It appeared that the recovery of SYUIQ-5 by diethyl ether was concentration-dependent with decreased recovery when its concentration increased (from 95.3 to 78.8%). This may be due to the high-affinity binding of the analyte to microsomal proteins and saturable extraction efficiency of diethyl ether.

3.5. Limits of quantification

The LOQ was defined as the lowest concentration that can be measured with acceptable precision and accuracy. The LOQ values was 1.0 μM with a CV of 4.8 and 7.6% for the precision and accuracy, respectively.

3.6. Application to enzyme kinetic study in rat liver microsomes

This method was applied to investigate the enzyme kinetics for SYUIQ-5 metabolism in liver microsomes from rats treated with control vehicle, β-naphthoflavone, phenobarbital, 20% ethanol and dexamethasone. Substrate depletion was measured and SYUIQ-5 relative turnover rates in different rat liver microsomes were examined. The kinetic parameters of SYUIQ-5 in different rat liver microsomes are listed in Table 3. Data fitting indicated that the metabolism of SYUIQ-5 in various rat liver microsomes followed Michaelis–Menton kinetics, with one-binding site model the best fit. The K_m and V_{max} for SYUIQ-5 metabolism in control rats were 12.26 μM and 2.01 nmol/min/mg protein, respectively. The kinetic constants in microsomes from rats treated with 20% ethanol or phenobarbital were similar to those of control rats, whereas the K_m in β-naphthofIavone-treated group or in dexamethasone-treated group was significantly lower than that in the three former group ($P < 0.05$, Table 3). As shown in Table 3, liver microsomes from rats treated with β-naphthoflavone or dexamethasone had significantly higher catalytic activities toward SYUIQ-5 than those in the control rats receiving control vehicle only. The V_{max} in rats treated with β-naphthoflavone or dexamethasone was about 1.60 and 1.55-fold higher than that in the control group ($P < 0.05$). The CL_{int} (V_{max}/K_m ratio, e.g., enzyme efficiency) was found to be 2.44- and 2.75-fold higher in β-naphthoflavone- or dexamethasone-treated rats than control rats ($P < 0.05$). These results indicated that different CYP enzymes played important roles in SYUIQ-5 metabolism in rat liver microsomes and that the β-naphthoflavone or dexamethasone-induced isoforms of CYP were more active than CYPs present in liver microsomes from rats treated with control vehicle, phenobarbital, or 20% ethanol.

Liver microsomes contain multiple forms of cytochrome P450 (P450 or CYP) that catalyze oxidation of numerous xenobiotic chemicals such as drugs, toxic chemicals, and carcinogens as well as endogenous chemicals including steroids, fatty acids, prostaglandins, and vitamins [10]. Their expression is regulated by both endogenous factors, such as gonadal and pitituary hormones, and a variety of exogenous compounds [10]. β-Naphthoflavone, phenobarbital, ethanol, and dexamethasone are typical inducer of CYP1A, CYP2B, CYP2E, and CYP3A, respectively [8,9]. Therefore, CYP1A and CYP3A may play important roles in SYUIQ-5 metabolism in rat liver microsomes, while CYP2B and CYP2E seemed to contribute to a minor extent. Since induction of CYPs by drugs are thought to have a major impact on drug metabolism, pharmacokinetics, and drug–drug interactions, it would be expected that various CYP inducers can increase the metabolism of SYUIQ-5 when used in combination. Further study in human liver microsomes is ongoing to explore the CYPs involved in the metabolism of SYUIQ-5. In our present assay condition, there was a major metabolite of SYUIQ-5 generated in rat liver microsomes. Further research is needed to determine its structure and the enzymes involved in its formation.

4. Conclusions

A sensitive assay for HPLC analysis of SYUIQ-5 in rat liver microsomes was developed. The analytes were extracted with diethyl ether and applied to HPLC with a reversed-phase C_{18} 5 μm column and detected at a wavelength of 278 nm. The total run time per sample was within 8 min. The developed HPLC method has been applied to investigate the metabolism of SYUIQ-5 in rat liver microsomes.

Acknowledgments

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