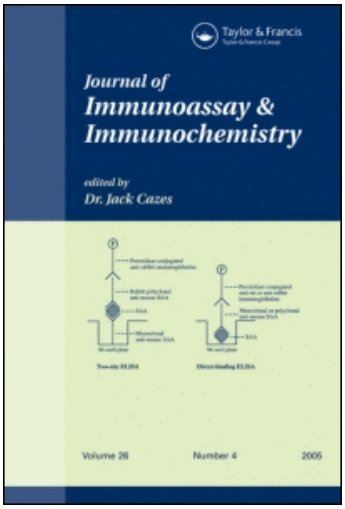

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RADIOIMMUNOASSAY FOR RAT B-TYPE NATRIURETIC PEPTIDE (BNP-45)

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ABSTRACT

Rat BNP-45 is the main circulating form of BNP in rat plasma. To understand the role of BNP in physiological and pathophysiological conditions, a specific radioimmunoassay (RIA) for the quantitative determination of the peptide in plasma and tissues is necessary. An assay using rBNP-45 as the standard in conjunction with antisera directed against this peptide has not been described in the literature, though some investigators have reported values ranging from 0.73-2.0 pmol/L using either BNP-26 or BNP-32 as the standard peptide. Unfortunately, these forms of BNP do not exist in rat plasma. In our studies, we have developed a specific RIA for rBNP-45 using rBNP-45 as the standard peptide and Tyr^{o}-rBNP-45 as the radioligand. We have used two specific antisera for assay purposes; one against rBNP-45, and the second to a peptide composed of the first 20 amino acids of rBNP-45 (rBNP[1-20]). The recovery of various amounts of rBNP-45 added to control plasma was 50-80% depending on the method of extraction and purification. The interassay and intraassay coefficients of variation were 12% and 6% respectively. Values obtained were similar for blood sampled by either cardiac puncture, decapitation, or aortic puncture. The method was used to measure rBNP-45 in the plasma of normal (WKY) and Spontaneously Hypertensive (SHR) rats. The values obtained were 5.46 ± 0.43 and 19.6 ± 2.36 pmol/L respectively. The rat atrial natriuretic peptide (ANP[99-126]) values in the same extracts were 23.2 ± 0.45 and 51.6 ± 3.16 pmol/L.

(Key Words: rat BNP, radioimmunoassay)

INTRODUCTION

Following the initial discovery of atrial natriuretic peptide (ANP) in 1981 by de Bold et al. (1), a number of natriuretic peptides have been identified. These new members of the natriuretic peptide family include brain or B-type natriuretic peptide (BNP) (2) and C-type natriuretic peptide (CNP) (3). The rat form of BNP was first purified and sequenced in our laboratory in 1989 (4), and was initially termed iso-rANP before its identification as one of the B-type natriuretic peptides (5). Although ANP and BNP are genetically distinct from each other (5), the two peptides possess striking homology in the nature of their biological activities (4).

Rat BNP-45 is the main circulating form of BNP in rat plasma (6,7). In order to understand the role of BNP in physiological and pathophysiological conditions, a specific radioimmunoassay (RIA) for the quantitative measurement of this peptide in plasma and tissues is necessary. Although some investigators have published values of BNP in rat plasma and tissue using radioimmunoassays with either rBNP-32 or rBNP-26 as the standard peptide (6,8,9), there appears to be variation in the values obtained. To address this discrepancy, we have established a radioimmunoassay for rBNP-45 using rBNP-45 as the standard peptide, antiserum to rBNP-45, and iodine-125 labelled Tyr°-rBNP-45 as the radioligand. No such assay has been described in detail in the literature. This method was evaluated in the present study for its precision, and we have compared the values obtained by our

method to the values obtained by the widely used rBNP-32 RIA kit from Peninsula Laboratories (Belmont, California). We have also compared these values to those obtained using an antibody directed against the N-terminal 20 amino acids of the rBNP-45 sequence. The application of our radioimmunoassay indicates that although different methods of blood sampling do not appear to affect the plasma values obtained, the procedure of plasma extraction can dramatically alter the recovery of rBNP. In addition, we have also demonstrated with this assay that the circulating plasma levels of BNP are higher in the spontaneously hypertensive rat (SHR) than in the normotensive Wistar-Kyoto animal (WKY).

MATERIALS AND METHODS

Materials

Rat BNP-45, rBNP-45 antiserum, and rBNP-32 RIA kits were purchased from Peninsula Laboratories (Belmont, CA, USA). Rat BNP-45 antiserum (# RAS 9080) crossreacts 100% with rBNP-45, rBNP-32, and rBNP[10-32], while it has 0% crossreactivity with proBNP[1-23], [24-45], ANP[99-126], human and porcine BNP, endothelin, and angiotensin II. The dilution used was as recommended by the manufacturer. The rBNP-32 kit consisted of rBNP-32 standard, antiserum to rBNP-32, and radiolabelled rBNP-32. The crossreactivity for rBNP-32 antiserum was similar to that of

rBNP-45. The protocol for RIA followed was as described by the manufacturer.

Rat BNP[1-20] and tyrosylated rBNP-45 (Y^{o}-rBNP) were synthesized by the core facility for Protein/DNA chemistry at Queen's University, Kingston, Canada. Peptides were prepared using standard solid-phase synthesis on a model 430 Applied Biosystems Peptide Synthesizer. Solvents used were high performance liquid chromatography (HPLC) grade and were obtained from BDH Chemicals (Toronto, Ont., Canada). All other chemicals were from Sigma Chemical Co. (St. Louis, MO, USA). C_{18} Sep-Pak cartridges were purchased from Waters Chromatography (Millipore, Milford, MA, USA).

Preparation of rBNP[1-20] Antisera

Synthetic rBNP[1-20] was conjugated to bovine thyroglobulin using a carbodiimide method and was used to immunize rabbits as previously described (10). The antisera crossreacts 100% with rBNP-45 and 0% with ANP[99-126], pro-ANP, AVP, endothelin, and angiotensin. The antiserum was used at a final dilution of 1:120,000.

Iodination of rBNP-45

Tyrosylated rBNP-45 was iodinated using a modification of the chloramine T method as reported previously (10). Briefly, 10 μg of peptide in

50 μl of 0.5 mol/L sodium phosphate buffer was mixed with 1 mCi of $I^{125}Na$ in a 1 ml conical glass vial. The reaction was initiated by the addition of 10 μl of a chloramine T solution (1 mg/ml in 50 mmol/L phosphate buffer, pH 7.6) and the vial was shaken gently for 15 seconds. The reaction was stopped by dilution with 0.5 ml of 0.1% trifluoroacetic acid (TFA). The diluted reaction mixture was then applied to a Sep-Pak cartridge that had been prepared by washing with 5 ml 100% acetonitrile (CH_3CN) and with 10 ml 0.1% TFA. The cartridge was washed with 20 mls of 0.1% TFA and eluted slowly with 3 mls of 80% CH_3CN in 0.1% TFA. The eluate was purified by reverse phase HPLC using a Vydac C_{18} column and a 60 minute gradient of 20-45% CH_3CN containing 0.1% TFA at a flow rate of 1.0 ml/minute. The purified radiolabelled peptide was stored at -70 °C in concentrations of 10^4 CPM/0.1 ml.

Blood sampling

Plasma was obtained using three different methods. For aortic and cardiac puncture samples, animals were first anaesthetized with Inactin at a dose of 100-120 mg/kg. The thoracic cavity of each animal was then opened via bilateral incisions through the rib cage along the mid-clavicular line. For cardiac puncture, blood was removed by inserting a heparinized 16-gauge needle into the apical region of the left ventricle. Aortic puncture samples

were obtained in a similar manner from the abdominal aorta just proximal to its bifurcation to form the common iliac arteries. Decapitated animals were bled over a funnel into a 10 ml glass test tube. All blood samples were added immediately to chilled tubes containing ethylene-diaminetetra-acetic acid (EDTA) (1mg/ml) and Aprotinin (100 KIU/ml), and centrifuged for 35 minutes at 1500 x g to obtain plasma. Plasma samples were divided into aliquots and stored at -70 °C until assayed.

Plasma extraction

Sep-Pak cartridges for plasma extraction were prepared by consecutive washes with 5 mls of 8 mol/L urea, 10 mls H_2O, 5 mls 80% CH_3CN in 0.1% TFA, and 10 mls 0.1% TFA. Frozen plasma samples (2-3 mls) were thawed and acidified with an equal volume of 0.1 N HCl. The acidified plasma was passed twice through washed Sep-Pak cartridges using a 10 syringe infusion pump (Harvard Apparatus, model 22) at a flow rate of 1 ml/min. Sep-Paks were then washed at a flow rate of 5 ml/min with 10 mls 0.1% TFA followed by 5 mls of 10% CH_3CN in 0.1% TFA. Samples were eluted with 3 mls of 80% CH_3CN in 0.1% TFA at a flow rate of 0.5 ml/min and dried using a Speed Vac. The resulting dry powder was stored at either -20 °C or -70 °C for RIA.

Recover of radiolabelled BNP-45 from plasma

Approximately 20,000 CPM of radiolabelled BNP-45 was added to 3 ml plasma and allowed to stand for one hour at 4°C. One set was then acidified with an equal volume of 0.1% TFA, the second set with 0.9% saline containing 0.15% EDTA (pH 6.0), and the third set with an equal volume of 0.1N HCl. The plasma samples were extracted with Sep Pak as described above, lyophilized, and dissolved in 650 μl RIA buffer. A 0.1 ml fraction was then counted to determine recovery.

RIA protocol

The dried extract was dissolved in 650 μl of RIA buffer consisting of 50 mmol/L sodium phosphate (pH 7.4), 0.25% w/v bovine serum albumin (BSA), 0.9% NaCl, 25 mmol/L EDTA, 0.1% (v/v) Triton X-100, and 0.01% NaN_3. One hundred μl of standard rBNP-45 solution or sample was then added to a 12 x 75 mm polystyrene tube and incubated with 100 μl of the appropriately diluted antisera for rBNP-45 or rBNP[1-20] in RIA buffer. After 24 hrs of incubation at 4 °C, 100 μl of the labelled peptide (8000-9000 cpm) in RIA buffer was added. After a further 24 hr incubation at 4 °C, free and bound fractions were separated by adding 25 μl of 5% normal rabbit serum and 100 μl of goat anti-rabbit gamma globulin (1:10). The mixture was incubated overnight at 4 °C, then centrifuged at 3000 x g for 35 minutes after

the addition of 100 μl 25% polyethylene glycol (PEG 8000) and 500 μl RIA buffer diluted 1:1 with H_2O. The resulting supernatants were aspirated and the remaining precipitate counted for radioactivity in a gamma counter (LKB Clinigamma) and values calculated using either a spline or logit log curve.

RESULTS

Fig. 1 shows typical standard curves obtained using rBNP-45 as the standard peptide and radiolabelled rBNP-45 against three different antisera. The effective range of the standard curve was 0.6 to 20 fmol, and the IC_{50} (half-maximum inhibition) for rBNP-45, rBNP-32, and $rBNP_{[1-20]}$ antisera were 5 fmol, 4.4 fmol, and 7 fmol/tube respectively. The minimum detection limit for the assay at 95% confidence was 0.6 fmol/tube. The interassay and intraassay coefficients of variation were 12.3 and 6.5% respectively.

Serial dilutions of plasma extracts paralleled the standard curve, suggesting that rBNP-45 present in plasma is immunologically indistinguishable from synthetic rBNP-45. Fig. 2 shows the reversed phase HPLC profile of immunoreactive rBNP-45 in 5 mls of pooled plasma extract using rBNP-45 and rBNP-32 antisera. The immunoreactive BNP-45 emerged at the elution time of synthetic rBNP-45, and no other peak was evident. These results substantiate the reported observation that rBNP-45 is the main circulating form of BNP in rat plasma (6,7).

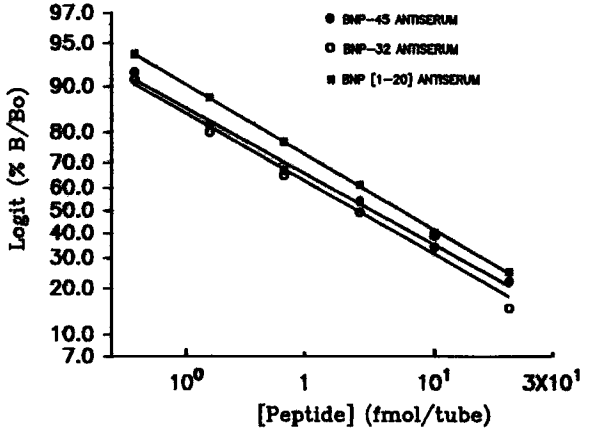

Figure 1 Standard curves with rBNP-45 as the standard and radiolabelled peptide against 3 different antisera.

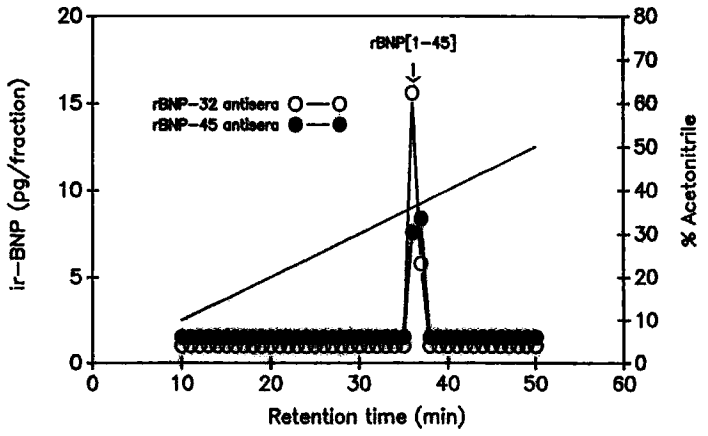

Figure 2 Reversed-phase HPLC profile of immunoreactive rBNP-45 in pooled rat plasma using rBNP-45 and rBNP-32 antisera.

TABLE 1

Effect of acidification of plasma on the recovery of radiolabelled rBNP-45 after Sep-Pak extraction

Method	Recovery (%)	Range (%)
I (RIA kit from Peninsula Lab) equal volume of 0.1% TFA; plasma pH = 6.8	50.5 ± 1.8 (n = 11)	45 - 62
II (Yokata et al.) equal volume of 0.9% saline + 0.15% EDTA at pH 6.0; plasma pH = 7.2	54.0 ± 2.1 (n = 8)	45 - 64
III (present study) equal volume of 0.1 N HCl; plasma pH = 3.0	80.0 ± 1.5* (n = 8)	76 - 82

Each recovery value represents the mean ± SEM. Asterisk denotes statistical significance of Method III from the other two groups at $P < 0.016$ by Bonferroni's modification of the Student's "t" test for multiple means.

As is evident from Table 1, the highest recovery of radiolabelled rBNP-45 (80%) was obtained when plasma was acidified with 0.1 N HCl as compared to the dilution with 0.1% TFA (50%) or 0.9% saline containing 0.15% EDTA (54%). This extraction method (acidification with 0.1 N HCl) gave similar recoveries when unlabelled peptide was added to pooled rat plasma (Table 2). As shown in Table 3, different methods of blood sampling did not appear to affect the plasma values of rBNP obtained.

TABLE 2

Recovery of rBNP-45 added to pooled rat plasma, acidified with an equal volume of 0.1 N HCl, and extracted with Sep Pak

rBNP-45 added (pmol/L)	Expected ir-BNP (pmol/L)	rBNP recovered (pmol/L)	Recovery (%)
0	6.72	-	-
5	11.72	8.56	73.0
10	16.72	13.14	78.5
20	26.72	18.16	68.0

TABLE 3

Effect of blood sampling method on the plasma values of rBNP-45

	Plasma level of rBNP-45 (pmol/L)		
Method	rBNP-32 antisera	rBNP-45 antisera	$rBNP_{[1-20]}$ antisera
Decapitation	6.12 ± 0.36	6.18 ± 0.38	8.56 ± 0.62
Aortic puncture	4.98 ± 0.39	5.26 ± 0.54	8.52 ± 0.29
Cardiac puncture (Lt. ventricle)	4.98 ± 0.68	6.20 ± 0.46	8.22 ± 0.7

Each value is the mean ± SEM; n = 4 for each sample.

TABLE 4

Plasma levels of endogenous immunoreactive rBNP in Sprague-Dawley, normotensive Wistar-Kyoto, and Spontaneously Hypertensive Rats using three different antisera

Rat species	Plasma level of rBNP-45 (pmol/L)		
	rBNP-32 antisera	rBNP-45 antisera	$rBNP_{[1-20]}$ antisera
Sprague-Dawley	-	3.08 ± 0.45 (n = 7)	4.08 ± 0.59 (n = 5)
Wistar-Kyoto	6.28 ± 0.54 (n = 8)	5.46 ± 0.43† (n = 8)	7.94 ± 0.77† (n = 8)
Spontaneously Hypertensive	24.80 ± 2.56* (n = 4)	19.60 ± 2.36* (n = 10)	23.22 ± 2.4* (n = 9)

Each value is the mean ± SEM. Statistical significance at $P < 0.016$ by Bonferroni's modification of the Student's "t" test for multiple means.
* significant from WKY and SD rats
† significant from SD rats

Table 4 shows the plasma levels of rBNP-45 in normotensive Wistar-Kyoto (WKY), Sprague Dawley (SD), and spontaneously hypertensive rats (SHR) using three different antisera. The values obtained using rBNP[1-20] antiserum were higher than rBNP-45 or rBNP-32 antiserum. The values obtained for SHR were significantly higher than those in WKY or SD rats. The levels of rBNP in SD rats were significantly lower than those in WKY.

DISCUSSION

To date no detailed radioimmunoassay method for the measurement of rBNP-45 in plasma has been reported using rBNP-45 as the standard peptide and rBNP-45 antisera. Some investigators have reported values ranging from 0.73 - 2.0 pmol/L using rat BNP-26 or rat BNP-32 antiserum, standard, and radiolabelled peptide. Since rat BNP-45 is the main circulating form of BNP in rat plasma (6,7), we have developed a method using antiserum for rBNP-45, radiolabelled BNP-45 and BNP-45 as the standard peptide. Using this method the levels of BNP-45 in normal rat plasma obtained were 3.0 - 6.0 pmol/L; these values are much higher than reported by others (7,12). Thibault et al. (9) have recently reported plasma levels of rat BNP-45 in the range of 2.0 - 4.0 pmol/L, and our values compare favourably with these. The values obtained using BNP[1-20] antiserum were higher than those obtained using BNP-45 or BNP-32 antiserum. Using a kit from Peninsula Laboratories with antibodies directed against rBNP-32 and using radiolabelled BNP-32 and BNP-32 standard peptide, we have demonstrated that the use of rBNP-32 instead of BNP-45 does not affect the measurement of plasma values of rBNP-45.

The underestimation of the plasma levels by various investigators may be due to the recovery of the peptide from plasma during Sep-Pak extraction. Our data indicate that the recovery of rBNP from plasma samples can be improved from 50% to over 80% by acidification of plasma with 0.1 N HCl

to pH 3.0 before extraction. Yokoto et al. (12) have used immunoaffinity columns to further purify the plasma extract. This method has some drawbacks however; a) low recovery, b) some of the antiserum may leach out of the column in the extract and may result in the underestimation of the actual value, c) it is difficult and expensive to perform the assay on large numbers of samples, and d) repeated use of the same column may further reduce the recovery of rBNP from plasma.

We have previously observed that in contrast with the measurement of arginine vasopressin (AVP), rANP values are not affected by the method of blood sampling (IR Sarda, DA Wigle, SC Pang, unpublished data). In the present study it appears that rBNP is also not affected (Table 3). This would indicate that the natriuretic peptide system does not react as quickly to haemorrhage or surgical techniques as AVP.

Using the method described in this paper, we measured levels of rBNP-45 plasma from normotensive WKY and SD rats, and from hypertensive SHR. The levels of rBNP were significantly higher in SHR plasma in comparison to normotensive rats. Interestingly, the levels in SD rats were lower than that present in WKY rats. This indicates that values may differ depending on the strain of animal used.

In summary, we have developed an RIA for the measurement of rBNP-45 in rat plasma. This assay uses radiolabelled rBNP-45 and rBNP-45 as the standard. We have also demonstrated that the method of blood sampling does

not affect the plasma values of rBNP-45. The application of this assay demonstrates higher circulating plasma levels of rBNP-45 in SHR rats.

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