Available online at www.sciencedirect.com
ScienceDirect
ELSEVIER
Journal of Chromatography B, 846 (2007) 162–168
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Development and validation of a liquid chromatography–tandem mass spectrometry for the determination of BPR0L075, a novel antimicrotuble agent, in rat plasma: Application to a pharmacokinetic study

Yi-Wei Chang, Wei-Cheng Chen, Ke-Ta Lin, Ling Chang, Hsien-Tsung Yao, Hsing-Pang Hsieh, Shih-Jung Lan, Chiung-Tong Chen, Yu-Sheng Chao, Teng-Kuang Yeh *

Division of Biotechnology and Pharmaceutical Research, National Health Research Institutes, 35, Keyan Road, Zhunan Town, Miaoli County, Taiwan, ROC

Received 7 March 2006; accepted 27 August 2006
Available online 26 September 2006

Abstract

A rapid and sensitive liquid chromatography–tandem mass spectrometric method (LC–MS/MS) had been developed and validated to determine the concentrations of BPR0L075 in rat plasma. After a simple protein precipitation of plasma samples by acetonitrile, BPR0L075 was analyzed on a C_8 column at a flow rate of 0.5 mL/min. The mobile phase consisted of a mixture of 10 mM ammonium acetate containing 0.1% formic acid and acetonitrile (20:80, v/v). Both BPR0L075 (analyte) and the internal standard (BPR0L092) were determined using electro-spray ionization and the MS data acquisition was via multiple reactions monitoring (MRM) in positive scanning model. The MS/MS ion transitions monitored are *m/z* 342.2/195.2 and 312.5/165.2 for BPR0L075 and BPR0L092, respectively. The low limit of quantitation was 0.5 ng/mL. Each plasma sample was chromatographed within 5 min. The method was validated with respect to linearity, accuracy, precision, recovery, and stability. A good linear relationship was observed over the concentration range of 0.5–1000 ng/mL ($r > 0.9994$). Absolute recoveries ranged from 63.45 to 68.34% in plasma at the concentrations of 2, 40, 400, and 800 ng/mL. The intra- and inter-day accuracy ranged from 92.04 to 111.80%. Intra- and inter-day relative standard deviations were 1.08–3.29% and 1.96–5.46%, respectively. This developed and validated assay method had been successfully applied to a pharmacokinetic study after intravenous injection of BPR0L075 in rats at a dose of 5 mg/kg.
© 2006 Elsevier B.V. All rights reserved.

Keywords: BPR0L075; LC–MS/MS; Bioanalysis; Validation; Pharmacokinetics

1. Introduction

BPR0L075, 6-methoxy-3-(3′,4′,5′-trimethoxy-benzoyl)-1H-indole (Fig. 1), is a synthetic indole compound, which was identified as a potential lead-based on extremely potent cytotoxicity with good pharmacological properties [1]. It is structurally designed from Combretastain A-4, a naturally occurring stilbene derived from the South African tree *Combretum caffrum*, inhibits tubulin polymerization by binding to tubulin at the colchicine-binding site [2]. BPR0L075 disrupts intracellular microtubule network and inhibits tubulin polymerization through binding to the colchicines-binding site [1]. BPR0L075 had been demonstrated that exerts broad spectrum of antitumor activity against human leukemia, glioblastoma, breast, gastric, colorectal, and liver cancer cells *in vitro* and human cervical carcinoma KB and human gastric carcinoma MKN-45 xenografts *in vivo* [1].

Since the last decade, pharmacokinetic screening has become an important well-recognized tool to select and optimize drug candidate in early drug discovery. In order to evaluate the pharmacokinetics and the bioavailability of BPR0L075 in rats, a LC–MS/MS assay method was developed. The development of a sound bioanalytical method is of paramount importance during the drug discovery stage due to the fact that it can be suitably modified and validated, and serve as a method of choice to begin with as the novel chemical entity for further preclinical development stage. The use of LC–MS/MS for bioanalytical determination in biological fluids has been increasing because of its sensitivity and selectivity. Currently, the application of LC–MS/MS is considered the best choice to support preclinical and clinical pharmacokinetic studies [3]. We have performed our

* Corresponding author. Tel.: +886 37 246 166x35757; fax: +886 37 586 456.
E-mail address: tkyeh@nhri.org.tw (T.-K. Yeh).

1570-0232/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2006.08.040

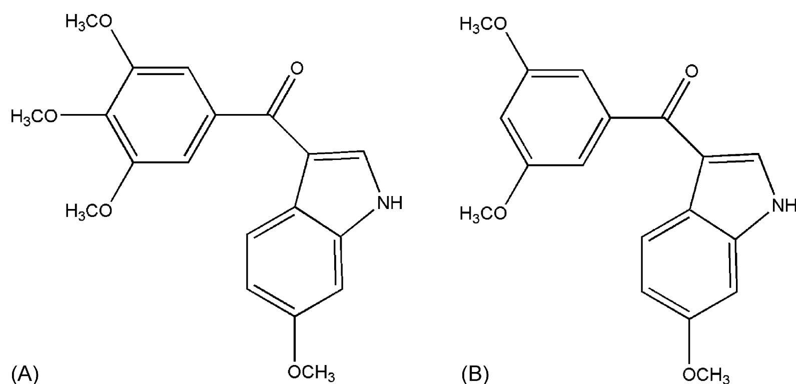

Fig. 1. Chemical structures of (A) BPR0L075 and (B) BPR0L092 (internal standard).

four major determinants of pharmacokinetic (absorption, distribution, metabolism and elimination) studies *in vitro* as well as *in vivo* by using LC–MS/MS during the drug discovery stage. Based on the best pharmacological, toxicological, and pharmacokinetic properties, BPR0L075 was chosen as the optimum drug candidate for further development [1,4,5].

In this paper, we described a rapid and highly sensitive liquid chromatography/tandem mass spectrometry (LC–MS/MS) method to quantify BPR0L075 in rat plasma. The sample preparation is simple and utilizes single one-step protein participation for sample extraction. This method was validated and successfully applied to a pharmacokinetic study in rats.

2. Experimental

2.1. Chemicals and reagents

BPR0L075, 6-methoxy-3-(3′,4′,5′-trimethoxy-benzoyl)-1H-indole and BPR0L092, 1-methyl-6-methoxy-3-(3′, 5′-dimethoxy-benzoyl)-1H-indole, as the internal standard (IS), were synthesized at the Division of Biotechnology and Pharmaceutical Research, National Health Research Institutes, Miaoli, Taiwan, ROC. The chemical purities of both compounds were all above 99%. Polyethylene glycol 400 (PEG 400) was purchased from Sigma (St Louis, MO, USA). Solutol was obtained from BASF (Ludwigshafen, Germany). All other chemicals were of reagent grade and were obtained commercially.

2.2. LC–MS/MS analysis

The HPLC system consisted of an Agilent 1100 series LC System (Palo Alto, CA, USA) and an Agilent ZORBAX Eclipse XDB-C_8 reverse-phase column (5 μm, 4.6 mm × 150 mm) interfaced to an AB Sciex API 3000 tandem mass spectrometer equipped with an ESI in the positive ion mode (Applied Biosystems, Foster City, CA, USA). Mobile phase consisted of 10 mM ammonium acetate containing 0.1% of formic acid and acetonitrile at 20:80 (v/v). The flow rate was 0.5 mL/min. Total running time was 5.0 min. The retention times of BPR0L075 and BPR0L092 (IS) were 1.6 and 1.7 min, respectively. Nitrogen was used as the nebulizing gas. The electrospray needle was maintained at 4.5 kV and heated-capillary temperature was set at 400 °C. Data acquisition was via multiple reactions monitoring (MRM). Ions representing the $[M+H]^+$ species for both the analyte and IS were selected in MS1 and collisionally dissociated with nitrogen gas to form specific product ions, which were subsequently monitored by MS2. The collision energy was 27 V for the analyte and 36 V for IS. The ions monitored for BPR0L075 (analyte) and BPR0L092 (IS) were m/z 342.2/195.2 and m/z 312.5/165.2, respectively. Plasma samples that had concentrations of BPR0L075 above the upper limit of quantitation (1000 ng/mL) were diluted proportionally with blank plasma prior to extraction with acetonitrile.

2.3. Preparation of calibration standards and quality controls

Primary stock solutions of BPR0L075 and IS, BPR0L092, were prepared separately in acetonitrile to yield standard solutions with a concentration of 1 mg/mL. These primary stock solutions were sealed and stored at −60 °C until used. The secondary working stock solution of BPR0L075 was prepared by diluting with acetonitrile from primary stock solution. The working internal standard solution was prepared by diluting the primary stock solution with acetonitrile giving a concentration of 250 ng/mL. Calibration standards of BPR0L075 were prepared by serial dilution with blank rat plasma yielding final concentrations of 0.5, 1, 5, 10, 50, 100, 500, and 1000 ng/mL. Because of a wide linear range of calibration curve, the quality control (QC) samples were prepared by spiking blank rat plasma with independently prepared BPR0L075 standard solutions to give concentrations of 2, 40, 400, and 800 ng/mL of BPR0L075.

2.4. Sample preparation

Fifty microlitres of rat plasma samples (blank plasma, calibration standards, QC samples, and pharmacokinetic plasma samples) were mixed with 100 μL acetonitrile containing 250 ng/mL of BPR0L092. The mixture was vortexed for 30 s and then centrifuged at 21,000 × g for 20 min in an Eppendorff Model 5417c centrifuge at room temperature. The supernatant was transferred to a clean tube and then a volume of 15 μL of the supernatant was injected onto LC–MS/MS.

2.5. Method validation

2.5.1. Linearity of calibration curves

Calibration curves were constructed from working standard solutions of BPR0L075 at concentration range 0.5–1000 ng/mL by plotting peak area ratio (y) of BPR0L075 to the internal standard, BPR0L092, versus BPR0L075 concentration (x). The BPR0L075 samples were prepared in five replicates. The regression parameters of slope, intercept, and correlation coefficient were calculated by weight ($1/x$) linear regression in Analyst 1.3 software used in Applied Biosystems API3000. The calibration curves require correlation coefficient $r > 0.999$.

2.5.2. Accuracy and precision

The accuracy and precision (presented as relative standard deviation, R.S.D.) of this analytical method were evaluated using QC samples. QC samples were prepared as described above. The solution of a certain concentration was injected onto LC–MS/MS for quantitative determination five times a day to evaluate intra-day precision. The same procedure was performed once a day for five consecutive days to determine inter-day precision. Accuracy was determined by comparing the calculated concentration using calibration curves to known concentration.

2.5.3. Recovery

The extraction recoveries of BPR0L075 using the protein precipitation procedure were calculated by comparing the peak areas of extracted plasma standards to those of postextraction plasma blanks spiked with corresponding concentrations. The absolute recoveries of BPR0L075 were determined by comparing the peak areas obtained from five extracted samples spiked with known amounts of standards with those obtained from the pure compounds of the same concentrations in the solvent. The concentration of BPR0L092 was 250 ng/mL. The recovery studies were assessed at least five replicates at four concentration levels (2, 40, 400, and 800 ng/mL).

2.5.4. Stability

To evaluate sample stability after three freeze-thaw cycles and room temperature, four replicates of QC samples at concentrations of 2, 40, 400 and 800 ng/mL were subjected to three freeze-thaw cycles or were stored at room temperature for 6 h before sample processing. Stability was assessed by comparing the mean concentration of the stored QC samples with the mean concentration of freshly prepared QC samples.

2.6. Pharmacokinetic study

The validated assay method was applied to a pharmacokinetic study of BPR0L075 in rats. This study was approved by Institutional Animal Care and Use Committee of National Health Research Institutes. A true solution of BPR0L075 (10 mg/mL) was prepared by dissolving appropriate amount compound in a mixture of PEG 400/dehydrated ethanol/Solutol (20:30:50, v/v/v) and was diluted with two parts of physiological saline to make the dosing solution of 3.3 mg/mL before dosing. Male Sprague-Dawley rats, weighing 250–350 g each (8–10 weeks old), were obtained from BioLASCO, Ilan, Taiwan. Single 5 mg/kg dose of BPR0L075 was separately administered to group of three male rats each intravenously by a bolus injection to the tail vein. The volume of dosing solution administered was adjusted according to the body weight recorded before dose administration. At 0 (prior to dosing), 2, 5, 15, and 30 min and at 1, 2, 4, 6, 8, 12, 24, 30, and 48 h after dosing, a blood sample (∼150 μL) was collected from each animal via the jugular-vein cannula and stored in ice (0–4 °C). Plasma was separated from the blood by centrifugation (14,000 × g for 15 min at 4 °C in a Beckman Model Allegra™ 6R centrifuge) and stored in a freezer (−60 °C). All samples were analyzed for the parent compound by LC–MS/MS. Data were acquired via multiple reactions monitoring. Plasma concentration data were analyzed with standard non-compartmental method with WinNonLin software program (version 3.1, Pharsight Corp, CA, USA).

3. Results and discussion

3.1. Mass spectrometry

The MS/MS parameters were optimized to the maximum response for the BPR0L075 and BPR0L092 under positive ion mode. Under electrospray ionization (ESI) condition, protonated molecular ions $[M+H]^+$ were the major peaks. Mass spectrum of BPR0L075 $[M+H]^+$ m/z 342.2 and the internal standard BPR0L092 $[M+H]^+$ m/z 312.5 are shown in Fig. 2. The MS/MS transition of 342.2/195.2 for BPR0L075 and 312.5/165.2 for BPR0L092 were selected.

3.2. Chromatography

Under optimized LC condition, the chromatographic retention times of BPR0L075 and BPR0L092 were 1.6 and 1.7 min, respectively (Fig. 3). The total run time was 5 min. LC–MS/MS is considered as powerful and selective instruments, which allow quantitation even of some co-eluenting peaks, it is generally agreed that it is not necessary to achieve a complete chromatographic separation of compounds [6]. There were no significant interferences from endogenous compounds observed at the retention times of the analytes as shown on Fig. 3.

3.3. Method validation

3.3.1. Linearity of calibration curves

Table 1 shows the summary of the individual standard data obtained in the five replicates. These calibration curves were found to be linear in the concentration range 0.5–1000 ng/mL in rat plasma with correlation coefficient ($r > 0.999$) when evaluated by weighted ($1/x$) linear regression. The mean slope of the calibration curves use in the method validation is 0.00368, with %CV of 4.08, and the typical calibration curve is $Y = 0.00368X + 0.00237$. The result indicated good linear relationships between the peak areas and concentrations. The

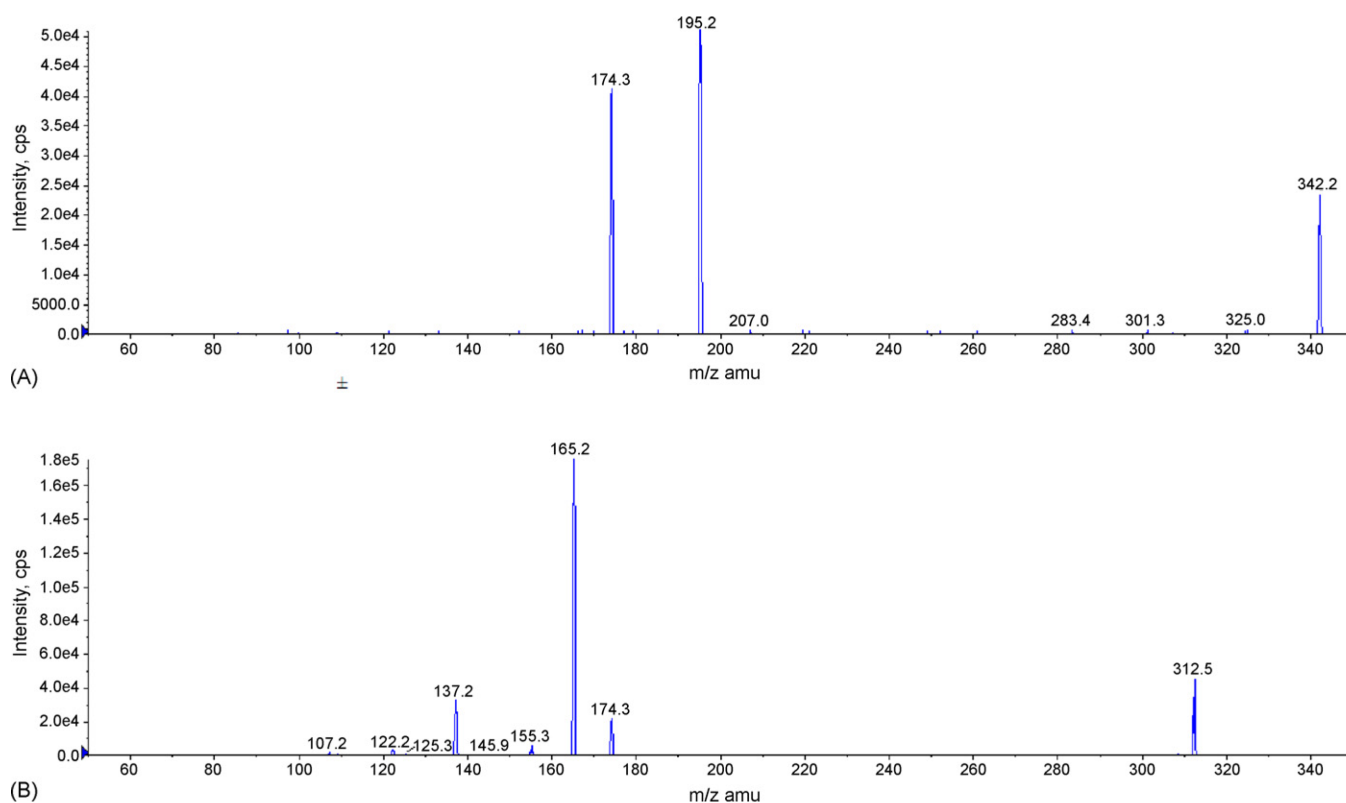

Fig. 2. Product ion mass spectra of $[M+H]^+$. (A) BPR0L075 ($[M+H]^+$, m/z 342.2) and (B) BPR0L092 ($[M+H]^+$, m/z 312.5).

Table 1
Summary of calibration curves ($n=5$)

Curve no.	Slope	Intercept	r
1	0.00358	0.00238	0.9999
2	0.00386	0.00265	0.9993
3	0.00370	0.00202	0.9998
4	0.00348	0.00221	0.9999
5	0.00378	0.00257	0.9997
Mean ± S.D.	0.00368 ± 0.00015	0.00237 ± 0.00026	0.99972 ± 0.00025
%CV	4.08	10.97	0.03

limits of quantitation (LOQ) and the limits of detection (LOD) were 0.5 and 0.1 ng/mL, respectively.

3.3.2. Accuracy and precision

The accuracy and precision of intra- and inter-day assay data for BPR0L075 were determined at concentrations of 2, 40, 400, and 800 ng/mL, respectively. Table 2 shows a summary of intra- and inter-day accuracy and precision for BPR0L075 in rat plasma. Accuracy was the percentage of the concentration found compared with the theoretical concentration. Precision was based on calculation of the R.S.D. The accuracy ranged from 92.04 to 111.80% throughout the four concentrations. The precision of BPR0L075 for the four concentrations examined was 3.29, 1.59, 1.32, and 1.08%, respectively. The inter-day accuracy and precision was studied over 5 days. The accuracy ranged from 95.40 to 107.55% and the precision ranged form 1.96 to 5.46% throughout the four concentrations examined.

Table 3
Absolute Recovery of BPR0L075 in rat plasma ($n=5$)

Concentration (ng/mL)	Recovery (mean ± S.D.)
2	67.52 ± 0.04
40	68.34 ± 0.01
400	63.45 ± 0.02
800	66.14 ± 0.01

Table 2
Accuracy and precision of BPR0L075 in rat plasma

Nominal concentration (ng/mL)	Intra-day precision ($n=5$)				Inter-day precision ($n=5$)			
	2	40	400	800	2	40	400	800
Measured concentration (ng/mL)	1.84 ± 0.06	44.80 ± 0.71	416.00 ± 5.48	801.00 ± 8.69	1.91 ± 0.07	43.05 ± 1.55	419.80 ± 8.23	811.10 ± 43.37
Accuracy (%)	92.04 ± 3.09	111.80 ± 1.92	103.80 ± 1.30	108.20 ± 1.23	95.40 ± 3.58	107.55 ± 3.80	106.75 ± 2.96	101.47 ± 5.55
R.S.D. (%)	3.29	1.59	1.32	1.08	3.68	3.61	1.96	5.46

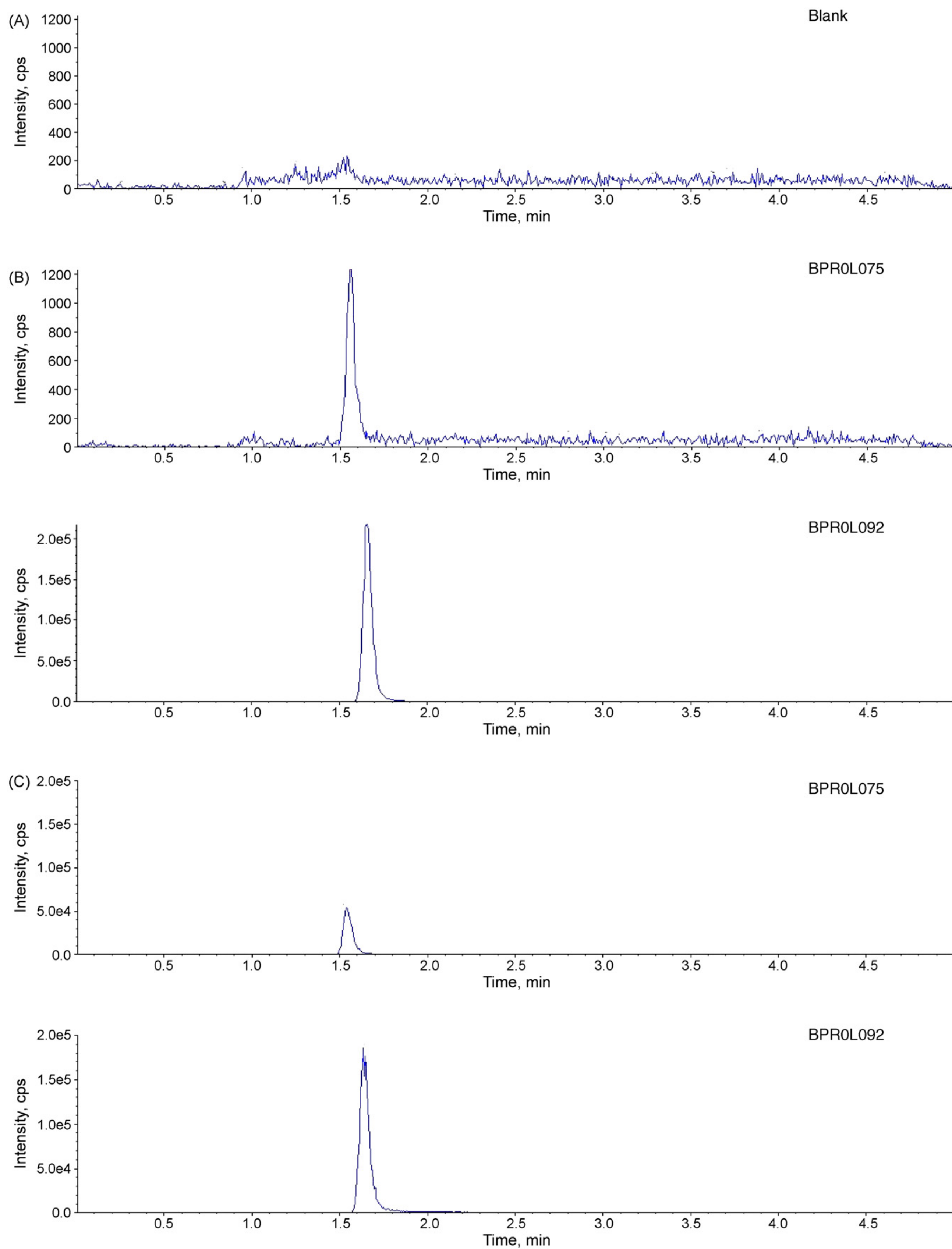

Fig. 3. Representative MRM Chromatograms of BPR0L075. (A) Blank rat plasma. (B) Blank rat plasma spiked with BPR0L075 (0.5 ng/mL) and internal standard (250 ng/mL). (C) Rat plasma sample 2 h after intravenous administration to a rat with 5 mg/kg dose of BPR0L075.

Table 4
Stability of BPR0L075 in rat plasma ($n = 5$)

Measured concentration (ng/mL)/storage condition	Nominal concentration (ng/mL)			
	2	40	400	800
Three freeze-thaw cycle stability (−60°C)				
Mean	1.88 ± 0.04	42.81 ± 1.82	422.53 ± 7.55	793.07 ± 31.65
R.S.D. (%)	2.16	4.25	1.79	3.99
6 h at room temperature				
Mean	1.84 ± 0.06	44.80 ± 0.71	416.00 ± 5.48	801.01 ± 8.69
R.S.D. (%)	3.29	1.59	1.32	1.08

3.3.3. Recovery and matrix effect

The mean extraction recovery of BPR0L075 using protein precipitation was 99.11–103.93% at concentrations of 2, 40, 400, and 800 ng/mL. Table 3 shows the absolute recoveries of BPR0L075 from rat plasma following acetonitrile extraction. The results showed that the absolute recoveries of BPR0L075 were in the range of 63.45–68.34% at concentrations of 2, 40, 400, and 800 ng/mL, suggesting that the recoveries of BPR0L075 were not concentration-dependent. The mean overall absolute recovery was relative low, indicating consistent matrix effect. Although the matrix effect causes approximately 35% suppression effect across all concentration levels of BPR0L075, however, the results showed that there was no significant lot-to-lot validation (Tables 1 and 3). Thus, no further attempts were pursued to compromise the matrix effect.

3.3.4. Stability

The stability of BPR0L075 in rat plasma under different storage conditions is summarized in Table 4. There was no significant degradation occurred at ambient temperature for 6 h and during the freeze-thaw cycles for BPR0L075 rat plasma samples, due to the concentrations deviated by no more than 4.25% relative to the reference nominal concentrations. These results indicated that BPR0L075 was stable under routine laboratory conditions.

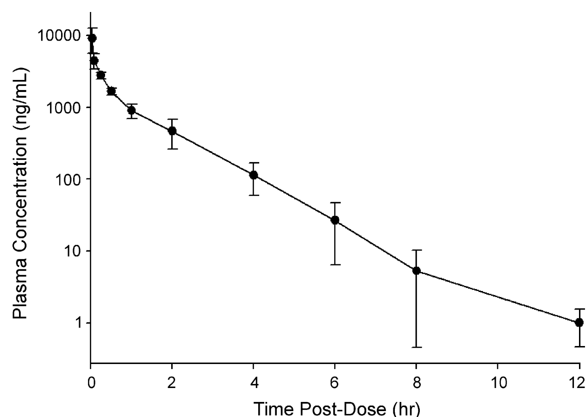

Fig. 4. Mean plasma concentration–time profile of BPR0L075 after an intravenous dose of 5 mg/kg to rats ($n = 3$).

Table 5
Pharmacokinetic parameters of BPR0L075 in male rats after a single 5 mg/kg intravenous dose (mean ± S.D., $n = 3$)

Parameter	Units	BPR0L075
$t_{1/2}$	h	0.9 ± 0.2
MRT	h	0.9 ± 0.3
CL	mL/min/kg	24.5 ± 6.0
V_{ss}	L/kg	1.7 ± 0.3
$AUC_{(0-12)}$	ng/mL × h	3880 ± 622
$AUC_{(0-\infty)}$	ng/mL × h	3888 ± 632

3.4. Pharmacokinetic study

The developed and validated method was applied to a pharmacokinetic study after single 5 mg/kg intravenous doses of BPR0L075 separately administered to group of three male rats. The concentration–time profile is shown in Fig. 4 and the pharmacokinetic parameters of BPR0L075 are summarized in Table 5. The concentration of BPR0L075 in plasma could be measured up to 12 h after dosing and averaged 1.0 ± 0.5 ng/mL at that time. The apparent elimination half-life ($t_{1/2}$) and the mean resident time (MRT) were about 1 h. The total body clearance (CL) and volume of distribution at steady state (V_{ss}) were 24.5 ± 6.0 mL/min/kg and 1.7 ± 0.3 L/kg, respectively. The corresponding value for $AUC_{(0-\infty)}$ was 3888 ± 632 ng/mL × h.

4. Conclusion

A rapid and sensitive LC–MS/MS assay method was developed and validated for the determination of BPR0L075 in rat plasma. This assay method showed a wide linear range of 0.5–1000 ng/mL, with excellent intra- and inter-day accuracy and precision. The optimum method was guaranteed enough sensitivity, selectively, accuracy, and precision. This method was successfully applied for the evaluation of pharmacokinetic profiles of BPR0L075 in rats at 5 mg/ml intravenous dose by using small sample volume (50 μL).

Acknowledgment

The authors are grateful to the National Health Research Institutes, Taiwan, Republic of China, for financial support.

References

[1] C.C. Kuo, H.P. Hsieh, W.Y. Pan, C.P. Chen, J.P. Liou, S.J. Lee, Y.L. Chang, L.T. Chen, C.T. Chen, J.Y. Chang, Cancer Res. 64 (2004) 4621.
[2] G.R. Pettit, S.B. Singh, M.R. Boyd, J. Med. Chem. 38 (1995) 1666.
[3] W.A. Korfmacher, K.A. Cox, M.S. Byrant, J. Veals, K. Ng, R. Watkins, C. Lin, Drug Discov. Today 2 (1997) 532.
[4] H.P. Hsieh, J.P. Liou, N. Mahindroo, Curr. Pharm. Des. 11 (2005) 1655.
[5] J.P. Liou, Y.L. Chang, F.M. Kao, C.W. Chang, H.Y. Tseng, C.C. Wang, Y.N. Yang, S.J. Lee, H.P. Hsieh, J. Med. Chem. 47 (2004) 4247.
[6] A. Liu, H. Lou, L. Zhao, P. Fan, J. Pharmaceut. Biomed. Anal. 40 (2005) 720.