Journal of Pharmaceutical and Biomedical Analysis 49 (2009) 447–450

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Development of a high performance liquid chromatography method for quantification of PAC-1 in rat plasma

Li-Na Fang[a], Xiao-Hui Chen[a], Zhen Song[a], Gang Wang[a], Xu Zhao[a], Lei Ren[a], Ping Gong[b], Kai-Shun Bi[a,*]

[a] School of Pharmacy, Shenyang Pharmaceutical University, Wenhua Road 103, Shenyang 110016, PR China
[b] School of Pharmaceutical Engineering, Shenyang Pharmaceutical University, Wenhua Road 103, Shenyang 110016, PR China

ARTICLE INFO

Article history:
Received 19 September 2008
Received in revised form
18 November 2008
Accepted 21 November 2008
Available online 27 November 2008

Keywords:
Anticancer agent
PAC-1
HPLC
Plasma
Pharmacokinetics

ABSTRACT

A sensitive and specific high performance liquid chromatography method with UV detection was developed and validated for the determination of PAC-1 in rat plasma. After extraction with ethyl acetate, the chromatographic separation was carried out on a Diamonsil C_{18} column (150 mm × 4.6 mm i.d., 5 μm particle size, Zhonghuida) protected by a ODS guard column (10 mm × 4.6 mm i.d., 5 μm particle size), using acetonitrile–methanol–phosphate buffer (pH 3.0, 30 mM) (31:3:66, v/v/v) as mobile phase at a flow rate of 1.0 mL/min, and wavelength of the UV detector was set at 281 nm. No interference from any endogenous substances was observed during the elution of PAC-1 and internal standard (IS, indapamide). The calibration curve was linear over the range of 0.05–20 μg/mL ($r > 0.99$). The lower limit of quantification was evaluated to be 50 ng/mL. The method was successfully applied to the pharmacokinetic study of PAC-1 after intravenous and oral administration in rats, respectively.

1. Introduction

PAC-1 (Fig. 1A), 1-Piperazineacetic acid, 4-(phenylmethyl)-2-[[2-hydroxy-3-(2-propen-1-yl)phenyl]methylene]hydrazide, was disclosed [1,2] by professor Hergenrother that directly activates procaspase-3 to caspase-3 *in vitro*, which activation places an important role in the apoptotic pathways, and induces apoptosis in cancerous cells isolated from primary colon tumors, in a manner directly proportional to the concentration of procaspase-3 inside these cells in which the expression of procaspase-3 is upregulated [3–7]. Professor Hergenrother found that PAC-1 retarded the growth of tumors in three different mouse models of cancer, including two models in which PAC-1 was administered orally. PAC-1 is the first small molecule known to directly activate procaspase-3 to caspase-3 [1]. The direct activation of executioner caspases is an anticancer strategy that may prove beneficial in treating the many cancers in which procaspase-3 concentrations are elevated.

In order to explore the value of the anticancer ability of PAC-1, its ADME characteristics should be determined first. But as far as we know, there is not any study to reveal the pharmacokinetic profile. HPLC method is a simple and sensitive method widely used in pharmacokinetic study [8,9]. Therefore, the rat plasma pharmacokinetics and the absolute bioavailability of PAC-1 were studied with a HPLC method. Both the development of the method for the determination and the pharmacokinetic study of PAC-1 were reported for the first time.

2. Experimental

2.1. Chemicals and reagents

PAC-1 (purity > 99.0%) was obtained from Sigma–Aldrich (Milwaukee, WI, USA); Indapamide (IS, Fig. 1B) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products of China. Acetonitrile and methanol (HPLC grade) were provided by Sinopharm Chemical reagent Co., Ltd. Phosphoric acid was purchased from Tianjin kermel chemical reagents development center. All the other reagents were of analytical grade. Distilled water, prepared with demineralized water, was used throughout the study.

2.2. Chromatographic system

The analyses were completed using an Agilent 1100 series HPLC system, including a quaternary pump, a variable wavelength UV detector, a column oven and an autosampler. The separation was performed on a Diamonsil C_{18} column (150 mm × 4.6 mm i.d., 5 μm particle size, Zhonghuida) protected by a ODS guard column (5 mm × 4.6 mm i.d., 5 μm particle size), using acetonitrile–methanol–phosphate buffer (pH 3.0, 30 mM) (31:3:66,

* Corresponding author.
E-mail address: bikaishun@yahoo.com (K.-S. Bi).

0731-7085/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2008.11.024

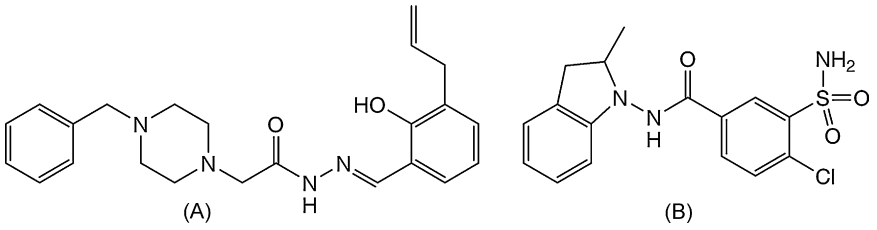

Fig. 1. Chemical structures of PAC-1 (A) and indapamide (B, IS).

v/v/v) as mobile phase at a flow rate of 1.0 mL/min. The wavelength was set at 281 nm and the column temperature was maintained at 40 °C. A personal computer equipped with an Agilent Chemstation program for LC systems was used to acquire and process chromatographic data. Peak area was evaluated as the analytical measurement. Centrifugation (TDL-16C, Shanghai Anting Medical Instrumental Factory, PR China) with calibrated centrifugal tubes was applied to accelerate the phase separation process.

2.3. Preparation of standards

Both of the stock solutions of PAC-1 and IS were prepared in methanol at concentration levels of 1 and 0.2 mg/mL, respectively. Working solutions were prepared by diluting the stock solutions above with methanol. The concentration of working solution for internal standard was 20 μg/mL. All PAC-1 and IS solutions were stored at 4 °C. The calibration curve at concentrations of 0.05, 0.15, 0.5, 2.5, 10, 15, and 20 μg/mL were prepared by spiking appropriate amount of the standard solutions in blank plasma. Three levels of QC samples (0.15, 2.5, and 15 μg/mL) in plasma were prepared separately in the same fashion.

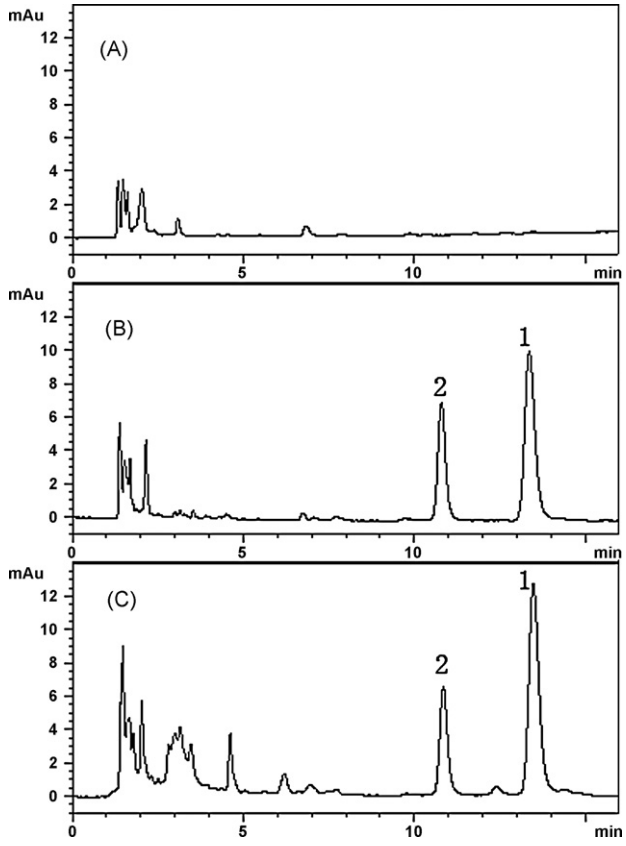

Fig. 2. Representative chromatograms of blank plasma (A); plasma spiked with PAC-1(2.5 μg/mL) and I.S. (5 μg/mL) (B); plasma samples obtained 60 min after a single i.g. treatment with 60 mg/kg of PAC-1 (C) from rats. Peak 1: PAC-1; Peak 2: IS.

2.4. Sample preparation

Plasma samples (200 μL) were spiked with 50 μL of internal standard, 20 μL of methanol and 200 μL of 2 M sodium hydroxide, and extracted for 5 min with 2 mL ethyl acetate. After centrifugation (4000 rpm, 5 min), the organic phase was transferred to another vial and evaporated to dryness at room temperature under a slight stream of nitrogen. Then the residue was reconstituted with 100 μL of mobile phase, 20 μL of which was used for analysis.

2.5. Method validation

The calibration curves were obtained by plotting the area ratios of PAC-1 and IS as a function of the PAC-1 concentration using least squares linear regression analysis. The LLOQ was defined as a reproducible lowest possible concentration, linear with the calibration curves having a relative error (R.E. %) below 20% and accuracy between 80 and 120% [10,11].

To assess the intra-day accuracy and precision of the method, 3 concentrations of PAC-1 (0.15, 2.5, and 15 ug/mL) were spiked into plasma, with 6 replicates independently prepared at each concentration. Similarly, the inter-day accuracy and precision was evaluated on 3 separate occasions, with 6 replicates at each concentration. Accuracy was defined as the relative error (R.E. %) [12] while precision was defined as the relative standard deviation (R.S.D. %).

The recovery of PAC-1 at 3 concentrations (0.15, 2.5, and 15 ug/mL) was determined in 6 occasions by comparing the peak areas of PAC-1 from extracted samples with those in post-extracted blank plasma samples spiked with PAC-1 at the same concentration. The recovery of IS was determined in the same way at concentration of 5 ug/mL.

The quality control (QC) samples (six replicates of QC samples at each of 0.15, 2.5, and 15 ug/mL concentrations) were assayed under several different conditions to assess the stability of PAC-1 in rat plasma [13]. The resulting concentrations were compared with their theoretical concentrations, and the relative error (R.E. %) was calculated. Stability samples were to be concluded stable if the relative error were within ± 15%.

2.6. Application to pharmacokinetic study

Male Wistar rats (220–250 g) were obtained from the Experimental Animal Center of Shenyang Pharmaceutical University. Animals were housed under controlled conditions (22 ± 2 °C, RH 50 ± 20%) with a natural light–dark cycle for 3 days before the experiment carried out. Before drug administration, they were fasted overnight and allowed free access to water. All procedures involving animals were in accordance with the Regulations of Experimental Animal Administration issued by the State Committee of Science and Technology of People's Republic of China.

Twelve rats divided randomly into two groups were fasted overnight. PAC-1, prepared at 1.5 mg/mL in ethanol–saline (2:98, v/v), was administered via tail vein as a bolus dose of 15 mg/kg to one group of rats. The other group of rats received a single dose of PAC-1(prepared at 3 mg/mL in ethanol-water (15:85, v/v))

Table 1
Precision and accuracy of PAC-1 determination in rat plasma (intra-day: 6 replicates at each concentration; inter-day: 18 replicates at each concentration).

Concentration (μg/mL)	Intra-day ($n=6$)		Inter-day ($n=3\times6$)	
	Precision (R.S.D. %)	Accuracy (R.E. %)	Precision (R.S.D. %)	Accuracy (R.E. %)
0.15	7.7	−2.7	2.5	−3.3
2.5	4.5	−4.3	4.8	−6.1
15	5.4	−1.6	5.3	0.6

Table 2
Recoveries of PAC-1 and IS ($n=6$).

Compound	Concentration (μg/mL)	Recovery (%) (mean ± S.D.)	R.S.D. (%)
PAC-1	0.15	84.4 ± 4.3	5.1
	2.5	83.9 ± 5.8	4.8
	15	79.7 ± 4.2	3.3
I.S.	5	89.9 ± 9.0	8.1

of 60 mg/kg by oral administration. According to the time schedules, typically at 0, 3, 5, 10, 15, 20, 30,45, 60, 90, 120, 180, 240, 360 min for intravenous administration and 0, 5, 10, 20, 30, 45, 60, 90, 120, 180, 240, 360, 480, 720 min for oral administration, respectively. Blood samples (0.5 mL) were collected from the ocular vein with heparinized tubes from each rat separately and then immediately centrifuged at 12,000 rpm for 5 min. The plasma obtained was stored frozen at −20 °C until analysis.

3. Results

3.1. Specificity, linearity and sensitivity

No endogenous interference was found at the retention times of PAC-1 and the IS. Representative chromatograms for blank rat plasma, rat plasma spiked with PAC-1 (2.5 ug/mL) and the IS (5 ug/mL) and plasma sample obtained at 60 min from Wistar rat after the i.g. treatment with 60 mg/kg of PAC-1 are shown in Fig. 2A–C, respectively. The IS and PAC-1 were well resolved with respective retention times of 10.8 and 13.4 min.

The standard calibration curve was linear over the concentration range of 0.05–20 ug/mL for PAC-1 with a correlation coefficient of above 0.99. The linear regression equation for PAC-1 rat plasma concentration was: $Y=(7.65\times10^{-1}\pm1.58\times10^{-2})X+(-1.15\times10^{-3}\pm1.97\times10^{-3})$ ($r=0.997$), where Y is the plasma concentration of PAC-1 and X is the peak area ratio of PAC-1 to the I.S. The LLOQ in rat plasma for PAC-1 was 50 ng/mL.

3.2. Precision and accuracy

The intra- and inter-day precision and accuracy of PAC-1 are presented in Table 1. The precision (R.S.D. %) was all less than 7.7%. The accuracy (R.E. %) of PAC-1 ranged from −4.3 to −1.6% for intra-day and −6.3 to 0.6% for inter-day, respectively. These results indicated that the present method had a good precision and accuracy.

3.3. Recovery

Table 2 shows the recoveries of PAC-1 and IS from rat plasma. The extraction efficacy of PAC-1 at three concentrations ranged from 79.7 to 84.4%, while the recovery of IS was 89.9%. This result suggested that there was no relevant difference in extraction recovery at different concentration levels.

3.4. Stability

The stability of PAC-1 in rat plasma under different storage condition is summarized in Table 3. PAC-1 was stable at room temperature for 4 h, at −20 °C for at least 15 days and its concentration did not show any significant change after 3 freeze and thaw cycles. In addition, the prepared samples in mobile phase with the IS in the autosampler was also stable at 4 °C for at least 12 h.

3.5. Application

The validated method was applicated to assay PAC-1 in rat plasma samples. Calibration levels were identical to those that used during validation procedures. Noncompartment model was used to calculate the pharmacokinetic parameters with DAS 2.0 software (Pharmacology Institute of China). The mean plasma concentration–time curves after oral administration at a dose of 60 mg/kg PAC-1 and intravenous administration at a dose of 15 mg/kg are shown in Fig. 3, respectively. The absolute bioavailability, F (%), is calculated as follows: F (%) = (AUC i.g. × D i.v.)/(AUC i.v. × D i.g.) × 100%, here D denoted the dose of administration to

Table 3
Stability of PAC-1 in rat plasma ($n=3$).

Spiked concentration (μg/mL)	Measured concentration (mean ± S.D.)	Accuracy (%)	R.S.D. (%)
Frozen for 15 days			
0.15	0.157 ± 0.010	104.5	6.5
2.5	2.520 ± 0.133	100.8	5.3
15	13.606 ± 0.631	90.7	4.7
Three freeze–thaw cycles			
0.15	0.151 ± 0.005	98.1	3.4
2.5	2.452 ± 0.094	98.1	3.9
15	15.158 ± 0.323	101.0	2.2
Room temperature for 4 h			
0.15	0.144 ± 0.015	96.3	10.6
2.5	2.394 ± 0.101	95.7	4.3
15	14.973 ± 0.702	99.8	4.7
Prepared samples in auto-sampler for 12 h			
0.15	0.149 ± 0.018	99.2	12.3
2.5	2.559 ± 0.082	102.4	3.3
15	15.113 ± 1.005	100.8	6.7

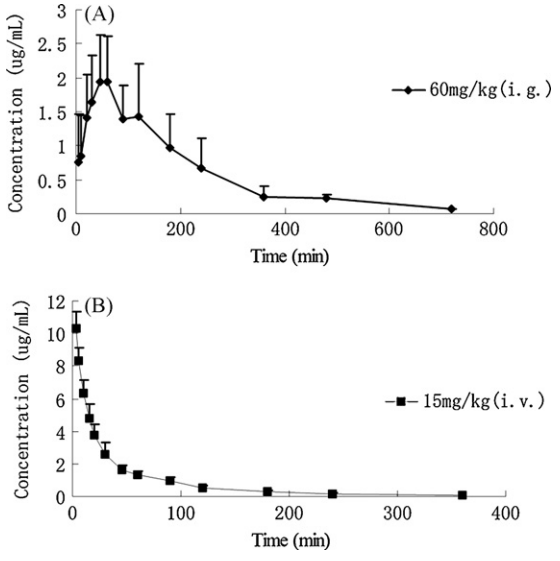

Fig. 3. Mean plasma concentration-time profile after oral administration of 60 mg/kg PAC-1 (A) and intravenous administration of 15 mg/kg PAC-1 (B), respectively.

Table 4
Pharmacokinetic parameters of PAC-1 in rat plasma.

Parameter	i.g. (mean ± SD)	i.v. (mean ± SD)
$AUC_{(0-t)}$ (μg/mL × min)	370.4 ± 186.5	323.2 ± 42.7
$AUC_{(0-\infty)}$ (μg/mL × min)	389.2 ± 187.8	333.7 ± 39.9
$MRT_{(0-t)}$ (min)	135 ± 32	51 ± 8
$MRT_{(0-\infty)}$ (min)	153 ± 29	66 ± 10
T_{max} (min)	50 ± 8	–
C_{max} (μg/mL)	2.1 ± 0.7	10.3 ± 1.1
t1/2z (min)	85 ± 21	83 ± 38
V_z/F (L/kg)	23.7 ± 15.3	5.5 ± 2.6
CL_z/F (L/min/kg)	0.208 ± 0.153	0.064 ± 0.005
F (%)	29.2	

the rats [14]. The absolute bioavailability of PAC-1 was 29.2% might due to first-pass effects. The obtained pharmacokinetic parameters of PAC-1 are shown in Table 4. The PK data analysis showed that PAC-1 has moderate clearance and a large volume of distribution. The plasma concentration–time curve of the compound exhibited double-peaks after oral administration and this might involve enterohepatic recirculation.

4. Discussion

To optimize the chromatographic conditions, the mobile phase systems including methanol–water, acetonitrile–water, acetonitrile–water containing acid, and methanol–acetonitrile–acidic buffer were investigated. The adoption of acidic buffer as a component of mobile phase was found to be essential to minimize the width of the PAC-1 peak. A slight change in the percentage of acetonitrile could lead to a comparatively obvious change in the retention time of PAC-1, while the involvement of methanol could meet the requirements. By increasing the amount of methanol or acetonitrile, the total elution time was decreasing but PAC-1 peak was closer to the endogenous interference.

The selection of the internal standard was an arduous and hard process. Nimodipine and indapamide had a suitable retention time and were well resolved from the target analyte. Indapamide was finally selected since nimodipine was not stable under light.

Liquid–liquid extraction and protein precipitation are the commonly used technique for sample preparation. Liquid–liquid extraction usually offers much cleaner sample that in turn makes the method more robust and scalable. In this study for the isolation of PAC-1 from plasma samples liquid–liquid extraction was used. Among the solvents such as diethyl ether, dichloromethane and ethyl acetate, dichloromethane gave the highest recovery. Because of the dichloromethane is the under layer, there is a little difficulty to transfer the organic phase. The adoption of sodium hydroxide was necessary because it can improve the recovery greatly. Therefore, ethyl acetate accompanied with sodium hydroxide used was proved to be a simple, efficient solvent for extracting PAC-1 from plasma since the recovery was almost equal to that of dichloromethane, and this solvent was also suitable for the extraction of IS. Finally the sample preparation was selected as above.

5. Conclusion

A specific, simple, sensitive and accurate HPLC method has been developed and validated for the quantitative determination of PAC-1 in rat plasma for the first time. The method has been successfully applied to a pre-clinical pharmacokinetics study of PAC-1.

References

[1] K.S. Putt, G.W. Chen, J.M. Pearson, J.S. Sandhorst, M.S. Hoagland, J.T. Kwon, S.K. Hwang, H. Jin, M.I. Churchwell, M.H. Cho, D.R. Doerge, W.G. Helferich, P.J. Hergenrother, Nat. Chem. Biol. 2 (2006) 543–550.
[2] P.J. Hergenrother, K.S. Put, G.W. Chen, WO 2006/128173 A2.
[3] S. Roy, C.I. Bayly, Y. Gareau, V.M. Houtzager, S. Kargman, S.L. Keen, K. Rowland, I.M. Seiden, N.A. Thornberry, D.W. Nicholson, Proc. Natl. Acad. Sci. U.S.A. 98 (2001) 6132–6137.
[4] A. Nakagawara, Y. Nakamura, H. Ikeda, T. Hiwasa, K. Kuida, M.S. Su, H. Zhao, A. Cnaan, S. Sakiyama, Cancer Res. 57 (1997) 4578–4584.
[5] K.F. Izban, T. Wrone-Smith, E.D. Hsi, B. Schnitzer, M.E. Quevedo, S. Alkan, Am. J. Pathol. 154 (1999) 1439–1447.
[6] R. Persad, C. Liu, T.T. Wu, P.S. Houlihan, S.R. Hamilton, A.M. Diehl, A. Rashid, Modern Pathol. 17 (2004) 861–867.
[7] P.A. Svingen, D. Loegering, J. Rodriquez, X.W. Meng, P.W. Mesner, S. Holbeck, A. Monks, S. Krajewski, D.A. Scudiero, E.A. Sausville, J.C. Reed, Y.A. Lazebnik, S.H. Kaufmann, Clin. Cancer Res. 10 (2004) 6807–6820.
[8] B.A. Bidlingmeyer, Practical HPLC Methodology and Applications, Wiley-Interscience, 1992.
[9] J.R. Larson, J.E. Tingstad, J.K. Swadesh, HPLC—Practical and Industrial Applications: Practical and Industrial Applications, CRC Press, 1997.
[10] W.B. Jung, J.K. Mi, C.G. Jang, S.Y. Lee, J. Chromatogr. B 859 (2007) 69–73.
[11] Q.H. Chen, S.X. Hou, J. Zheng, Y.Q. Bi, Y.B. Li, X.J. Yang, Z. Cai, X.R. Song, J. Chromatogr. B 858 (2007) 199–204.
[12] X. Liu, Y.W. Huang, J. Li, X.B. Li, K.S. Bi, X.H. Chen, J. Pharm. Biomed. Anal. 43 (2007) 371–375.
[13] Guidance for industry, Bioanalytical method validation (updated 2001), http://www.fda.gov/cder/guidance/index.htm.
[14] L.B. Zhao, X.M. Wang, J.H. Wu, Y.S. Jia, W.Q. Wang, S.H. Zhang, J.L. Wang, J. Chromatogr. B 857 (2007) 341–346.