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A sandwich ELISA for assessment of pharmacokinetics of HSA-$(BNP)_2$ fusion protein in mouse plasma

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ABSTRACT

Brain natriuretic peptide (BNP) is a circulating hormone of cardiac origin that plays an important role in the regulation of intravascular blood volume and vascular tone. HSA-$(BNP)_2$, derived from the joining of human BNP to the C-terminus of human serum albumin (HSA), has been developed to prolong the BNP pharmacodynamic action. For the analysis of pharmacokinetics of the new drug, a novel sandwich enzyme-linked immunosorbent assay (ELISA) was established and validated to quantify HSA-$(BNP)_2$ fusion protein in mouse plasma. The ELISA method was calibrated with 1:10 and 1:100 dilutions of blank mouse plasma spiked with HSA-$(BNP)_2$ standard and validated with respect to parallelism, precision (intra- and inter-assay variation), accuracy (recovery), specificity and stability. The practical working range was estimated to be 31.2–2000 ng/ml with the limit of detection was 7.8 ng/ml. Recoveries ranged from 80.5 to 108.4%, while the intra- and inter-assay precisions were <2.73% and <4.32%, respectively. The terminal half-life of HSA-$(BNP)_2$ was 2.14 h, which had extended more than 40 times compared to 3.1 min half-life of BNP monomer in mouse.

1. Introduction

Brain natriuretic peptide (BNP) was firstly isolated from porcine brain by Sudoh in 1988, and it has a striking similarity to atrial natriuretic peptide (ANP) with regard to both its amino acid sequence and pharmacological spectrum [1]. In later studies, BNP was found to be mainly synthesized in ventricular myocardium and causes effects such as diuresis, natriuresis, vasodilation, and inhibition of aldosterone synthesis and renin secretion as a circulating hormone, and it played an important role in regulating blood pressure and blood volume [2,3].

Food and Drug Administration (FDA) had approved intravenous therapy with a recombinant form of human BNP, nesiritide (Natrecor, Scios Inc., Fremont, CA), as a therapeutic approach for acutely decompensated human congestive heart failure [4–6]. However, due to its rapid clearance and the need for continuous intravenous infusion, current use of BNP is limited to decompensated acute heart failure in the hospital setting [7,8]. As heart failure is a progressive disease, it is necessary to establish continual hemodynamic profile favorable to heart failure condition and to maintain a neurohormonal balance to protect the heart from further deterioration. BNP has properties that are particularly valuable for long-term use. Unlike other heart drugs that are helpful in short-term but harmful in long-term, BNP has the long-term safety profile for it is not inotropic, chronotropic, and proarrhythmic [9,10].

Human serum albumin (HSA) is the most abundant natural carrier protein in plasma and has a half-life exceeding 19 days in human. BNP and HSA fusion could reduce renal filtration, NPR-C receptor mediated clearance and proteolytic BNP cleavage by steric hindrance than single BNP peptide [11–13]. Wang et al. [8] had constructed AlbuBNP and BNP(2x)/HSA, which created by human BNP or duplicated BNP moiety seamlessly fused to N-terminus of HSA and expressed in 293F cells for sustained *in vivo* BNP activities. The dose–response relationship of AlbuBNP, BNP(2x)/HSA and BNP were determined by the NPRA/cGMP assay, suggesting the maximal activities of AlbuBNP and BNP(2x)/HSA approached that of BNP.

A new fusion protein, HSA-$(BNP)_2$, derived from the joining of human BNP to the C-terminus of HSA, has been developed. However, the available assays for BNP quantification were all aimed to the single BNP peptide and were not suitable to detect the content of fusion protein molecule. In this paper, a sandwich ELISA method for the quantification of fusion protein HSA-$(BNP)_2$ in mouse plasma was established to analysis pharmacokinetic parameters. Two different antibodies, aimed to BNP and HSA, respectively, were used in this method, insuring the detection of the complete fusion protein molecule.

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2. Materials and methods

2.1. Standard preparation

HSA-$(BNP)_2$ fusion protein (molecular weight approximately 73 kDa) was produced as a recombinant protein composed of recombinant HSA genetically fused at its C-terminus to the N-terminus of recombinant diad BNP. It was expressed in *Pichia pastoris* GS115 using the multi-copy gene plasmid pPIC9K (Invitrogen, Taastrup, Denmark).

The recombinant protein was expressed in *pichia* according to the manufacturer's recommendations (Multi-Copy *Pichia* Expression Kit, Invitrogen). The culture was centrifuged at $8000 \times g$ for 10 min at 4 °C and the supernatant was filtered through a 0.45 μm filter. After that, the supernatant was enriched 10-fold by ultrafiltration devices with Biomax-10 membrane (Pellicon XL 50 Cassette and Labscale TFF System, Millipore, Massachusetts, USA). The concentrated solution was loaded onto a 25 ml Blue Sepharose 6 Fast Flow affinity column (Amersham Pharmacia Biotech, Sweden) which had been equilibrated with start buffer (0.02 M phosphate, 0.15 M NaCl, pH 7.2). The column was first eluted with elution buffer 1 (0.02 M phosphate, 2 M NaCl, pH 7.2) and then eluted with elution buffer 2 (0.02 M phosphate, 2 M NaCl, 50% ethylene glycol, pH 7.2). Fractions were collected and analyzed by SDS-PAGE (Mini Protean 3 cell, Bio-Rad, California, USA). The fractions containing relative pure interest protein were joined together and dialyzed against water in MD34-14 dialysis tubing and freeze-dried (Labconco FreeZone, Missouri, USA). The protein was resuspended in distilled water and separated by size exclusion chromatography on HiLoad 16/60 Superdex 75 prep grad column (Amersham Pharmacia Biotech, Sweden) which had been equilibrated with start buffer (0.05 M sodium phosphate, 0.1 M NaCl, pH 7.2). Fractions were collected and analyzed by SDS-PAGE. The fractions containing a single protein bond at 73 kDa were joined together, extensively dialyzed and freeze-dried. The obtained protein power was regarded as the standard of HSA-$(BNP)_2$ and stored at −80 °C.

2.2. Standards and quality control samples

The standard powder of HSA-$(BNP)_2$ was resuspended in PBST (0.01 mol/l, pH 7.6 PBS with 0.05% Tween 20) and the content was determined by the Urine Microalbumin Kit (Mind Bioengineering, Shanghai, China). The highest concentration used was 4000 ng/ml and from this dilution a 12-step, 2-fold serial dilution with PBST was made. Calibration standards were prepared by 1:10 and 1:100 dilutions of blank mouse plasma spiked with HSA-$(BNP)_2$ standard at the same concentrations. The final concentrations of standard curve samples were 1, 2, 3.9, 7.8, 15.6, 31.2, 62.5, 125, 250, 500, 1000, 2000 and 4000 ng/ml, respectively. Quality control samples were prepared at 125, 250, 500 and 1000 ng/ml with blank mouse plasma in 1:10 and 1:100 dilutions, respectively.

2.3. Sandwich ELISA procedure

Ninety-six-well ELISA plates (Greiner Bio-One, Germany) were coated with 100 μl/well capture antibody (goat polyclonal anti-HSA antibody, ab19180, Abcam, London, UK) diluted in coating buffer (0.05 M carbonate buffer, pH 9.5) at 4 °C overnight. Plates were washed three times with 200 μl/well PBST, then blocked with 200 μl/well 5% skimmed milk powder (Nestle, Switzerland) in PBST for 1 h at 37 °C. After washing, 100 μl/well standards, controls or samples were then added in triplicate to the plates. The plates were incubated at 37 °C for 1 h. After washing, 100 μl/well of the detector antibody (mouse monoclonal anti-BNP antibody, 24C5, HRP, ab14699, Abcam, London, UK) diluted in PBST were added to the plates and the plates were incubated for 1 h at 37 °C. After a final washing, the plates were developed with 100 μl/well TMB substrate reagent set (Tiangen Biotech, Beijing, China) and incubated for 10 min at room temperature in the dark. The reaction was stopped by adding 50 μl/well 2 M H_2SO_4, and the absorbance was read at 450 nm using an ELISA plate reader (Thermo Labsystems, Thermo Fisher Scientific, New Hampshire, USA).

2.4. Optimization

To develop a highly sensitive and specific ELISA, the assay was optimized according to the procedures described by Crowther [14]. The dilutions of capture antibody and detector antibody were decided by checkerboard titration (CBT). The capture antibody was diluted with coating buffer in a 2-fold dilution ranged from 1:100 to 1:12,800. The detector antibody was diluted with PBST in a 2-fold dilution ranged from 1:1000 to 1:64,000. The HSA-$(BNP)_2$ standard at 2000 ng/ml was applied as positive antigen.

2.5. Assay validation

2.5.1. Parallelism and linearity

Standard of HSA-$(BNP)_2$ was 2-fold diluted ranging from 1.0 to 4000 ng/ml and calibration standards were prepared by 1:10 and 1:100 dilutions of blank mouse plasma spiked with HSA-$(BNP)_2$ standard at the same concentrations. The standard curves were prepared according to the procedure of HSA-$(BNP)_2$ sandwich ELISA. All these samples were analyzed in a triplicate setup.

2.5.2. Precision

Intra-assay precision, the repeatability of HSA-$(BNP)_2$ sandwich ELISA, was evaluated by testing quality control samples containing different amounts of HSA-$(BNP)_2$ standard at levels of 125, 250, 500 and 1000 ng/ml in matrices of blank mouse plasma in 1:10 and 1:100 dilutions. These samples were repeated six times in an intra-assay run and the coefficient variations (CV) were determined. Inter-assay precision, the reproducibility of HSA-$(BNP)_2$ sandwich ELISA, was evaluated by testing the same quality control samples in triplicate analyses on 3 days.

2.5.3. Accuracy

The accuracy of HSA-$(BNP)_2$ sandwich ELISA was evaluated by performing recovery studies using quality control samples. 1:10 and 1:100 dilutions of blank mouse plasma spiked with HSA-$(BNP)_2$ standard at levels of 125, 250, 500 and 1000 ng/ml were tested by HSA-$(BNP)_2$ sandwich ELISA. The samples were analyzed in triplicate, while the mean values of recovery and the coefficient variations (CV) were calculated.

2.5.4. Specificity

Some proteins which have the similar structure of HSA-$(BNP)_2$ were tested in this sandwich ELISA to assess the specificity of this method. These proteins including BNP, HSA, proBNP, IFN_β-HSA, $(GLP)_2$-HSA and IL_2-HSA were diluted to 4000 ng/ml with PBST and tested according to the procedure of HSA-$(BNP)_2$ sandwich ELISA.

2.5.5. Sample stability

To test sample stability under different conditions, three of mouse plasma samples were suffered five cycles of freezing and thawing, or stored at 4 °C for 7, 30, 60 days. Samples were assayed after each cycle and tested in a triplicate setup.

2.6. Pharmacokinetic experiments in mice

Animal handling procedures were in accordance with the National Institutes of Health Guide for Care and Use of Laboratory

Animals. A total of 24 Kunming mice (male:female = 1:1) weighting between 18 and 22 g were obtained from Experimental Animal Center (Zhejiang, China). The mice were randomly divided into four groups, and in each group, there were six mice. HSA-$(BNP)_2$ fusion protein was dissolved in sterile normal saline for intravenous administration at a concentration of 100 μg/ml. Each group of mice were mainlined from the tail vein with the intravenous dose of 375, 750 and 1500 μg/kg. Control group received sterile normal saline injections. The blood samples were taken from the tail vein at 0 (5 min postdose), 1, 2, 4, 8, 16 h and centrifuged at 3500 × *g* for 10 min to collect the plasma. The blank mouse plasma were taken by exposing the bulb of eye and centrifuged at 3500 × *g* for 10 min. Plasma samples were diluted in 1:100 ratio with PBST before tested by HSA-$(BNP)_2$ sandwich ELISA. In cases when assayed concentrations exceeded the limit of quantification, 31.2 ng/ml, samples were reanalyzed in 1:10 dilution ratio.

3. Results

3.1. Standard preparation

The fermentation supernatant contained a number of distinct protein bands including a 100 kDa protein which maybe the native products expressed by *P. pastoris*, the interest protein of HSA-$(BNP)_2$ at 73 kDa and the degradation products of interest protein lower than 73 kDa. The 100 kDa protein could not affinity with blue sepharose and had been removed by affinity chromatography. The degradation products of interest protein could be mostly removed, as it was mostly eluted by elution buffer 2. Another gel filtration chromatography was applied to remove small amount of degradation products and relatively pure (>95%) HSA-$(BNP)_2$ fusion protein with the molecular weight of 73 kDa was obtained (Fig. 1). This was regarded as standard of HSA-$(BNP)_2$ fusion protein in this paper.

3.2. Optimization

Each of the column contains a constant level of capture antibody but different dilutions of detector antibody. Different values of OD_{450} between the columns, where there is a constant dilution of detector antibody, reflects the effect of altering the capture antibody concentration. There is no effect on values when capture antibody is coated at 1:100, 1:200, 1:400 and 1:800 dilutions. When diluting capture antibody at 1:3200 and detector antibody at 1:2000, the reaction was most sufficient and the OD_{450} value reached 1.547 (Table 1).

3.3. Standard curves of HSA-$(BNP)_2$ sandwich ELISA

Standard curve of HSA-$(BNP)_2$ sandwich ELISA was obtained by a 2-fold dilution of HSA-$(BNP)_2$ standard ranging from 1.0 to 4000 ng/ml (Fig. 2A). The working range of the assay was defined as the part of the curve which had a linear correlation coefficient of $R^2 = 0.9931$. Generally, it included seven standard points, ranging from 31.2 to 2000 ng/ml (Fig. 2B).

Parallelism between standard curve and calibration curves were observed. The calibration curve prepared by 1:10 dilution of blank mouse plasma was lower but parallel with standard curve indicated that mouse plasma would weaken this ELISA reaction but still has a linear correlation coefficient of $R^2 = 0.9968$ in the working range, which included six calibration points, ranging from 62.5 to 2000 ng/ml. The calibrating curve of 1:100 dilution was coincident with standard curve suggested the weak effect contributed by mouse plasma was disappeared when further diluted the matrix to 1:100 dilution (Fig. 2).

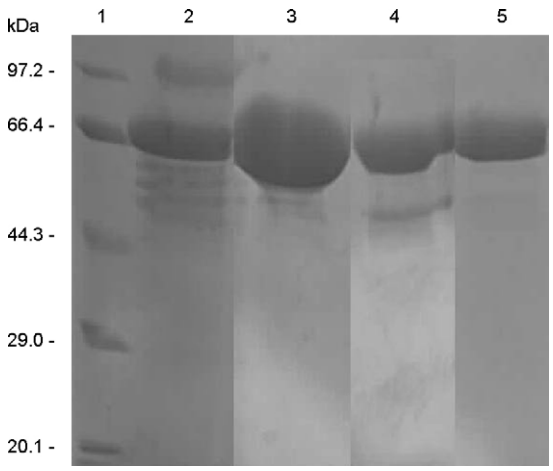

Fig. 1. Coomassie-stained SDS-PAGE of samples during purification process of HSA-$(BNP)_2$ fusion protein. Lane 1: molecular weight marker, protein sizes (kDa) are indicated on the left side of the gel; lane 2: concentrated supernatant; lane 3: eluate of Blue Sepharose 6 Fast Flow affinity column with elution buffer 1 (0.02 M phosphate, 2 M NaCl, pH 7.2); lane 3: eluate of Blue Sepharose 6 Fast Flow affinity column with elution buffer 2 (0.02 M phosphate, 2 M NaCl, 50% ethylene glycol, pH 7.2); lane 4: standard of HSA-$(BNP)_2$ fusion protein (eluate of HiLoad 16/60 Superdex 75 prep grad column).

3.4. Limit of detection and limit of quantitation

The limit of detection (LOD) was determined as the average of triplicate blank samples plus two times the standard deviations (SD). In this experiment, the LOD was 7.8 ng/ml. The limit of quantitation (LOQ) was defined as the HSA-$(BNP)_2$ concentration in the lowest working range. The LOQ was 31.2 ng/ml in 1:100 dilution of blank mouse plasma and 62.5 ng/ml in 1:10 dilution of blank mouse plasma.

Table 1
Checkerboard titration of HSA-$(BNP)_2$ sandwich ELISA.

	Capture antibody								
	1:100	1:200	1:400	1:800	1:1600	1:3200	1:6400	1:12,800	CBS[a]
Detector antibody									
1:1000	1.414	1.712	1.539	1.810	1.310	1.461	1.350	1.398	0.133
1:2000	1.273	1.267	1.323	1.572	1.197	1.547	1.495	1.177	0.133
1:4000	1.063	0.980	1.133	0.930	0.980	1.153	0.946	0.861	0.104
1:8000	0.427	0.630	0.633	0.712	0.545	0.678	0.593	0.513	0.081
1:16,000	0.300	0.346	0.406	0.369	0.361	0.348	0.327	0.284	0.089
1:32,000	0.211	0.250	0.275	0.229	0.210	0.170	0.208	0.210	0.088
1:64,000	0.134	0.158	0.172	0.170	0.152	0.157	0.140	0.138	0.078
PBST[b]	0.078	0.076	0.082	0.076	0.081	0.070	0.077	0.082	0.078

[a] No capture antibody in coating buffer.
[b] No detector antibody in PBST solution.

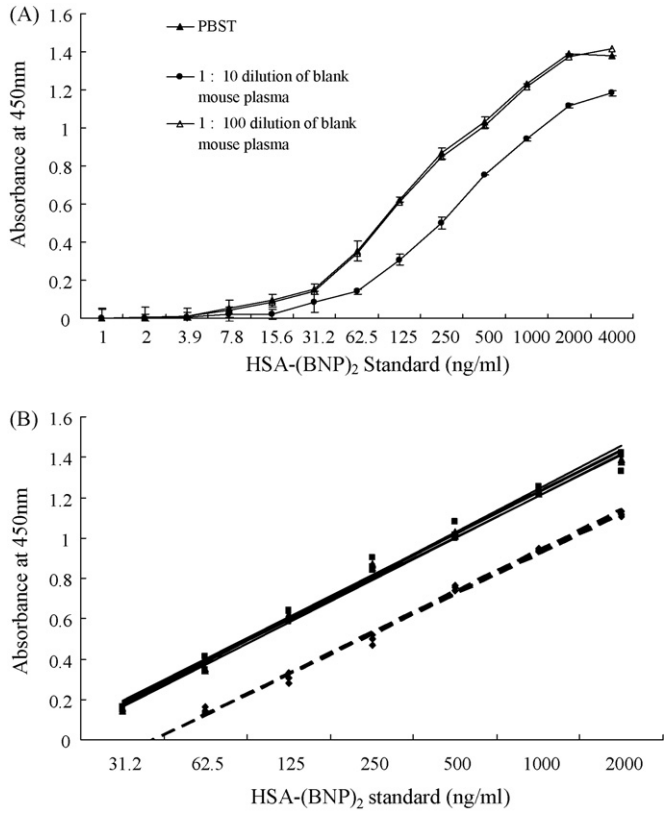

Fig. 2. The standard curves obtained in HSA-$(BNP)_2$ sandwich ELISA. (A) Parallelism of standard curve and calibration curves prepared by PBST, 1:10 and 1:100 dilution of blank mouse plasma spiked with HSA-$(BNP)_2$ standard ranging from 1.0 to 4000 ng/ml. In the absence of spiking, no signal was detected. The graph shows the average values of triplicates in one experiment and the standard deviations (CV). (B) The solid lines represent the seven-point linear calibration curve of PBST and 1:10 dilution of blank mouse plasma spiked with HSA-$(BNP)_2$ standard ranging from 31.2 to 2000 ng/ml ($R^2 = 0.9931 \pm 0.0042$). The dash lines represent the six-point linear calibration curve of 1:10 dilution of blank mouse plasma spiked with HSA-$(BNP)_2$ standard ranging from 62.5 to 2000 ng/ml ($R^2 = 0.9968 \pm 0.0018$). The lines show the independent values obtained from the triplicate runs.

3.5. Precision

Quality control samples containing different amounts of HSA-$(BNP)_2$ standard at levels of 125, 250, 500 and 1000 ng/ml in 1:10 and 1:100 dilutions of blank mouse plasma were evaluated for precision analysis in HSA-$(BNP)_2$ sandwich ELISA. The CV of intra-assay ranged from 0.92 to 2.73% in 1:10 dilution of blank mouse plasma and 0.74–1.70% in 1:100 dilution. The CV of inter-assay ranged from 2.18 to 4.32% in 1:10 dilution matrix and 1.42–3.97% in 1:100 dilution (Table 2).

Table 2
Precision of HSA-$(BNP)_2$ sandwich ELISA.[a].

Spiking concentration (ng/ml)	Intra-assay CV (%)		Inter-assay CV (%)	
	1:10 dilution	1:100 dilution	1:10 dilution	1:100 dilution
125	0.92	0.76	3.71	3.53
250	1.32	1.70	3.96	3.62
500	2.73	0.74	4.32	3.97
1000	2.63	1.00	2.18	1.42

[a] Samples were repeated six times in an intra-assay run and tested in triplicate analyses on 3 days in an inter-assay run.

Table 3
Recovery test of blank mouse plasma spiked with HSA-$(BNP)_2$ standard.[a].

Spiking concentration (ng/ml)	1:10 dilution		1:100 dilution	
	Recovery (%)	CV (%)	Recovery (%)	CV (%)
125	80.5 ± 3.5	4.35	84.5 ± 1.3	1.54
250	96.4 ± 1.8	1.87	99.1 ± 2.5	2.52
500	105.1 ± 2.9	2.76	108.4 ± 3.1	2.86
1000	100.5 ± 2.3	2.29	101.1 ± 2.7	2.67

[a] Recovery is calculated as the ratio of HSA-$(BNP)_2$ content predicted by the calibration curve and the amount of HSA-$(BNP)_2$ spiking into the sample. Values represent the average of triplicate in one experiment and are reported as mean ± standard error of the mean (S.E.M.).

3.6. Accuracy

1:10 and 1:100 dilutions of blank mouse plasma spiked with HSA-$(BNP)_2$ standard at levels of 125, 250, 500 and 1000 ng/ml were tested by sandwich ELISA. Recovery is calculated as the ratio of HSA-$(BNP)_2$ content predicted by the calibration curve and the amount of HSA-$(BNP)_2$ spiked into the sample. The recovery ranged from 80.5 to 105.1% was observed in 1:10 dilution of mouse plasma with the CV ranged from 1.87 to 4.35%. The recovery of 1:100 dilution was slightly higher, ranged from 84.5 to 108.4%, with the CV ranged from 1.54 to 2.86% (Table 3).

3.7. Specificity

Proteins of BNP, HSA, proBNP, IFN_β-HSA, $(GLP)_2$-HSA and IL_2-HSA which have the similar structure of HSA-$(BNP)_2$ were tested by HSA-$(BNP)_2$ sandwich ELISA. The OD_{450} values of these proteins were 0.109, 0.125, 0.113, 0.130, 0.118 and 0.097, respectively, which were all below the limit of detection (LOD) compared with 0.114 of the blank sample's value. It was concluded that there was no cross-reactivity with other similar structure proteins in HSA-$(BNP)_2$ sandwich ELISA.

3.8. Sample stability

The ELISA assay was influenced by repeated freezing and thawing and prolonged storage at 4 °C. There was no significant change after a single freeze thawing procedure, but the samples gradually lose the activity after repeated freezing and thawing experiments. After five repeated freeze thawing procedure, samples lose approximately 35% of the activity. There was a slight loss of sample activity when samples were stored at 4 °C for 7 days, but a significant loss of activity was observed when they were stored at 4 °C for 30 days and 60 days. Thus, we conclude that it is essential to store samples at −20 °C for long-term storage, and repeated freezing and thawing should be avoided.

3.9. Pharmacokinetic analysis in mice

The well-validated HSA-$(BNP)_2$ sandwich ELISA method was successfully applied to determine the plasma concentrations of HSA-$(BNP)_2$ in mice after a single intravenous administration of 375, 750 and 1500 μg/kg. The mean plasma concentration–time profiles are illustrated in Fig. 3. The results showed that the concentration of HSA-$(BNP)_2$ in mouse plasma achieved the peak value simultaneously at 5 min after intravenous administration and the terminal half-life ($t_{1/2}$) was 2.14 h.

4. Discussion

Recombinant human BNP has been licensed to patent medicine as nesiritide (Natrecor, Scios Inc., Fremont, CA) by FDA since 2001,

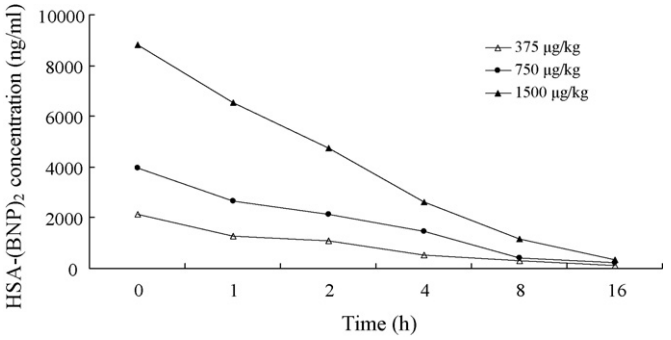

Fig. 3. Concentration–time profiles of HSA-$(BNP)_2$ after a single intravenous administration of 375, 750 and 1500 μg/kg in mice plasma. The line represents the mean data obtained from six mice sampled at each time point.

for the intravenous treatment of patients with acutely decompensated congestive heart failure. Since the half-life ($t_{1/2}$) of nesiritide is only 18 min in human [15,16], a fusion protein of HSA-$(BNP)_2$, derived from the joining of human BNP to the C-terminus of HSA, has been developed for long-term activity. For the pharmacodynamic and pharmacokinetics analysis of the new drug, a novel method specifically for the new molecule should be established.

A sensitive ELISA method has been developed and validated to quantify HSA-$(BNP)_2$ fusion protein in mouse plasma. Different from other methods only detect the single BNP molecule [17–20], this method specifically aimed to the intact fusion protein molecule which had both epitopes of BNP and HSA. Polyclonal antibody against HSA was coated into microplates for capturing the protein with HSA epitope and the captured protein has the BNP epitope was again conjugate with BNP monoclonal antibody with HRP labeled. Since then, only the protein has the two epitopes of BNP and HSA could be captured and detected.

Since the drug of HSA-$(BNP)_2$ fusion protein was a new molecule and could not be purchased from other companies, it was necessary to prepare the standard drug by ourselves. The new protein expressed in *P. pastoris* was purified by two steps of chromatography, Blue Sepharose affinity chromatography and Superdex 75 size exclusion chromatography. After that, the protein with the purity above 95% was obtained and was acceptable as a standard.

The working range of the standard curve included seven standard points, ranging from 31.2 to 2000 ng/ml. Parallelism between standard curve and calibration curves was also observed, which demonstrate that the novel sandwich ELISA setup could be used for measuring of HSA-$(BNP)_2$ concentrations in the mouse blood. The calibration curve same with standard curve was obtained by dilution the mouse plasma in 1:100 ratio, suggesting that 3.12–200 μg/ml of HSA-$(BNP)_2$ could be detected in mouse blood. The limit of quantitation was not low enough to monitor the entire pharmacokinetics progress, and we calibrated the curve of mouse plasma in 1:10 dilution spiked with HSA-$(BNP)_2$ standard. Though the matrix of 1:10 dilution of mouse plasma had a slight depressant effect on the ELISA method, it was parallel with standard curve and had a working range of six calibration points, ranging from 62.5 to 2000 ng/ml. Therefore, the quantitation limit of this ELISA method in blood reached 0.625 μg/ml, which had 5-fold decreased from 3.12 μg/ml.

Precision represents the ability to yield the same result when the sample is tested in intra- or inter-assays with the same lot of manufacturing tests. The repeatability of the HSA-$(BNP)_2$ sandwich ELISA as measured by intra-assay precision was <2.73% and the reproducibility as measured by inter-assay precision was <4.32% in four quality control samples. The method worked reliable both at low and high concentrations and the performance precision was considered to be satisfactory.

The accuracy of an assay states the effect of a substance present in an analytical system which causes a deviation of the measured value from the true value. Accuracy of this method as determined for the matrixes of mouse plasma recovery experiments was good with variances (S.E.M. <20%). In both 1:10 and 1:100 plasma dilutions, the recovery at the lowest spiking level (125 ng/ml) was lower than other concentrations. Only approximately 80% of spiked HSA-$(BNP)_2$ was recovered at 125 ng/ml, but the S.E.M. were all below 10% in other three concentrations. This could be speculated that the matrix have larger effect on the ELISA method when lower amounts of protein contained.

The novel ELISA method presented here is based entirely on commercially available antibodies. The polyclonal antibody of HSA was coated into microplates and blocked with 5% skimmed milk powder. The plates were freeze-dried and stored at −20 °C for 30 days and the results showed no difference with the plates coated right now. The commercially antibodies were freezing and thawing for several times, and there were no significant change when they were freezing and thawing within five times, but the activity of the antibodies were slightly descent when frozen and thawed above six times. There was no evidence for a harmful effect when the antibodies stored at 4 °C for 60 days. So the antibodies should be subpackaged and stored at −70 °C and the one for present use should be stored at 4 °C. The antigen could be stored at 4 °C within 1 week, but if longer storage is required, it is recommended that they should be re-frozen and stored at −20 °C.

In conclusion, the novel sandwich ELISA was developed to determine the fusion protein of HSA-$(BNP)_2$ in mouse plasma and successfully applied to study pharmacokinetics of HSA-$(BNP)_2$ in mice after intravenous administration. The terminal half-life of HSA-$(BNP)_2$ evaluated by this method was 2.14 h, compared to 3.1 min half-life of BNP monomer in mouse [8], the terminal half-life of fusion protein had extended more than 40 times. Though the BNP activity of HSA-$(BNP)_2$ may decrease by the steric hindrance, the prolonged half-life would make up this deficient and produce long-acting therapeutic effect.

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References

[1] T. Sudoh, K. Kangawa, N. Minamino, H. Matsuo, A new natriuretic peptide in porcine brain, Nature 332 (1988) 78–81.
[2] J.A. de Lemos, D.K. McGuire, M.H. Drazner, B-type natriuretic peptide in cardiovascular disease, Lancet 362 (2003) 316–322.
[3] T. Nishikimi, N. Maeda, H. Matsuoka, The role of natriuretic peptides in cardioprotection, Cardiovasc. Res. 69 (2006) 318–328.
[4] W.S. Colucci, U. Elkayam, D.P. Horton, W.T. Abraham, R.C. Bourge, A.D. Johnson, et al., Intravenous nesiritide, a natriuretic peptide, in the treatment of decompensated congestive heart failure, Nesiritide Study Group, N. Engl. J. Med. 343 (2000) 246–253.
[5] S. Iyengar, D.S. Feldman, R. Trupp, W.T. Abraham, Nesiritide for the treatment of congestive heart failure, Expert Opin. Pharmacother. 5 (2004) 901–907.
[6] H.H. Chen, M.M. Redfield, L.J. Nordstrom, D.P. Horton, J.C. Burnett Jr., Subcutaneous administration of the cardiac hormone BNP in symptomatic human heart failure, J. Card. Fail. 10 (2004) 115–119.
[7] A. McGregor, M. Richards, E. Espiner, T. Yandle, H. Ikram, Brain natriuretic peptide administered to man: actions and metabolism, J. Clin. Endocrinol. Metab. 70 (1990) 1103–1107.
[8] W. Wang, Y. Ou, Y.G. Shi, AlbuBNP, a recombinant B-type natriuretic peptide and human serum albumin fusion hormone, as a long-term therapy of congestive heart failure, Pharmacol. Res. 21 (2004) 2105–2111.
[9] J.G. Lainchbury, J.C. Burnett, D. Meyer, M.M. Redfield, Effects of natriuretic peptides on load and myocardial function in normal and heart failure dogs, Am. J. Physiol. Heart Circ. Physiol. 278 (2000) H33–H40.

[10] M.R. Burger, A.J. Burger, BNP in decompensated heart failure: diagnostic, prognostic and therapeutic potential, Curr. Opin. Investig. Drugs 2 (2001) 929–935.
[11] J.A. Marques, J.K. George, I.J. Smith, V. Bhakta, W.P. Sheffield, A barbourin–albumin fusion protein that is slowly cleared in vivo retains the ability to inhibit platelet aggregation in vitro, Thromb. Haemost. 86 (2001) 902–908.
[12] B.L. Osborn, H.S. Olsen, B. Nardelli, J.H. Murray, J.X. Zhou, A. Garcia, G. Moody, L.S. Zaritskaya, C. Sung, Pharmacokinetic and pharmacodynamic studies of a human serum albumin-interferon-alpha fusion protein in cynomolgus monkeys, J. Pharmacol. Exp. Ther. 303 (2002) 540–548.
[13] C. Sung, B. Nardelli, D.W. LaFleur, E. Blatter, M. Corcoran, H.S. Olsen, C.E. Birse, O.K. Pickeral, J. Zhang, D. Shah, G. Moody, S. Gentz, L. Beebe, P.A. Moore, An IFN-beta albumin fusion protein that displays improved pharmacokinetic and pharmacodynamic properties in nonhuman primates, J. Interferon Cytokine Res. 23 (2003) 25–36.
[14] J.R. Crowther, The ELISA guidebook, Methods Mol. Biol. 149 (2000) 1–143.
[15] R.E. Hobbs, R.M. Mills, Therapeutic potential of nesiritide (recombinant B-type natriuretic peptide) in the treatment of heart failure, Expert Opin. Investig. Drugs 8 (1999) 1063–1072.
[16] V.K. Munagala, J.C. Burnett Jr., M.M. Redfield, The natriuretic peptides in cardiovascular medicine, Curr. Probl. Cardiol. 29 (2004) 707–769.
[17] L.B. Yap, H. Ashrafian, D. Mukerjee, J.G. Coghlan, P.M. Timms, The natriuretic peptides and their role in disorders of right heart dysfunction and pulmonary hypertension, Clin. Biochem. 37 (2004) 847–856.
[18] B.J. Kim, S.J. Hwang, K.C. Sung, B.S. Kim, J.H. Kang, M.H. Lee, J.R. Park, Assessment of factors affecting plasma BNP levels in patients with chronic atrial fibrillation and preserved left ventricular systolic function, Int. J. Cardiol. 118 (2007) 145–150.
[19] H. Sakai, T. Tsutamoto, C. Ishikawa, T. Tanaka, M. Fujii, T. Yamamoto, H. Takashima, M. Horie, Direct comparison of brain natriuretic peptide (BNP) and N-terminal pro-BNP secretion and extent of coronary artery stenosis in patients with stable coronary artery disease, Circ. J. 71 (2007) 499–505.
[20] O.W. Nielsen, P.J. Cowburn, A. Sajadieh, J.J. Morton, H. Dargie, T. McDonagh, Value of BNP to estimate cardiac risk in patients on cardioactive treatment in primary care, Eur. J. Heart Fail. 9 (2007) 1178–1185.