Analytical Methods

Development and validation of a reversed liquid chromatographic method with fluorescence detection for the pharmacokinetic study of a new chimeric peptide

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ARTICLE

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The objective of this research is to develop a sensitive bioanalytical method and investigate the pharmacokinetics of a new chimeric peptide (BN-9) in rats. According to the analysis criteria, a reversed phase high performance liquid chromatography method with fluorescence detection (HPLC-FLD) was established and employed in monitoring the compound in rat plasma using doxorubicin (DOX) as internal standard (IS). Fluorescent BN-9 and IS were extracted from plasma using dehydrated alcohol. The mobile phase was composed of acetonitrile and potassium dihydrogen phosphate buffer containing 0.05% trifluoroacetic acid (TFA) (pH 7.4; 0.02 M) (30:70, v/v) at 0.7 mL/min. Fluorescence detection was conducted at 490 nm (excitation wavelength) and 520 nm (emission wavelength). The lower limit of quantification (LLOQ) was 0.009 μg/mL. The intra-batch and inter-batch precision was less than 8.116%, and the accuracy was within 5.632%. Blood samples were collected after intravenous administration at the doses of 5, 10, and 20 mg/kg fluorescent BN-9 to rats. The main pharmacokinetic parameters were obtained by non-compartmental and compartmental analysis with DAS 2.1.1 software. The $t_{1/2\alpha}$ values were 9.584 ± 5.137, 8.548 ± 2.093 and 9.621 ± 0.3224 min, respectively. The results showed that the method was successfully applied to investigate the pharmacokinetic profiles of fluorescent BN-9 following intravenous administration to rats.

1. Introduction

Morphine as a powerful analgesic plays an important role in the treatment of acute and chronic pain. However, long-term use of morphine carries varying degrees of side effects such as addiction, tolerance and both physical and psychological dependence.[1-4] Hence, there is a need to develop a novel antinociceptive agent to surmount its deficiencies. Much of the research has elucidated that neuropeptide FF (NPFF, Phe-Leu-Phe-Gln-Pro-Gln-Arg-Phe-NH_2) is characterized as analgesia-modulating peptide in 1985.[5-7] Biphalin ((Tyr-D-Ala-Gly-Phe-$NH)_2$), a novel bivalent opioid tetrapeptide, is a highly potent enkephalin analogue with a biological profile similar to endogenous enkephalins.[8-10] Based on this foundation, BN-9 (Tyr-D-Ala-Gly-Phe-Gln-Pro-Gln-Arg-Phe-NH_2) is synthesized by R. Wang *et al*.[11] The chimeric peptide (BN-9) is prepared by using both Biphalin and NPFF as chemical templates. In the pharmacological experiments, BN-9 has more central analgesic potency than morphine. It is found that the peptide possesses little effect on gastrointestinal motility and is free from analgesic tolerance.[11] Hence it availably overcomes opioid drugs' drawbacks such as tolerance and constipation.

Although the analgesic activity of BN-9 has been well verified, there is no report concerning the pharmacokinetics so far. Literature survey reveals that pharmacokinetic studies of protein and polypeptide drugs face many challenges because of their low content in organs[12]and a short half-life.[13] But so far, there is no alternative analytical method to quantify BN-9 in vivo. Hence, it is extremely indispensable to establish a simple and selective method for the determination of BN-9 in plasma.

Several analytical methods have been proposed for the determination of polypeptide, including bioassay method,[14] immunological method,[15] isotope tracing technique[16] and high performance liquid chromatography (HPLC).[17-22] Compared to ultraviolet instrumentation, a reversed liquid chromatographic

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method with fluorescence detection is more selective and sensitive. Although the mass spectrometric method offers superior sensitivity, it is not readily available in most laboratories due to its high equipment cost. Consequently, it is of great feasibility to develop a HPLC-FLD assay based on the fluorescence properties of materials. Nevertheless, BN-9 has weak fluorescence intensity. To enhance the fluorescence intensity, the fluorescence markers exert a tremendous fascination on us. Fluorescein isothiocyanate (FITC) is an amine-reactive derivative of fluorescein dye which has extensive utilization in biochemistry.[23] It has been widely available to label peptides and proteins.[24-26] The labeled molecules can be detected with very high sensitivity.[27] Consequently, fluorescent BN-9 was synthesized by our laboratory.

Therefore, the focus of our study was to develop a convenient and high-efficiency HPLC-FLD assay for the determination of BN-9 in rat plasma. The composition of mobile phase and available internal standard were explored. In addition, the synthesis, purification and characterization of fluorescent BN-9 were also examined in this research. HPLC-FLD method was established, validated and successfully applied to the pharmacokinetic study of fluorescent BN-9 in rats.

2. Materials and methods

2.1. Materials

Fmoc protected amino acids, N-Ethyldiisopropylamine and N-Hydroxybenzotrizole were obtained by GL Biochem Ltd (Shanghai, China). Rink Amide MBHA resin was supplied by Tianjin Nankai Hecheng Science & Technology Co., Ltd, China. Trifluoroacetic acid was from Tianjin reagent factory. Methanol and acetonitrile (HPLC grade) were provided by Shandong Yuwang Co., Ltd. (Shandong, China). Dehydrated alcohol was from Tianjin Concord Technology Co., Ltd. (Tianjin, China). Fluorescein isothiocyanate (FITC, purity>98%) was gained from Aladdin reagent Co., Ltd. (shanghai, China). Doxorubicin hydrochloride (DOX, purity>98%) was purchased from the Shanghai Sangon Biological Engineering Technology Co., Ltd. (Shanghai, China). Other chemicals used in the study were of the analytical reagent grade.

2.2. Synthesis and purification of fluorescent BN-9

BN-9 was assembled on the Rink Amide MBHA resin by stepwise solid-phase method. According to the report by W. Rui et al[11], the process was as follows: (1) DMF wash (×3, 2 min); (2) 20% piperidine/DMF (×4, 2 min); (3) DMF wash (×4, 2 min); (4) Na-Fmoc-Amino Acid (3 eq.)/HBTU (3 eq.)/HOBt (3 eq.)/DIEA (6 eq.) in DMF (×1), 1 h; (5) DMF wash (×3, 2 min); (6) Kaiser ninhydrin Test. The synthesis of BN-9 was finished after the coupling of the Tyr. Before labeling with FITC, amino hexanoic acid was coupled to the last amino acid in the same way. FITC (1.3 eq.) was dissolved in DMF and DIEA (6 eq.), the solution was added dropwise to the resin. Then the mixture was stirred overnight in the dark. The fluorescent BN-9 was cleaved from the resin with a mixture of 95% TFA, 2.5% TIS and 2.5% H_2O for 3 h at room temperature and separated with cold ethyl ether by precipitation. All crude peptides were desalted by Gel filtration (Sephadex G-25). Preparative reversed-phase HPLC-UV was performed to purify the reaction mixture in the Waters Delta 600 system. Fractions encompassing the purified peptides were pooled and lyophilized. The purity of BN-9 was confirmed by analytical HPLC which was carried out with Waters 1525 instrument equipped with a YMC-Pack ODS-A analytical column (150×4.6 mm, 5 μm, Japan); absorbance was monitored at a wavelength of 220 nm. The solvents for elution consisted of acetonitrile (containing 0.1% TFA) and water (containing 0.1% TFA) in the ratio 40:60 (v/v) with a flow rate of 1 mL/min. The retention time was recorded as t_R (min). The molecular weight was characterized by an electrospray ionization mass spectrometer (Mariner ESI-TOF MS, Applied Biosystems, CA).

2.3. Chromatographic conditions

The Shimadzu HPLC system consisted of two LC-10ATVP pumps (Shimadzu Corp., Kyoto, Japan), a DGU-12A vacuum degasser, a CTO-10ASvp column oven, a manual sample valve injector with a 20 μL loop, a RF-10AXL fluorescence detector (FD) and a SCL-10Avp system controller. The data were gathered with Shimadzu CLASS-VP software (Ver.6.1). Chromatographic separation was performed on a YMC-Pack ODS-A analytical column (150×4.6 mm, 5 μm, Japan) coupled with a Shimadzu guard column (10.0 mm×4.0 mm, 5 μm, Japan). The composition of mobile phase was acetonitrile and 0.02 M phosphate buffer (including 0.05 % TFA, pH adjusted to 7.4) in the ratio of 30:70 (v/v). DOX (Fig. 1) was served as an internal standard (IS). The samples were separated favorably at a flow rate of 0.7 mL/min and the column temperature was controlled at 35 °C. Fluorescence detection was performed with excitation and emission wavelengths at 490 nm and 520 nm, respectively.

2.4. Preparation of standard solutions, calibration standards and quality control samples

The stock solution of fluorescent BN-9 was prepared in dehydrated alcohol at a concentration of 100 μg/mL, and the internal standard (IS) stock solution was attained by dissolving doxorubicin in methanol at 100 μg/mL. The fluorescent BN-9 stock solution was serially diluted with dehydrated alcohol to obtain working standard solutions with concentrations of 0.048, 0.097, 0.195, 0.390, 0.781, 1.562, 3.125, 6.250, 12.500,

25.000, 50.000, 100.000 μg/mL. Each calibration standard plasma sample was prepared separately by mixing blank rat plasma (100 μL) with 20 μL each working standard solution to obtain the following concentrations: 0.009, 0.019, 0.039, 0.078, 0.156, 0.312, 0.625, 1.250, 2.500, 5.000, 10.000 and 20.000 μg/mL. In order to reduce deviation, there should be two calibration curves in the method validation for each analytical run in the concentration ranges of 0.009-0.312 and 0.312-20.000 μg/mL, respectively. Quality control (QC) samples were prepared as above at final concentrations of 0.019, 0.781, 0.312, 1.250 and 5.000 μg/mL. The calibration standards and QCs were processed as described below.

2.5. Sample processing

An aliquot of 100 μL plasma sample was extracted with 400 μL dehydrated alcohol after the addition of 20 μL IS solution (100 μg/mL). After through vortex mixing for 1 min on a vortex mixer, the sample mixture was centrifuged at 12,000 rpm for 5 min. The supernatant was then transferred into another centrifuge tube and a 20 μL aliquot of the supernatant was injected into the HPLC system for analysis.

2.6. Validation

The methodology validation was in accord with the FDA guidance on bioanalytical method validation.[28]

2.6.1. Linearity and Lower limit of detection (LLOD) and quantification (LLOQ)

To assess the linearity of the method, a series of standards were prepared as described above covering a range of 0.009-0.312 and 0.312-20.000 μg/mL. The standard curves were established by plotting the peak area ratios of fluorescent BN-9 to IS versus the analyte concentration in rat plasma. The curve equations and the correlation coefficient (*r*) were calculated by linear regression. The linear model was valid if the *r* was greater than 0.99. The LLOD and LLOQ represented the sensitivity of methods and were described as the concentration of the analyte yielding a signal to noise of 3:1and 5:1, respectively. The relative standard error of LLOQ should be within 20 %.

2.6.2. Selectivity

To investigate the possible endogenous interferences, blank plasma samples from individual rats were processed and analyzed during method validation. The selectivity of the method was examined by comparing chromatograms of blank plasma samples, blank plasma spiked with fluorescent BN-9 and IS and rat plasma sample after intravenous administration. The retention times of the compounds were determined under the chromatographic conditions.

2.6.3. Accuracy and precision

Intra-day and inter-day accuracy and precision were evaluated by QC samples at 0.019, 0.781, 0.312, 1.250 and 5.000 μg/mL concentration levels. For every concentration, five replicate QC samples were prepared. The evaluations of within-batch accuracy and precision were performed on three consecutive days. Assay accuracy was explained in terms of relative error and precision was explained via relative standart error.

2.6.4. Relative and absolute recovery

The absolute recovery of fluorescent BN-9 in plasma was calculated by comparing the mean peak areas of extracted QC samples with unprocessed reference solutions. QC samples were made at five concentration levels. Relative recovery of fluorescent BN-9 was assessed by comparing the nominal and measured concentration of five QC samples. In addition, the recovery of internal standard was evaluated at the concentration of 100 μg/mL in the same manner.

2.6.5. Stability

Samples stability was investigated by analyzing five replicates of QC samples at 0.019, 0.781, 0.312, 1.250 and 5.000 μg/mL concentrations under various conditions. For short-term stability, QC samples were placed at room temperature for 12 h before extracting as described in the “Sample Processing” section. To evaluate freeze-thaw stability, QC samples were exposed to three cycles from -20 °C to 25 °C. In each freeze-thaw cycle, the QCs were frozen at -20 °C for 24 h and then thawed naturally at the room temperature. Long-term stability was assessed by kept QC samples at -20 °C for three months. Stability of the extracted QC samples was also evaluated at room temperature for 8 h. These samples were determined and the results compared with the freshly prepared samples.

2.7. Pharmacokinetics study

2.7.1. Animals

Male Sprague Dawley (SD) rats (220 ± 10 g) were obtained from the Experimental Animal Center of Lanzhou University. All animals were kept in conformity with the European Community guidelines for the use of experimental animals (86/609/EEC). All the procedures of the animal test were approved by the Ethics Committee of Lanzhou University, China.

2.7.2. Drug administration and sampling

The fluorescent BN-9 was dissolved in phosphate buffer saline solution containing 30% 1, 2-propylene glycol (v/v) at 1.5, 3.0, and 5.5 mg/mL for dosing. Twelve rats were randomly divided into three groups. For the pharmacokinetics studies, the fluorescent BN-9 was injected into tail vein at doses of 5, 10 and 20 mg/kg. After intravenous administration, the rats were anaesthetized with anhydrous ether, then blood samples were collected approximately 300 μL from retro-orbital venous plexus puncture into heparinized tubes at 0.033, 0.083, 0.166, 0.25, 0.333, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 6 and 8 h , respectively. Plasma samples were centrifuged at 4500 rpm for 15 min, and then the supernatant plasma were frozen immediately at -20 °C until analysis.

2.7.3. Pharmacokinetics data analysis

Compartmental and non-compartment pharmacokinetic analysis were performed using DAS Version 2.1.1 (Drug and Statistics 2.1.1, the Committee of the Mathematic Pharmacology, the Chinese Society of Pharmacology, Hefei, China). The pharmacokinetic parameters were the distribution half-life ($t_{1/2\alpha}$), the elimination half-life ($t_{1/2\beta}$), apparent volume of distribution of the central compartment (V_1), the clearance (CL), the area under the curve (AUC), the elimination rate constant of the central compartment (K_{10}), distribution rate constant transported from the central compartment to the peripheral compartment (K_{12}), constant distributed from the peripheral compartment to the central compartment (K_{21}) and maximum concentration (C_{max}). All parameters were expressed as mean ± SD. Statistical differences of pharmacokinetic parameters among different doses were assessed by the one-way ANOVA ($p < 0.05$) with the SPSS 19 software.

3. Results and Discussion

3.1. Peptide synthesis

The fluorescent peptide was synthesized by solid-phase methods. For TFA-mediated cleavage, N-terminal FITC-labeled peptides undergo the removal of the last amino acid. This can be avoided by the introduction of amino hexanoic acid as spacer between fluorescein isothiocyanate and the peptide.[29] Based on the difference in their molecular weight, organic salts were separated from the crude peptide by gel sephadex filtration. Furthermore, the objective peptide was purified using preparative RP-HPLC to remove free FITC and other impurities. The gradient elution was reasonable because the polarity between the synthetic peptide and by-products was closed. Through evaporation of the solvents under vacuum condition, the peptides could be stably stored for a period of time. The chemical purity determined by analytical RP-HPLC is above 95%.The ESI-MS result (Fig. 1) is in accord with the theoretical value (calculated MW 1613.68; observed MH^+ 1614.68).

3.2. Optimization of HPLC conditions

The composition of the mobile phase was optimized after several attempts to achieve symmetric peak shapes and adequate resolution of analytes. Initially, the mobile phase of acetonitrile and 0.1% TFA in water was tested, but a low response was observed. Then acetonitrile and water (containing 0.1% TFA, 0.2% triethylamine) was used as mobile phase, the problem of low response was solved. Nevertheless, the addition of triethylamine caused significant baseline shifting. The mobile phase consisting of acetonitrile and 0.01 M potassium dihydrogen phosphate (pH adjusted to 7.4) was also tried, it was found that the peak shape of fluorescent BN-9 was poor. Finally, these problems were solved by using acetonitrile and 0.02 M potassium dihydrogen phosphate (containing 0.05% TFA, pH adjusted to 7.4) as the mobile phase. Under this chromatographic conditions, a symmetric peak shape of target compounds were obtained with the addition of 0.05% TFA in potassium dihydrogen phosphate. Moreover, analytes also possessed stable fluorescence intensity as well as adequate resolution. Actually, fluorescence property of fluorescein is strongly pH-dependent.[30] In the mobile phase, the pH value of aqueous phase was adjusted to 7.4 because FITC has intense fluorescence intensity in weak alkaline solution.[30] The potassium dihydrogen phosphate buffer as the mobile phase could provide a relatively stable pH environment to maintain the stability of the fluorescence intensity. In this study, an isocratic mobile phase consisting of acetonitrile and 0.02 M phosphate buffer (30:70, v/v) was chosen.

3.3. Optimization of the emission and excitation wavelengths

Fluorescent BN-9 possessed strong spontaneous fluorescence property, which contributed to the improvement of sensitivity. The optimal wavelengths for fluorescent BN-9 were generated by fluorescence spectrophotometer through the measurement of excitation and emission spectra respectively. The results of fluorescence scanning for fluorescent BN-9 are shown in Fig. 2. The excitation spectra of fluorescent BN-9 showed maxima at 490 nm, and emission spectra showed the highest intensity at 520 nm. In this work, the wavelengths of fluorescence detection were set at 490 nm (Ex) and 520 nm (Em), respectively.

3.4. Selection of the internal standard

In general, an internal standard should be structural and chemical similarity to the analyte and well separated from the

analyte, and it should not react with the latter. By searching for documents,[31-34] drugs such as doxorubicin (DOX) and emodin were candidates of internal standard according to fluorescence spectra. As a result, emodin was resolved in the mobile phase specified in "2.3.". DOX showed strong fluorescence intensity, an appropriate retention time and good stability under the chromatographic condition. In addition, the recovery of DOX in plasma was 87.403% as shown in Table 2. It was also sufficiently separated from fluorescent BN-9, and peak shape exhibited well in the present method. Therefore, DOX was selected as IS with high recovery presented in the method.

3.5. Selection of extraction solvent

The ideal extraction solvents should produce clear supernatants and high recovery. For this purpose, initially a liquid-liquid extraction was tested by adding 0.5 mL acetonitrile to the spiked sample. Thereafter, moderate sodium chloride was added to the sample. The mixture was vortex-mixed. After centrifugation, the supernatant was transferred into another eppendorf tube and evaporated under nitrogen stream at 35 °C. Then the residue was dissolved in 100 µL methanol. However, the extraction recovery was unsatisfactory. Later, 10% perchloric acid was used to extract fluorescent BN-9 from rat plasma. The sample had a clear supernatant but did not show any analytical signal. Thereafter, the extraction solvent was saturated sodium sulfate. The supernatant of the processed sample was not plain. After several trials, dehydrated alcohol was selected as extraction solvent on account of its highest extraction efficiency and reproducibility for both BN-9 and IS. Also, dehydrated alcohol remarkably enhanced the fluorescence intensity of the peptide.

3.6. Validation of the method

3.6.1. Linearity, LLOD and LLOQ

The standard curves were linear covering the concentration range from 0.0097 to 20.000 µg/mL. The regression equations were $y = 6.085x - 0.067$ (0.009-0.312 µg/mL) with the r value of 0.9990 and $y = 10.880x - 1.903$ (0.312-20.000 µg/mL) with the r value of 0.9935, where y and x represented fluorescent BN-9 concentration and the peak area ratio of fluorescent BN-9 to IS, respectively. The LLOD was 0.005µg/mL, and the LLOQ was established at 0.009 µg/mL. The precision of other concentration levels were not more than 15%, and all calibration curves satisfied the acceptance standard.

3.6.2. Selectivity

Specificity was assessed by distinguishing the analyte from substances in the plasma. As shown in Fig. 3 (b), the chromatograms showed that there were no endogenous interfering peaks observed at the retention times of the BN-9 and IS in the blank plasma samples. In addition, the retention times for two fluorescent isomers conjugated to BN-9 and IS were at 5.0, 6.9 min and 15.1 min, respectively. The resolution between BN-9 and IS conformed to the requirements.

3.6.3. Accuracy and precision

To assess the accuracy and the intra-and inter-day precision of the method, we carried out replicated analyses of spiked analytes at 0.019, 0.781, 0.312, 1.250 and 5.000 µg/mL in five separated analytical runs. The accuracy and precision are shown in Table 1. In all cases, the intra- and inter-day precision was less than 8.116%, and accuracy was within 5.632% deviation. The results demonstrated that the analysis was reliable and reproducible.

3.6.4. Recovery

The results of extraction efficiency are summarized in Table 2. The recoveries of BN-9 and IS ranging from 87.403% to 91.067% were high and reproducible. The simple protein precipitation employed in this experiment was capable of effectively recovering analytes from plasma.

3.6.5. Stability

The analyte stability in rat plasma was assessed under a variety of exposures. The results of stabilities are presented in Table 3. All RSD values were within 9.045% in stability tests. For short-term stability, QC samples left at room temperature for 12 h were not affected. Additionally, there was no significant degradation when samples suffered from three freeze-thaw cycles as well as at -20 °C for 90 days. Extracted compounds were stable at ambient temperature for 8 h. The results indicated that samples were allowed to be analyzed under the tested conditions without distinct loss of drugs.

3.7. Pharmacokinetics study

The method was successfully applied to quantify plasma concentration of fluorescent BN-9 at different time after intravenous injection in rats. The mean plasma concentration-time curves are shown in Fig. 4. The pharmacokinetic parameters generated by a two-compartment model with a weight of 1/c are summarized in Table 4. The plasma concentration of fluorescent BN-9 decreased significantly after administration. The $t_{1/2\alpha}$ was 9.584 ± 5.137, 8.548 ± 2.093, 9.621 ± 0.3224 min at 5, 10, 20 mg/kg doses, respectively,

which revealed that the drug was rapidly distributed. The elimination time was short in vivo with the $t_{1/2\beta}$ of 55.455 ± 7.172, 49.581 ± 4.238, 53.345 ± 7.634 min. In the 20 mg/kg group, the plasma C_{max} values of fluorescent BN-9 were remarkably higher than those observed in 5 and 10 mg/kg groups ($p < 0.05$). Moreover, the value of *AUC* in 20 mg/kg group was also greater than that discovered in 5 and 10 mg/kg groups ($p < 0.01$). It was noteworthy that C_{max}, AUC_{0-t} and $AUC_{0-\infty}$ increased proportionately with the increase of dose of fluorescent BN-9 with correlation coefficients of 0.987, 0.983 and 0.980 respectively. The phenomenon indicated that these parameters of fluorescent BN-9 showed a dose-dependent profile. And there were no significant differences in other pharmacokinetic parameters including $t_{1/2\alpha}$, $t_{1/2\beta}$, *CL* and V_1 by ANOVA ($p > 0.05$). Therefore, fluorescent BN-9 showed linear pharmacokinetic characteristics in rats at the doses of 5, 10, 20 mg/kg.

4. Conclusion

In this study, fluorescent BN-9 was synthesized by the solid-phase peptide synthesis method with high purity. A rapid, selective and specific HPLC-FLD method was developed for determination of fluorescent BN-9 in rat plasma. A simple protein precipitation method was established with favorable recovery. The method exhibited high sensitivity, specificity and repeatability with the LLOQ of 0.009 μg/mL. The validated method was successfully used to study pharmacokinetics of the fluorescent BN-9. Following intravenous administration of fluorescent BN-9 at 5, 10 and 20 mg/kg, the pharmacokinetics in rats manifested linear pharmacokinetic characteristics. The findings of bioanalytical method and pharmacokinetics might contribute to the determination of fluorescent BN-9 in vivo and provide initial pharmacokinetic profiles of the drug for clinical studies in the future.

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Table 1

Intra- and inter-day accuracy and precision for fluorescent BN-9 determination in plasma (*n = 5*)

Nominal concentration (μg/mL)	Intra-day			Inter-day		
	Measured concentration (μg/mL)	RSD (%)	Bias (%)	Measured concentration (μg/mL)	RSD (%)	Bias (%)
0.019	0.019 ± 0.002	7.764	-1.436	0.019 ± 0.001	6.872	-1.641
0.078	0.079 ± 0.006	7.440	1.434	0.078± 0.005	6.628	0.324
0.312	0.330 ± 0.027	8.116	5.632	0.321 ± 0.024	7.684	2.954
1.250	1.299 ± 0.087	6.669	3.963	1.275 ± 0.066	5.245	2.023
5.000	5.023 ± 0.142	2.827	0.469	5.019 ± 0.136	2.728	0.387

Table 2

Relative and absolute recovery of fluorescent BN-9 in rat plasma samples (*n = 5*)

Analytes	Concentration (μg/mL)	Relative Recovery (%)	Absolute Recovery (%)
fluorescent BN-9	0.019	92.307 ± 5.699	89.350 ± 4.086
	0.078	91.446 ± 9.791	90.394 ± 2.672
	0.312	92.134 ± 6.719	90.215 ± 1.358
	1.250	93.003 ± 5.810	90.035 ± 1.434
	5.000	94.303 ± 4.878	91.067 ± 4.539
DOX	100.000		87.403 ± 1.577

Table 3

The results of sample stabilities under various storage conditions (*n = 5*)

Conditions	Concentration (μg/ml)		RSD (%)
	Spiked	Measured (mean ± SD)	
Short-term (8h, 25 °C)	0.019	0.018 ± 0.001	5.509
	0.078	0.081 ± 0.007	9.045
	0.312	0.286 ± 0.021	7.219
	1.250	1.253 ± 0.097	7.794
	5.000	5.117 ± 0.195	3.827
Post-term (8h, 25 °C)	0.019	0.018 ± 0.001	5.093
	0.078	0.076 ± 0.006	7.841
	0.312	0.314 ± 0.028	8.993
	1.250	1.341 ± 0.098	7.381
	5.000	5.124 ± 0.413	8.071
Three freeze-thaw cycles	0.019	0.019 ± 0.002	11.622
	0.078	0.080 ± 0.005	6.693
	0.312	0.298 ± 0.022	7.449
	1.250	1.241 ± 0.089	7.213
	5.000	5.324 ± 0.345	6.481
Long-term (-20 °C for 90 days)	0.019	0.020 ± 0.001	7.539
	0.078	0.081 ± 0.006	8.122
	0.312	0.301 ± 0.017	5.891
	1.250	1.246 ± 0.092	7.399
	5.000	5.176 ± 0.098	1.907

Table 4

Pharmacokinetic parameters of fluorescent BN-9 in rat plasma after intravenous administration (*n* = 4)

Parameters	5 mg/kg	10 mg/kg	20 mg/kg	P value
$t_{1/2\alpha}$ (min)	9.584 ± 5.137	8.548 ± 2.093	9.621 ± 0.3224	> 0.05
$t_{1/2\beta}$ (min)	55.455 ± 7.172	49.581 ± 4.238	53.345± 7.634	> 0.05
V_1 (L/kg)	0.890 ± 0.074	0.873 ± 0.173	0.982 ± 0.078	> 0.05
CL (L/min/kg)	0.026 ± 0.003	0.036 ± 0.006	0.035 ± 0.004	> 0.05
AUC_{0-t} (mg/L*min)	172.229± 34.158	267.205± 52.429	542.122± 63.443	< 0.01
$AUC_{0-\infty}$ (mg/L*min)	189.313± 25.775	288.435± 52.642	583.867± 68.503	< 0.01
K_{10} (L/min)	0.033 ± 0.003	0.038 ± 0.008	0.035 ± 0.004	> 0.05
K_{12} (L/min)	0.113 ± 0.045	0.042 ± 0.029	0.029 ± 0.019	> 0.05

K_{21} (L/min)	0.056 ± 0.024	0.025 ± 0.004	0.014 ± 0.001	> 0.05
C_{max} (mg/L)	4.928 ± 0.088	9.957 ± 0.077	17.433 ± 0.648	< 0.01

Figure 1

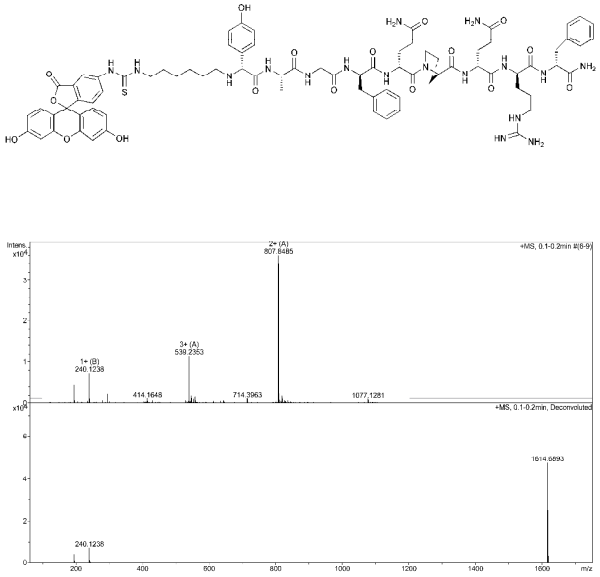


Fig. 1 Chemical structure and Mass spectrum of fluorescent BN-9

Figure 2

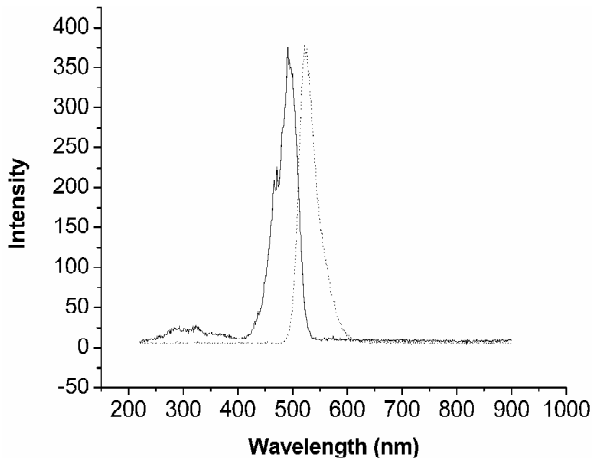


Fig. 2 Fluorescence spectra of fluorescent BN-9 when excitation wavelength is 492 nm (---) and emission wavelength is 530 nm (–)

Figure 3

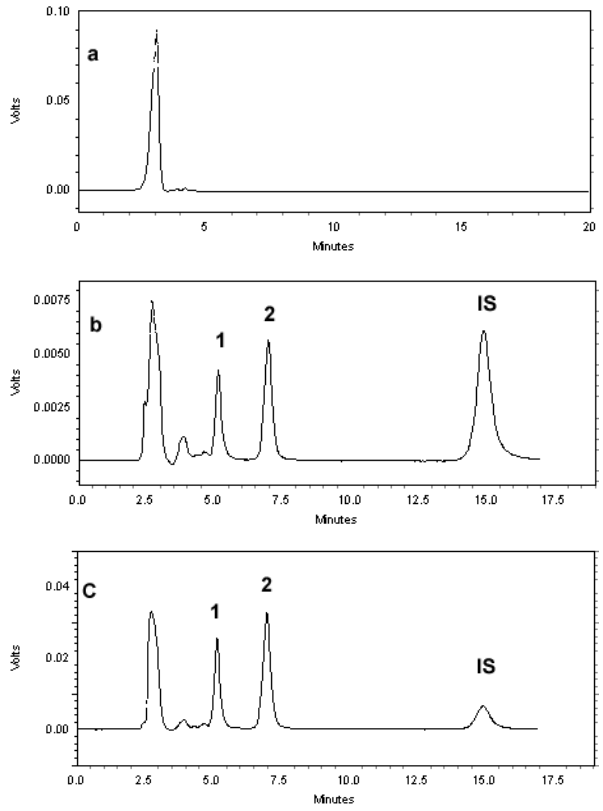


Fig. 3 The HPLC chromatograms obtained from a blank rat plasma (a), a blank plasma sample spiked with 0.3125 µg/ml fluorescent BN-9 (1, 2) and 100 µg/ml IS (b) and 90 min plasma samples from a rat after intravenous administration of 10 mg/kg (c).

Figure 4

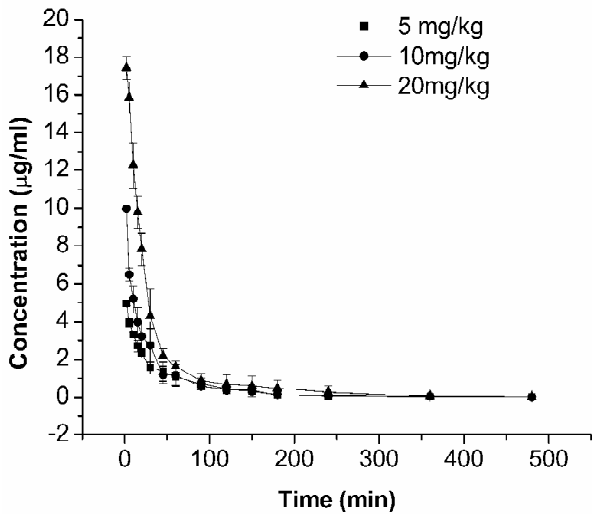
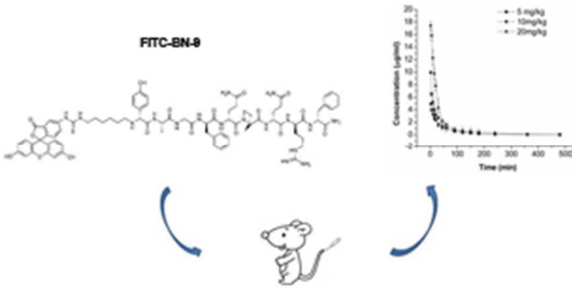


Fig. 4 Mean (± SD) plasma concentration-time profiles of fluorescent BN-9 in the plasma of healthy rats following intravenous administration at doses of 5, 10, 20 mg/kg (*n* = 4)



A reversed phase high performance liquid chromatography method with fluorescence detection (HPLC-FLD) was validated and employed in the pharmacokinetics of FITC-BN-9 in rats.