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Short communication

A liquid chromatography–tandem mass spectrometry method for the quantification of PAC-1 in rat plasma

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ABSTRACT

A sensitive and specific liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS) method 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 an ACQUITY UPLC™ BEH C_{18} column, with acetonitrile and water (39:61 (v/v) both containing 0.1% formic acid) as mobile phase at a flow rate of 0.20 mL/min. The detection was performed on a triple quadrupole tandem mass spectrometer by multiple reaction monitoring (MRM) mode via electrospray ionization (ESI) source. The calibration curve was linear over the range of 10–1500 ng/mL ($r > 0.99$). The LOQ was evaluated to be 0.3 ng/mL. The method described herein is sensitive, selective and faster than other existing method, and was successfully applied to the pharmacokinetic study and gender difference investigation of PAC-1 after oral administration in rats.

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1. Introduction

Cancer is a leading cause of death worldwide. Despite the latest statistics has not been revealed, it accounted for 7.4 million deaths (around 13% of all deaths) in 2004, and deaths from cancer worldwide are projected to continue rising, with an estimated 12 million deaths in 2030, according to WHO.

A compound called procaspase-activating compound 1 (PAC-1, Fig. 1A), 1-Piperazineacetic acid, 4-(phenylmethyl)-2-[[2-hydroxy-3-(2-propen-1-yl)phenyl]methylene]hydrazide, was reported by professor Hergenrother [1,2] that enhances the activity of procaspase-3 *in vitro* and induces apoptotic death in cancer cells and cells from primary tumor isolates. PAC-1 also retarded the growth of tumors in three different mouse models of cancer, including two models in which PAC-1 was administered orally. *In vitro* study [3] on mechanism showed that PAC-1 activates procaspase-3 *in vitro* through chelation of inhibitory zinc ions. Zinc inhibits the procaspase-3-catalyzed processing of peptidic substrates and the autoactivation of procaspase-3, while PAC-1 binds quite tightly to zinc with a dissociation constant of approximately 42 nM. The binding of PAC-1 to zinc sequester the inhibitory zinc ions, thus allowing procaspase-3 to autoacticate itself to caspase-3. Evaluation of 26 PAC-1 derivatives suggested [4] an intimate link between the metal chelating properties of the compounds and their ability to induce caspase-3 activation *in vitro* and to induce death in U-937 cell in culture. The small-molecule-mediated activation of procaspases has great therapeutic potential, which is an anticancer strategy that may prove beneficial in treating the many cancers in which procaspase-3 concentrations are elevated [5–9].

A kinetic study of the degradation [10] and a structure elucidation of degradation products [11] of PAC-1 as well as characterization of the *in vivo* and *in vitro* metabolic profile [12] of PAC-1 were carried out in our previous work, and an HPLC method has been developed for the determination of PAC-1 in rat plasma [13], but the method established required a total run time of 15 min, and was not sensitive enough when the dosing is relatively lower. Gender differences in pharmacokinetics and pharmacodynamics [14] have been proved many years ago. Some drugs [15–18] were found to undergo sex-related physiological disposition. According to our unpublished research, tissue distribution of PAC-1 showed a significant gender differences in rat liver, heart, slung, kidney, etc. Thus a new method depending on tandem mass spectrometer was developed and validated for the quantification of PAC-1 in rat plasma, and a pharmacokinetic profile of PAC-1 as well as gender difference study after oral administration was revealed in this work.

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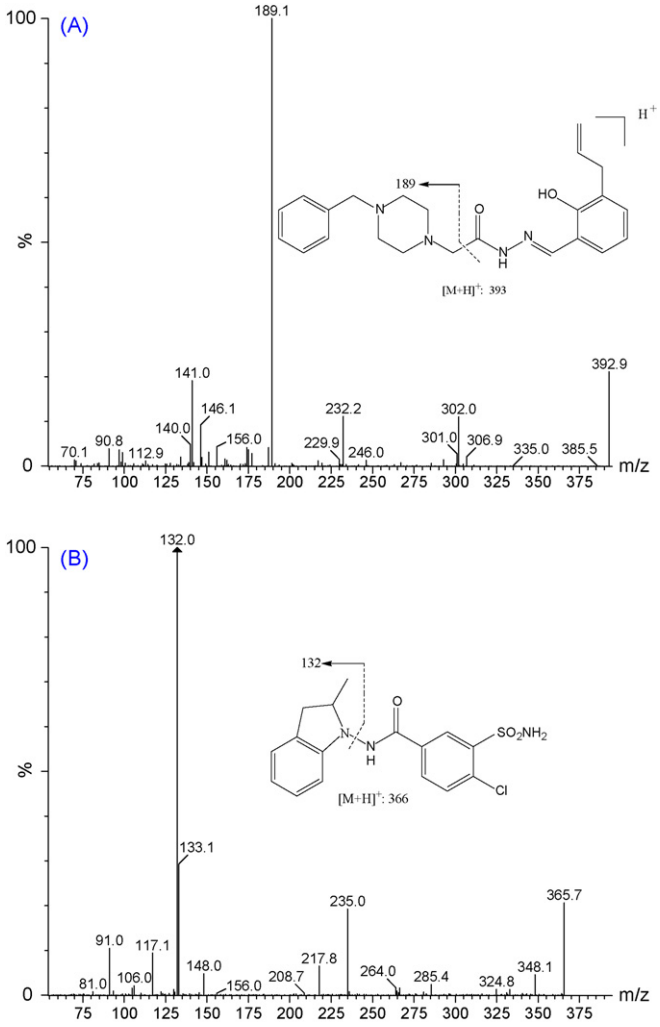

Fig. 1. Full-scan mass spectra of ion product of PAC-1 (A) and IS (B).

2. Experimental

2.1. Chemicals and reagents

PAC-1 (purity > 99.6%) 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. Acetonitrile and methanol (both were of HPLC grade) were provided by Fisher Scientific (Fair Lawn, NJ), while formic acid (HPLC grade) was purchased from YuWang Group (Shangdong, China). All the other reagents were of analytical grade. Distilled water, prepared with demineralized water, was used throughout the study.

2.2. Chromatographic system

Samples were analyzed using an ACQUITY UPLC system (Waters Corp., Milford, MA, USA) coupled to a Quattro Micro™ API mass spectrometer (Waters Corp. Milford, MA, USA). The UPLC system consisted of a quaternary pump, an autosampler and a column oven. The chromatographic separation was performed on a BEH C_{18} column (50 mm × 2.1 mm i.d., 1.7 μm particle size) with acetonitrile and water (39:61 (v/v) both containing 0.1% formic acid) as mobile phase at a flow rate of 0.20 mL/min. The column temperature was maintained at 35 °C. The autosampler was conditioned at 4 °C and the injection volume was 5 μL.

A triple quadrupole tandem mass spectrometer equipped with an electrospray ionization (ESI) interface was used for analytic detection. The ESI source was set in positive ionization mode. Parent ions ($[M+H]^+$) were identified as 393 and 366. The data were collected using multiple reaction monitoring (MRM) mode of transitions of m/z 393 → 189 for PAC-1 (Fig. 1A), and m/z 366 → 132 for indapamide (IS, Fig. 1B), respectively. The MS parameters were set as follows: capillary voltage at 0.5 kV, cone voltage at 60 V, extractor voltage at 3 V, RF lens at 0 V, source temperature at 120 °C, desolvation temperature at 350 °C, multiplier at 650 V and dwell time at 0.30 s. Nitrogen was used as the desolvation and cone gas with a flow rate of 650 and 50 L/h, respectively. Argon was used as the collision gas at a pressure of 2.1×10^{-3} Torr to form specific product ions. Cone voltage and collision energy were optimized at 60 V and 22 eV for PAC-1, respectively. For the IS, the two parameters were 30 V and 15 eV, respectively. All data were processed using MassLynx V4.1 software (Waters Corp., Milford, MA, USA).

2.3. Preparation of standards

The stock solutions of PAC-1 and working solution of IS were prepared in methanol at concentration levels of 0.2 mg/mL, respectively. Working solutions of PAC-1 were prepared by diluting the stock solutions above with methanol. All PAC-1 and IS solutions were stored at 4 °C. The calibration curve at concentrations of 10, 25, 100, 500, 1000, and 1500 ng/mL were prepared by spiking appropriate amount of the standard solutions in blank plasma. Three levels of QC samples (20, 200, and 1200 ng/mL) in plasma were prepared separately in the same fashion.

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, 5 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 limit of detection (LOD) was defined as the concentration at which the signal-to-noise (S/N) of the instrument response was 3, while the limit of quantification (LOQ) as 10. The lower limit of quantification (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%.

To assess the intra-day accuracy and precision of the method, 3 concentrations of PAC-1 (20, 200, and 1200 ng/mL) were spiked into plasma, with 5 replicates independently prepared at each concentration. Similarly, the inter-day accuracy and precision was evaluated on 3 separate occasions, with 5 replicates at each concentration. Accuracy was defined as the relative error (R.E. %) while precision was defined as the relative standard deviation (R.S.D. %).

The recovery of PAC-1 at 3 concentrations (20, 200, and 1200 ng/mL) was determined in 5 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 125 ng/mL.

The quality control (QC) samples (six replicates of QC samples at each of 20, 200, and 1200 ng/mL concentrations) were assayed

under several different conditions to assess the stability of PAC-1 in rat plasma [19]. 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 was within ±15%.

2.6. Application to pharmacokinetic study

Wistar rats (200–220 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. Blood samples (0.5 mL) were collected from the ocular vein with heparinized tubes before and 5, 10, 20, 30, 45, 60, 90, 120, 180, 240, 360, 480 min after oral administration (15 mg/kg), and then immediately centrifuged at 12,000 rpm for 5 min. The plasma obtained was stored frozen at −20 °C until analysis.

2.7. Data processing and statistical analysis

Noncompartment model was used to calculate the pharmacokinetic parameters with DAS 2.0 software (Pharmacology Institute of China). The mean maximum plasma concentration of PAC-1 (C_{max}) and the time to maximum concentration (T_{max}) were determined from inspection of the individual PAC-1 concentration–time data. The terminal elimination rate constant (λ_z) was estimated by linear regression of the terminal portion of the concentration–time curve, and the elimination $t_{1/2z}$ was calculated as $0.693/\lambda_z$. The area under the mean plasma concentration–time curve (AUC_{0-8}) was estimated using the trapezoidal method. The apparent oral clearance (CL_z/F) was calculated as Dose/$AUC_{0-\infty}$.

All results from the study were expressed as mean ± SD. All analyses were performed with SPSS software (ver. 16.0; SPSS, Chicago, IL). The pharmacokinetic parameters between different gender groups were compared by independent samples *t*-test after their natural log-transformation, or Mann–Whitney test. $P < 0.05$ was considered statistically significant for all the tests.

3. Results and discussion

3.1. LC–MS/MS selection and chromatographic system optimization

Although both ESI and APCI sources produced significant signal for quantification, ESI was chosen because the sensitivity and linearity for the analyte were better than that of the APCI. In the parent ion full-scan spectra in positive ESI mode, the most abundant ions were parent molecules $[M+H]^+$ *m/z* 393 and 366 for PAC-1 and IS, respectively. Parameters such as desolvation temperature, ESI source temperature, capillary and cone voltage, flow rate of desolvation gas and cone gas were optimized to obtain highest intensity of parent ion of PAC-1. MS/MS experiments were performed on the parent molecules of PAC-1 and IS at *m/z* 393 and 366, respectively. The major fragments were found at *m/z* 189 and 132, respectively, which were used for PAC-1 and IS quantification in rat plasma. In addition, product ion spectra of *m/z* 189 and 132 are presented in Fig. 1A and B, along with a suggested fragmentation.

Formic acid was a requisite to the ionization of PAC-1, while the content had a little affected in the sensitivity of the IS. The elution order of PAC-1 and IS was related to the percentage of acetonitrile in the mobile phase. Methanol or acetonitrile without water served as solvent may lead to peak splitting of PAC-1, thus mobile phase was used to resolve the sample after evaporation during sample preparation.

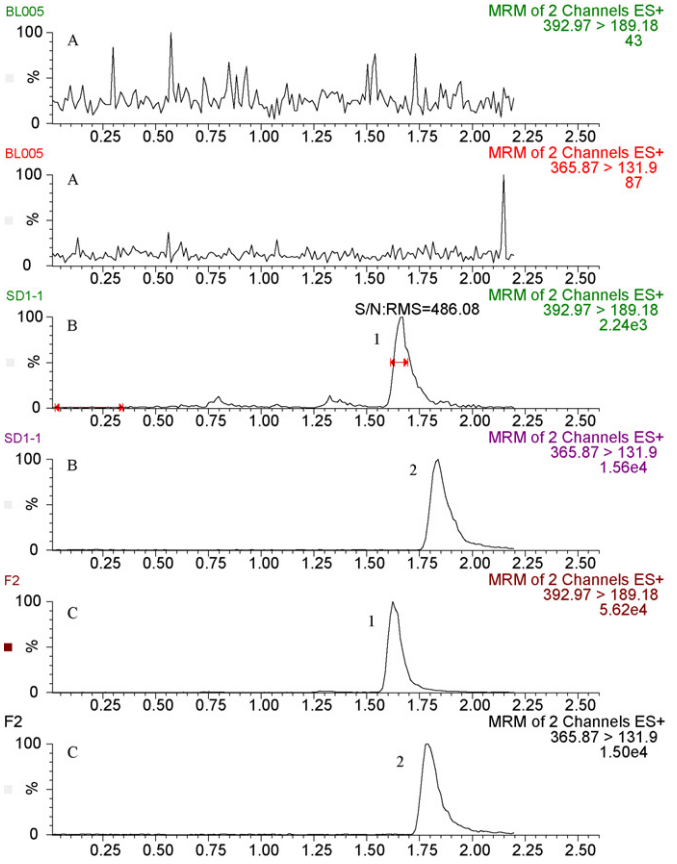

Fig. 2. Representative chromatograms of blank plasma (A); plasma spiked with PAC-1 (LLOQ, 10 ng/mL) and IS (B); plasma samples obtained 10 min after a single i.g. treatment with 15 mg/kg of PAC-1 (C) from a male rat. Peak 1: PAC-1; Peak 2: IS.

Matrix effect was evaluated based on direct comparison of the peak areas of PAC-1 and IS injected directly in mobile phase, and spiked samples originating from blank plasma. Preliminary investigation showed that the matrix effect was absent under the present chromatographic condition.

3.2. Method validation

3.2.1. Specificity, linearity and sensitivity

No endogenous interference was found at the retention times of PAC-1 and the IS. Representative MRM chromatograms for blank rat plasma, rat plasma spiked with PAC-1 (10 ng/mL, LLOQ) and the IS (125 ng/mL) and plasma sample obtained at 10 min from male Wistar rat after the i.g. treatment with 15 mg/kg of PAC-1 are shown in Fig. 2A–C, respectively. The respective retention times of PAC-1 and IS were 1.62 and 1.79 min, much shorter than our previous work [13] reported as 13.4 and 10.8 min. Retention times could not be shortened because of a significant matrix effect (data not shown).

The standard calibration curve was linear over the concentration range of 10–1500 ng/mL for PAC-1 with a correlation coefficient of above 0.99. The linear regression equation for PAC-1 rat plasma concentration was: $Y = (3.15 \times 10^{-3} \pm 0.36 \times 10^{-3})X + (3.57 \times 10^{-3} \pm 4.43 \times 10^{-3})$ ($r = 0.9957 \pm 0.0015$), where Y is the plasma concentration of PAC-1 and X is the peak area ratio of PAC-1 to the IS.

Table 1
Precision and accuracy of PAC-1 determination in rat plasma (intra-day: 5 replicates at each concentration; inter-day: 15 replicates at each concentration).

Concentration (ng/mL)	Intra-day ($n = 5$)		Inter-day ($n = 5 \times 3$)	
	Precision (R.S.D %)	Accuracy (R.E. %)	Precision (R.S.D %)	Accuracy (R.E. %)
20	5.9	10.9	6.2	10.9
200	7.2	9.3	1.9	9.8
1200	5.5	−6.6	6.8	12.4

The sensitivity of the present method was significantly improved compared with our previous HPLC method [13]. Being defined as the concentration at which S/N = 10, the LOQ of this UPLC–MS/MS method was found to be 0.3 ng/mL, about 150 times increased compared with that of HPLC method reported as 50 ng/mL. The LLOQ (10 ng/mL) for PAC-1, with an S/N not less than 480, was not as low as LOQ, but sensitive enough for the pharmacokinetics study of PAC-1 in the present study.

3.2.2. 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 7.2%. The accuracy (R.E. %) of PAC-1 ranged from −6.6 to 10.9% for intra-day and 9.8 to 12.4% for inter-day, respectively. These results indicated that the present method had a good precision and accuracy.

Sample preparation by protein precipitation with small volume of rat plasma (50 μL) was investigated and significant matrix effect was observed because of the complexity of matrix. Liquid–liquid extraction offers much cleaner sample that in turn makes the method more robust and sensitive. In view of the complex procedures involved in liquid–liquid extraction, a relative larger volume of rat plasma may assure the accuracy and precision of the method.

3.2.3. Recovery

The extraction efficacy of PAC-1 at three concentrations ranged from 83.9 to 89.3%, while the recovery of IS was 86.8%, with R.S.D. less than 6.1%. This result suggested that there was no relevant difference in extraction recovery at different concentration levels.

3.2.4. Stability

The stability of PAC-1 in rat plasma under different storage conditions is summarized in Table 2. PAC-1 was stable at room temperature for 4 h, at −20 °C for at least 30 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.

Table 2
Stability of PAC-1 in rat plasma ($n = 3$).

Spiked concentration (ng/mL)	Measured concentration (mean ± SD)	Accuracy (R.E. %)	R.S.D. %
Frozen for 30 days			
20	19.0 ± 0.7	−7.4	3.6
200	198.5 ± 14.4	−8.9	7.3
1200	1202 ± 109	10.3	9.1
Three freeze–thaw cycles			
20	19.0 ± 1.0	−9.5	5.5
200	204.4 ± 14.0	6.8	6.8
1200	1161 ± 84	−9.2	7.3
Room temperature for 4 h			
20	19.3 ± 0.7	−7.4	3.6
200	202.0 ± 8.5	−3.6	4.2
1200	1203 ± 83	−7.1	6.9
Prepared samples in autosampler for 12 h			
20	19.5 ± 1.2	−9.4	6.3
200	209.6 ± 11.1	8.4	5.3
1200	1244 ± 44	5.9	3.6

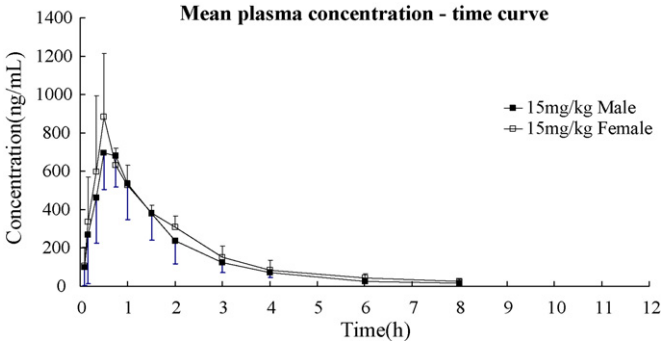

Fig. 3. Mean plasma concentration–time profile of male rats and female rats after oral administration of 15 mg/kg PAC-1, respectively.

Table 3
Pharmacokinetic parameters of PAC-1 in rat plasma.

Parameters	Units	Male	Female
$AUC_{(0-t)}$	μg·h/L	1284 ± 226	1479 ± 297
$AUC_{(0-\infty)}$	μg·h/L	1303 ± 226	1522 ± 325
$AUMC_{(0-t)}$		2202 ± 477	2739 ± 858
$AUMC_{(0-\infty)}$		2381 ± 485	3176 ± 1171
$MRT_{(0-t)}$	h	1.71 ± 0.14	1.84 ± 0.36
$MRT_{(0-\infty)}$	h	1.83 ± 0.17	2.05 ± 0.46
$T_{1/2z}$	h	1.24 ± 0.42	1.39 ± 0.47
T_{max}	h	0.51 ± 0.13	0.47 ± 0.07
CL_z/F	L/(h kg)	11.8 ± 2.0	10.2 ± 1.9
V_z/F	L/kg	21.6 ± 9.4	20.1 ± 7.5
C_{max}	μg/L	788.8 ± 158.8	916 ± 376

3.3. Application

The validated method was applied to assay PAC-1 in rat plasma samples. Calibration levels were identical to those that used during validation procedures. The mean plasma concentration–time curves after oral administration at a dose of 15 mg/kg PAC-1 is shown in Fig. 3. The obtained pharmacokinetic parameters of PAC-1 are shown in Table 3. PAC-1 could be detected 5 min right after drug administration and reached its maximum concentration in plasma after a median of 0.50 h (0.33–0.75 h); C_{max} were 788.8 ± 158.8 and 916.0 ± 375.8 ng/mL for the male rats and female rats, respectively. The estimated half-lives were found to be 1.24 ± 0.42 and 1.39 ± 0.47 h for male and female rats, respectively, while the pharmacokinetic study was carried out for 8 h, which corresponds to at least three elimination half-lives. The result proves the method developed here has adequate sensitivity for the intended application. The PK data analysis showed that PAC-1 has moderate clearance and a large volume of distribution. No significant differences ($P > 0.05$) were observed for the major pharmacokinetic parameters between male and female rats, indicating that there was no gender difference in pharmacokinetics of PAC-1.

4. Conclusions

A specific, simple, accurate, more sensitive and faster HPLC–MS/MS method has been developed and validated for the quantitative determination of PAC-1 in rat plasma, and has been

successfully applied to a pre-clinical pharmacokinetics study of PAC-1. There is no gender difference in pharmacokinetics of PAC-1. Method established is suitable for the determination of PAC-1 in bile and urine.

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