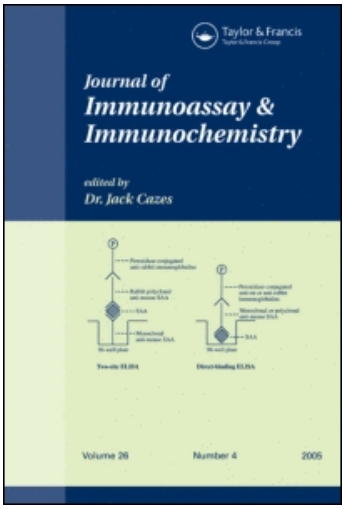

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N. Wilson[a]; J. R. Ledsome[a]; R. Keeler[a]; A. J. Rankin[a]; J. P. Wade[ab]; C. A. Courneya[a]
[a] Department of Physiology, University of British Columbia, Vancouver, B.C. [b] Department of Medicine, Faculty of Medicine, University of British Columbia, Vancouver, B.C.

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HETEROLOGOUS RADIOIMMUNOASSAY OF ATRIAL NATRIURETIC POLYPEPTIDE IN DOG AND RABBIT PLASMA

N. Wilson, J.R. Ledsome, R. Keeler,
A.J. Rankin, J.P. Wade* and C.A. Courneya
Department of Physiology and *Department of Medicine,
Faculty of Medicine,
University of British Columbia
2075 Wesbrook Mall
Vancouver, B.C.
V6T 1W5

ABSTRACT

Atrial natriuretic peptide (ANP) was measured in plasma of dogs and rabbits by radioimmunoassay (RIA) using a commercially available anti α-ANP serum and compared to our measurements of ANP in rats and humans. Plasma concentration of ANP in dog coronary sinus (234.9 ● 41.0 pg/ml) was significantly greater than in systemic arterial blood (81.2 ± 8.4 pg/ml). Gel filtration of dog coronary sinus plasma resulted in an ANP peak with the elution volume (V_e) of synthetic atriopeptin III (AIII) and a minor peak eluting with the void volume (V_o). Rabbit systemic arterial plasma ANP was 53.3 ● 4.3 pg/ml and yielded one peak, with a V_e of AIII. Ion exchange chromatography of dog and rabbit atrial extracts (AE) resulted in a major ANP region which resembled AIII. Gel filtration of AE showed larger molecular species as well as AIII. Dilutions of dog and rabbit plasma and AE were parallel with the AIII standard in radioimmunoassay.

(Key Words: Atrial natriuretic, radioimmunoassay, dog, rabbit)

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INTRODUCTION

Extracts of mammalian atria contain polypeptides which have been shown to induce natriuresis (1), smooth muscle relaxation, including vasorelaxation (2,3,4), and to inhibit aldosterone (5,6) and renin (7) secretion. The subject has been reviewed (8,9).

Biological assays for atrial natriuretic peptide (ANP) include natriuresis in the rat (1), relaxation of the chick intestine and vasorelaxation of rabbit thoracic aorta (3). Radioimmunoassay (RIA) systems for ANP have been reported by four groups of workers up to the present date (10,11,12,13,14, 15). Rat plasma ANP (pANP) levels were measured in three of the above reports. The pANP concentration was reported as 156 fM/ml [423 pg/ml; 156 pM] (10), 54.6 ± 13.9 pg/ml [21.4 pM] (12), and 0.83 ± 0.10 to 1.61 ● 0.14 ng/ml [830-1610 pg/ml; 290-562 pM] (15). Concentrations within square brackets were calculated taking into the account differences in RIA standards used by these authors.

Measurements of circulating ANP in plasma of mammalian species are necessary to establish the physiological role and control of secretion of this hormone. Multiple measurements required for such experiments make RIA systems a practical alternative to biological assays. The structural characteristics of dog and rabbit ANP molecules are at present not known, but immunoreactivity in atria and plasma of both species can be

detected using the commercially available rabbit anti-atrial natriuretic polypeptide serum (Peninsula Laboratories, Belmont, CA, USA). We have adapted this antiserum to measurements of immunoreactive plasma iANP in dog and rabbit, and compared them to our measurements of plasma levels in humans and rats.

MATERIALS AND METHODS

1. Radioimmunoassay (RIA) Standards were prepared from atriopeptin III (rat, no. 8799, lot 006101) and from α-atrial natriuretic polypeptide, human, 28 amino acid (no. 8798, lot 006046). The concentrations were corrected for °/₀ peptide content. A stock solution of peptide (0.5μg/μl) was made in 0.1M HOAc, aliquoted and stored at -20°C. For each RIA a fresh set of standards was prepared, containing 1, 2.5, 5, 10, 25, 50, 100 and 250 pg/100 μl in the RIA buffer (0.1M sodium phosphate pH 7.4; 0.05 M NaCl; 0.1°/₀ BSA; 0.1°/₀ Triton X-100; 0.01°/₀ NaN_3; 1.5°/₀ Trasylol, Miles, 100,000 KIU/ 10ml).

Iodination was performed using the method developed for vasopressin in this laboratory (16). Ten μl 0.05M HOAc, and 20 μl phosphate buffer (0.5M, pH 7.4) were added to 10 μg of lyophylized peptide (same as used for standards). This was followed by addition of 10 μl chloramine T (Eastman Kodak no. 1022, 1 mg/ml in water), and 1 - 1.5mCi of $Na^{125}I$ (Amersham, IMS-30). The reaction was allowed to proceed for 45-60 sec.

One hundred µl of BSA solution (50 mg/200 µl saline; Pentex, Miles Labs Inc., 81-001-2) was then added, followed by 50 mg AGI-X10 (Biorad, no. 9995) in 200 µl water. The mixture was centrifuged for 3 min, and the supernate transferred to an SP Sephadex C-25 column at 4°C as described for purification of rat ANP by Currie et al. (19). Purified iodinated material was stored at 4°C. The specific radioactivity of iodinated ANP (peak tube) was calculated using self-displacement (17).

The RIA incubation mixture consisted of 100 µl of antiserum and 100 µl of diluted (or undiluted) sample at 4°C. In early experiments the antiserum (RAS8798, Peninsula, Inc., CA) was diluted as directed by the manufacturer. After preincubation for 36-48 hrs, 100 µl of ^{125}I-ANP (approx. 5000 dpm) was added, and the incubation continued for 24 hrs. At the end of this period, separation of antibody-bound from free ^{125}I-ANP was achieved using either the second antibody system (goat anti-rabbit IgG serum, GARGG-500, Peninsula Laboratories Inc., lot 006025) and allowing the precipitate to form for 2 hrs at 20°C, or with polyethylene glycol (Carbowax PEG 8000, Fisher Sci., 100 g plus 400 ml H_2O, 600 µl/tube) and γ-globulin (Sigma BG II, 8 mg/ ml, 100 µl/tube). Second antibody precipitation was used for human, dog, and rat samples. Both bound and free fractions were counted (LKB Clinigamma 1272 Quatro), and the results corrected for non-specific binding.

RIA of column eluates of atrial extracts were analyzed in triplicates using the equilibrium RIA system described earlier (18). One hundred µl of diluted column fraction (1:10 in RIA buffer) was mixed with 100 µl antibody and 100 µl I^{125}-ANP (approx 8000 dpm). Non-specific binding was measured in duplicate for each fraction. The standard curve was not always run to allow for measurement of maximal number of samples within one RIA. In these experiments, the results were expressed as percent depression of maximum binding. This method, although non-ideal, allows for frequent sampling of the column eluent.

2. Sample Preparation

Blood was drawn into a chilled syringe, transferred into a chilled test tube containing aprotinin (Trasylol, Miles, 10,000 KIU/ml, 20 µl per 10 ml of blood) or other inhibitors (see Results), and centrifuged at 4°C for 3 min at x 3,000g.

Blood samples were obtained from the femoral arteries and/or coronary sinuses of 13 mongrel dogs (10 males and 3 females, weighing 16.2 ± 0.9 kg) following the administration of morphine, 0.5 mg/kg, s.c. and α-chloralose, i.v. (10 ml/kg, 1g/100 ml saline). Dog blood for stability studies (150 ml) was obtained from 5 dogs (14.5 ± 0.9 kg, 3 males and 2 females). Rat blood was obtained from carotid arteries of rats anesthetized with Inactin (100 mg/kg, i.p.). Rabbit blood was

drawn from femoral arteries of 18 male New Zealand white rabbits, following anesthesia with urethane (1 g/kg) and α-chloralose (100 mg/kg). Normal human blood samples were obtained by venipucture at 10 a.m. from seated healthy human volunteers of either sex, aged 20-52 years. Samples from patients with congestive heart failure were obtained after informed consent at the Coronary Care Unit, Vancouver General Hospital.

Extracts of atrial (AE) and control tissues were prepared in cold 1.0M HOAc by disruption in a tissue homogenizer (2 x 15", Polytron, Luzern). The extracts were centrifuged at 4°C, 30 min, x 48,200 g, divided into aliquots and frozen.

3. Column Chromatography

(a) Ion-exchange. SP-Sephadex C-25 (20 x 220 mm) was eluted at 4°C with a gradient from 25 mM to 1.0 M NH_4OAc, both in 0.5 M HOAc (19), following an initial wash with the starting buffer to elute the unadsorbed material. Each chamber contained 250 ml buffer.

(b) Gel filtration. Sephadex G-15 (25 x 150 mm, 20°, 0.2 M HOAc, 1.5 ml/min) was used for desalting of crude atrial extracts. Sephadex G-100 (40-120 μ, 9 x 686 mm, 4°C, 1 M HOAc, 6.9 ml/hr) was used for partial purification of desalted atrial extracts. For chromatography of plasma, Sephadex G-100 was equilibrated and eluted with the radioimmunoassay buffer (0.1M

sodium phosphate pH 7.4; 0.05M NaCl; 0.1°/₀ BSA; 0.1°/₀ Triton X-100; 0.01°/₀ NaN_3; 1.5°/₀ Trasylol). Plasma samples with high ANP values were used for gel filtration.

RESULTS

Two times greater dilution of the antiserum than that recommended by the manufacturer was found to be more economical, and resulted in a more sensitive assay. The 80°/₀ and 50°/₀ binding (where 100°/₀ is the maximum binding in the absence of standard) of ^{125}I-ANP was 4.6 ± 0.8 and 13.3 ± 2.0 (n=3) and 2.6 ± 0.6 and 7.0 ● 0.5 (n=32) pg/tube for the two dilutions respectively (second antibody separation, both groups). The values for PEG separation (increased dilution) were 2.0 ± 0.1 and 6.8 ● 0.2 (n=18) pg/tube. The intra- and interassay errors (CV°/₀) were 7.5 ● 0.8 (n=32) and 7.9 ± 1.2 (n=24) respectively for the second antibody separation, and 7.9 ± 1.1 (n=19) and 7.6 ± 1.1 (n=8) for PEG. Interassay errors were calculated only on assays set up on the same day, because of the unstable nature of iANP (vide infra). Recovery of 5 pg AIII/tube in seven separate experiments was 5 ± 0.2 pg.

Elution of iodinated atriopeptin III (AIII) and human ANP from SP-Sephadex C-25 is shown in Fig. 1. Elution of 125-I-AIII occurs in the region corresponding to that described for non-iodinated atriopeptin in the same ion-exchange system

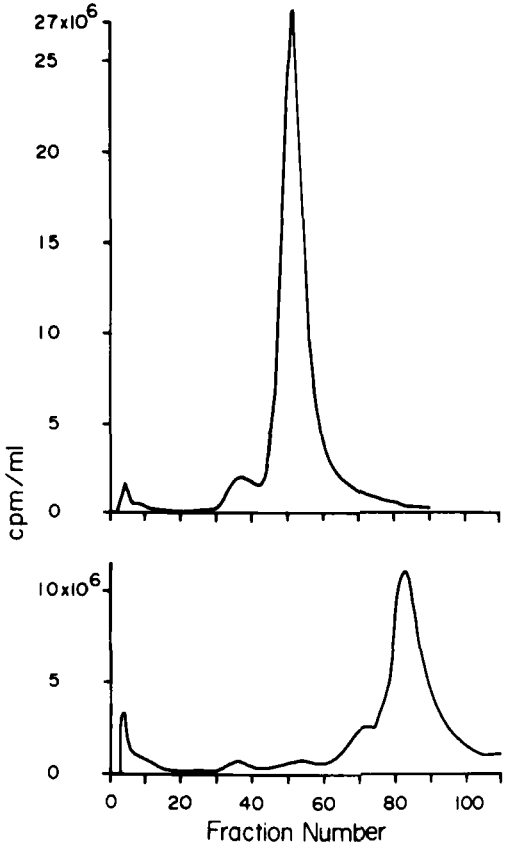

Fig. 1. Elution of iodinated rat AIII (above) and human (below) ANP from SP-Sephadex C-25 (20 x 220 mm), 4°C, 25 mM to 1.0 M NH_4OAc gradient in 0.5 M HOAc. Both chambers 250 ml each. Fractions: 4 ml.

(19). Our experiments suggest that the elution of 125-I-AIII is retarded in comparison to AIII. Specific radioactivity (s.r.) measurements performed on 7 individual fractions across the peak showed that s.r. increased along the ascending limb (from 93 to 910 μCi/μg in the fraction), reached a maximum

(1300 μCi/mg) and thereafter decreased gradually on the descending portion of the limb. We believe that the purification system was capable of separating iodinated from non-iodinated AIII molecules. The fraction with the highest number of counts was used for RIA. Human 125-I-ANP eluted at a higher salt concentration as would be expected for a molecule with an additional positively charged tetrapeptide sequence. Iodination of human ANP yielded preparations with s.r. in the 200-300 μCi/μg range in the tube with the maximum number of counts.

The comparison of human ANP with AIII within the same assay, with iodinated AIII as the tracer (not shown) yielded identical curves. In each case the concentration was corrected for °/₀ peptide: 86°/₀ for rat AIII (Peninsula Labs. No. 8799, Lot 006101) and 66°/₀ for human α-ANP (Peninsula Labs No. 8798, lot 006046). Dilutions of dog and rabbit plasma and of atrial extracts, are shown in Figs. 2 and 3 respectively. Dilutions of atrial extracts and plasma of both species were parallel to rat AIII standards.

Table 1 depicts levels of iANP in tissue extracts of dog and rabbit. In both dog and rabbit the highest concentration of iANP was found in the atria, exceeding the immunoreactivity in other tissues 100-1000 fold. Table 2 shows the comparison of plasma iANP levels in several species. The basal levels of iANP in the four species studied were found to be in the 50-100

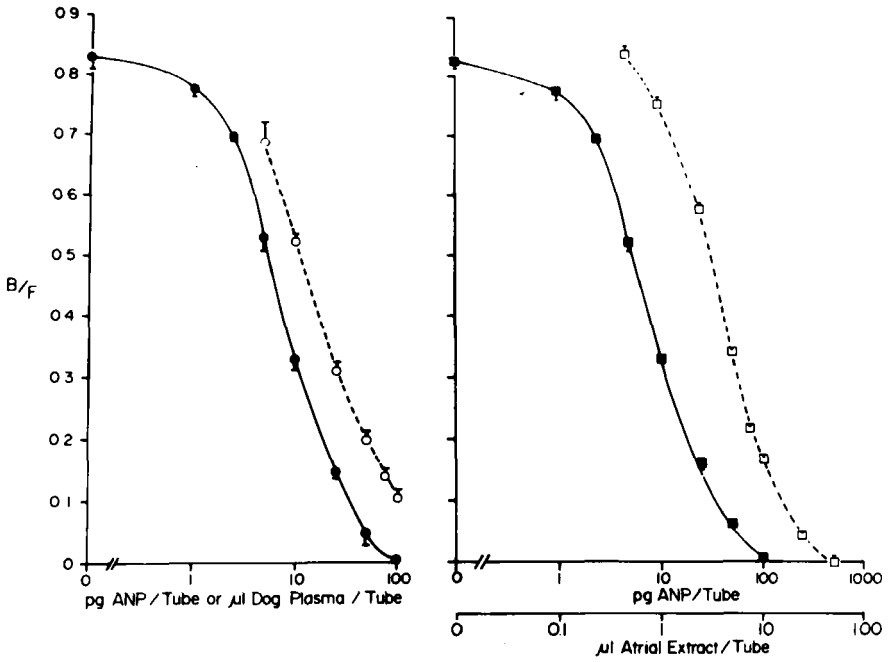

Fig. 2. Dilutions of dog plasma (L) and of dog atrial extract (R) (broken lines) compared to rat AIII standard (solid lines). Dog plasma obtained from the coronary sinus.

pg/ml [20-40 pM] range. Elevated levels were seen in the dog coronary sinus and in patients with congestive heart failure (CHF).

Table 3 shows the stability, or the lack of it, of iANP in dog plasma stored at -20°C, in comparison to the samples measured on the day of collection. Each plasma pool was measured 6 times in triplicate, using a different pipette tip for each of the 6 triplicate determinations. Of the four dogs studied, three showed marked loss of iANP following one week of storage

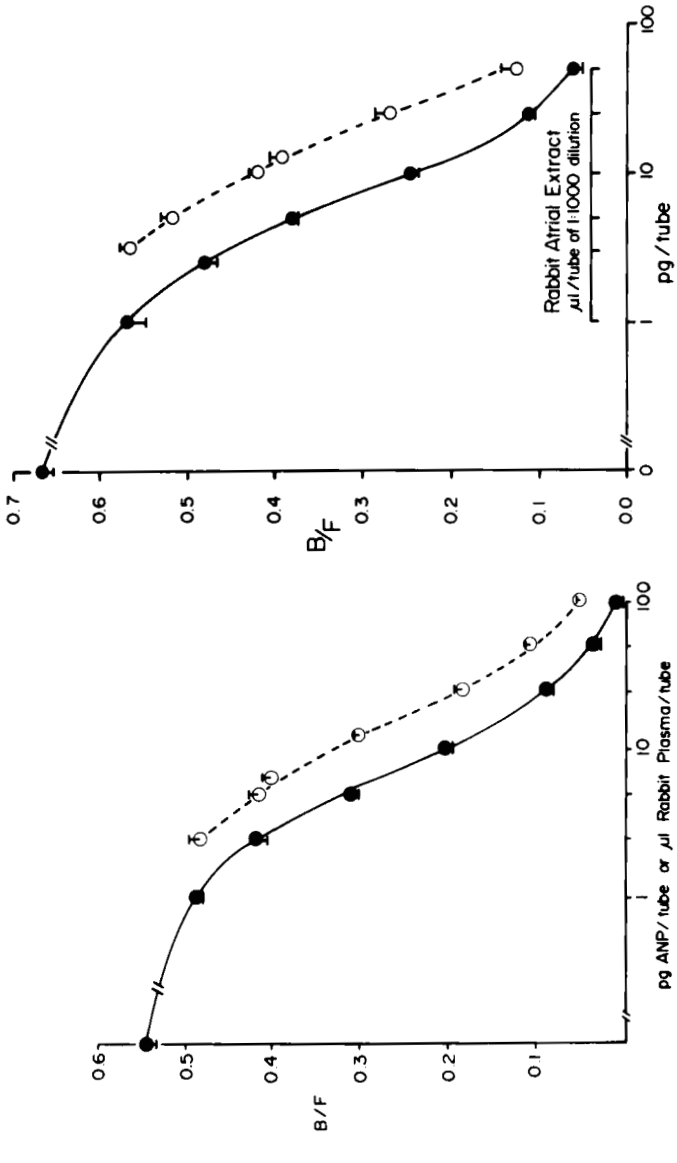

Fig. 3. Dilutions of rabbit plasma (L) and of rabbit atrial extract (R) (broken lines) compared to rat AIII standard (solid lines).

TABLE 1

Immunoreactive ANP (iANP) in Dog and Rabbit Tissues

	iANP ng/g*
DOG:	
kidney	8 ± 0.5
liver	8 ± 0.7
muscle (hind limb)	2 ± 0.1
lung, left	34 ± 1
atrium, right	1926 ± 23
atrium, left	4780 ± 34
ventricle	15 ± 1
RABBIT:	
kidney	9 ± 0.6
liver	15 ± 1
muscle (hind limb)	20 ± 1
lung, left	N.D.
atria (L and R)	9860 ± 51
ventricle, left	N.D.

* Mean ± SE. ND - not detectable.

TABLE 2

Comparison of Plasma iANP in Different Species

SPECIES	N	iANP ** pg/ml plasma
rat	13	105 ± 2
human (normal)	13	70 ± 5
human (CHF)	20	317 ± 32
rabbit	18	53 ± 4
dog	13	77 ± 4
dog: femoral a.	5*	81 ± 8
dog: coronary s.	5*	235 ± 41

* 32 pairs of simultaneous femoral artery and coronary sinus samples obtained from 5 dogs. ** M ± SE. CHF - Congestive Heart Failure

RIA was performed on the day of sample collection for rat and human normal samples; after 24 hr. at -20°C for dog samples; after < one week at -20°C for rabbit and human CHF samples.

at -20°C. This loss of immunoreactivity cannot be ascribed to repeated freezing and thawing, because plasma was stored in 1 ml aliquots, and each aliquot thawed only once. Also, the storage freezer was of a manual defrost type. Since aprotinin used in these experiments did not prevent the loss of iANP in storage, other preservatives were compared using plasma from

TABLE 3

Dog Plasma iANP* During Storage at -20°C (Trasylol**)

Storage time	Dog 8 0	Dog 9 0	Dog 10 0	Dog 12 0
Day 0	46 ± 1	67 ± 2	78 ± 3	--
Day 1	51 ± 2	60 ± 1	75 ± 2	71 ± 2
Day 7 - 12	46 ± 2	51 ± 2	48 ± 2	53 ± 1
Day 33 - 48	51 ± 2	45 ± 2	45 ± 1	64 ± 1
Month 7	45 ± 1	42 ± 1	43 ± 1	56 ± 1

* M ● SE n=6 in each group. ** Trasylol (Miles; 100,000 KIU in 10 ml; 20 µl/10 ml blood.

one dog (Table 4). None of the agents prevented the loss of iANP, although least degradation occurred in presence of EDTA.

Elution of immunoreactive material in dog and rabbit atrial extracts from SP-Sephadex is shown in Fig. 4. In each experiment the extract was co-chromatographed with ^{125}I-ANP tracer, since a new column of ion-exchanger was prepared for each run. Elution of the major peak of immunoreactivity from both dog and rabbit atrial extracts preceeded the elution maxi-

TABLE 4

Dog No. 13(0)

Dog Plasma iANP (pg/ml*) Storage at -20°C with Inhibitors

Storage Time	Capto-pril	EDTA	Soybean t.i.	Hen egg t.i.	Baci-tracin	None
0	155 ± 2	167 ± 7	144 ± 5	145 ± 3	163 ± 4	135 ± 4
1 month	85 ± 4	103 ± 2	83 ± 2	75 ± 2	91 ± 1	64 ± 5
5 months	37 ± 1	81 ± 4	77 ± 3	66 ± 2	77 ± 3	--

* M ± SE n=6 in each group.

Captopril (E.R. Squibb, Sq 14,225, 70 mg/10 ml 0.04 M HOAc) 10 µl/5ml plasma; EDTA (F. Smith) 0.5 mg/ml plasma; Soybean trypsin inhibitor (Worthington, 1 mg 1.57 mg trypsin, 5 mg/ml H_2O) 10 µl/ml plasma; Hen egg trypsin inhibitor (Boehringer 1089313, March 1, 1981, 5 mg/ml H_2O) 10 µl/ml plasma; Bacitracin (Upjohn DIN 030708 05-0233-01, 100 mg/10 ml H_2O) 10 µl/ml plasma.

mum of ^{125}I-AIII. Comparison of Sephadex G-100 elution profile of Atriopeptin III with those of dog and rabbit atrial extracts is shown in Fig. 5. Gel filtration resulted in the elution of multiple peaks of iANP, especially from the dog AE. A smaller immunoreactive region in each extract was found to correspond to V_e of AIII. Elution of AIII (Peninsula Labs, No. 8799, lot 006101) resulted in twin peaks when 1 M HOAc was

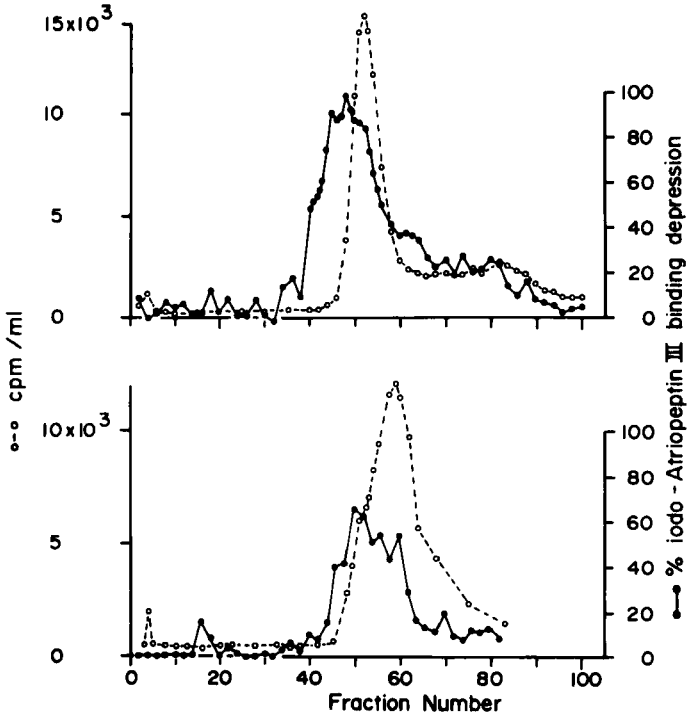

Fig. 4. Elution of atrial extracts (solid lines) of dog (upper) and rabbit (lower) co-chromatographed with ^{125}I-AIII on SP-Sephadex C-25, as Fig. 1.

the eluent. When the RIA buffer was the eluent (Fig. 6) AIII was eluted as a single peak. Gel filtration of dog plasma (coronary sinus) from the same system (Fig. 6) demonstrated that the major part of immunoreactivity corresponded to V_e of AIII. A small amount of iANP was eluted in V_0 of the column. Gel filtration of rabbit plasma (Fig. 6)(arterial sample) yielded one immunoreactive region with $V_e > V_t$.

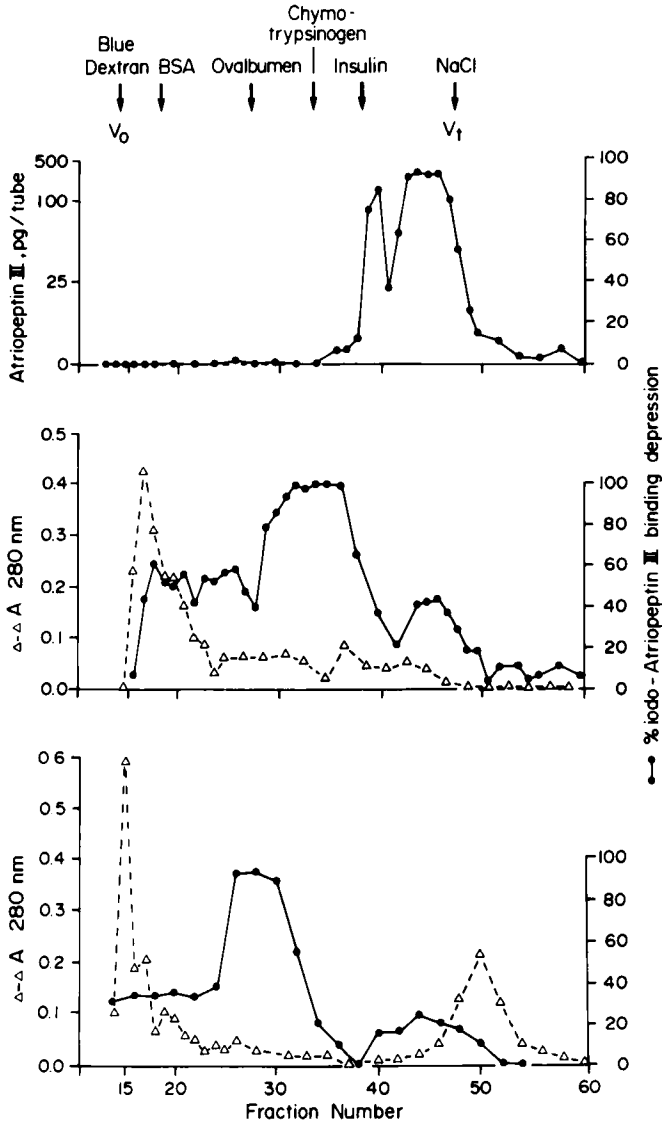

Fig 5. Elution of AIII (upper), dog atrial extract (middle) and rabbit atrial extract (lower) from Sephadex G-100 (40-120 μ), 9 x 686 mm, 4°C, 1 M HOAc, 6.9 ml/hr. Fractions: 1 ml. Solid line: iANP. Broken line: A_{280nm}.

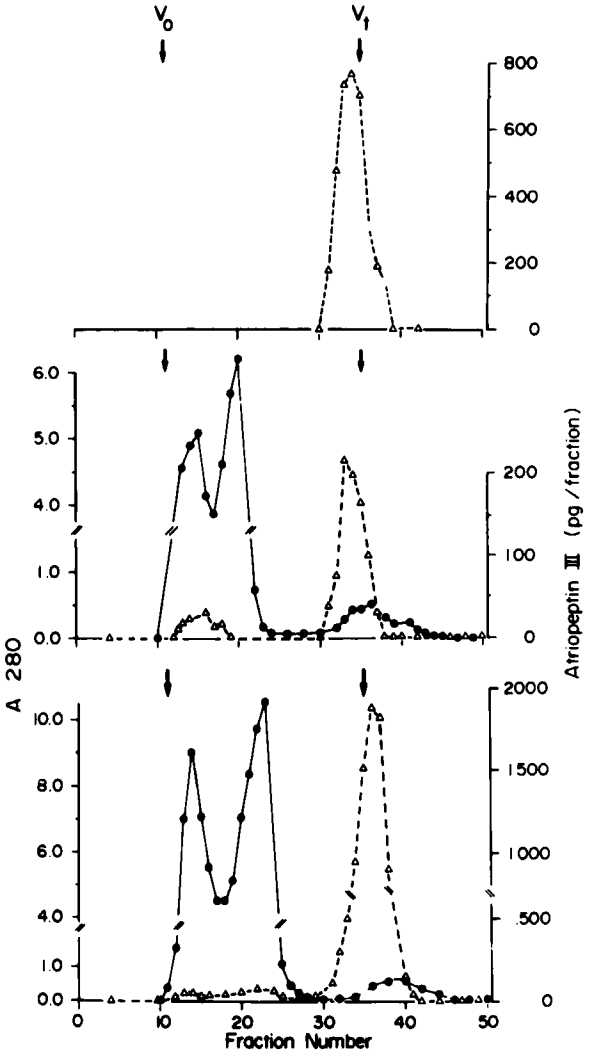

Fig. 6. Elution of AIII (upper), dog coronary plasma (middle) and rabbit plasma (lower) from Sephadex G-100 (40-120μ), 9x680 mm, 4°C, in RIA buffer, 7.0 ml/hr. Fractions: 1 ml. Solid line: A_{280nm}. Broken line: iANP.

DISCUSSION

The sensitivity of the RIA system used in this study exceeds the values reported in the literature (10,11,12,14) and that claimed by the manufacturer of the antibody (21). Higher specific activity consistently achieved in our preparations (800-1000 μCi/μg) as compared to those reported earlier (10,11,12,14) may be due to the separation of iodinated from non-iodinated AIII in the purification system used. On the basis of these experiments we cannot predict whether our 125-I-AIII preparations were mono- or di-iodinated. The iodination of ANP was performed using the same method which we use for iodination of vasopressin (16). This method, by avoiding the addition of a reducing agent following oxidation with chloramine T, favours the preservation of the disulfide bridge in the molecule. The peak radioactivity fraction stored at 4°C was used for preparation of dilutions of ^{125}I-ANP for addition to the RIA. Iodinated AIII was stable for 6 weeks under these conditions.

The same level of specific activity could not be reached when the human peptide was iodinated using the same iodination procedure as for AIII, or using the lactoperoxidase method (20). Human ANP contains a methionyl residue within the disulfide ring, which may be oxidized during the iodination procedure. However, the antiserum is directed toward the C-terminus of the molecule (22). The human ANP (α-atrial natriuretic poly-

peptide, Peninsula Labs, lot 006046) used for iodination contained 66% peptide. It is possible that the remaining material (34%) interfered with optimal iodination. It is also possible that separation of iodinated from non-iodinated human ANP in the system used was not as efficient as it was for AIII.

The concentrations of iANP found in the atrial extracts (Table 1) are difficult to compare with the other literature reports, as different antisera, standards, and extraction methods were used. Concentrations of iANP in dog and rabbit atria (Table 1) were lower than those reported for the rat (10,11,13,14).

Concentrations of iANP in dog, rabbit and human plasma were not available in the literature for comparison with our study at (Table 2).

We sampled dog coronary sinus blood in the course of atrial distension experiments (23). If the heart was indeed the source of iANP, the vascular drainage of this organ should reflect that fact. Higher coronary iANP concentrations were found in 30 out of 32 sample pairs (Table 2). High levels of iANP were also seen in congestive heart failure, a disease state characterized by salt and water retention and increased atrial volume and pressure.

Parallelism between the dilutions of dog and rabbit plasma, atrial extracts, and AIII standards suggests, but does

not conclusively prove, a similiar molecular structure iANP of in those species and the rat. Comparison of the elution profiles of rabbit and dog atrial extracts from the SP-Sephadex C-25 column with those of the iodinated rat and human ANP suggests that both rabbit and dog iANP resemble the rat more than the human molecule in their charge (Fig. 1 and Fig. 4). Elution profiles of AE from Sephadex G-100 show that the major immunoreactive components in dog and rabbit AE corresponded in their elution volumes to chymotrypsinogen (m.wt. 25,000) and ovalbumen (m.wt. 47,000) respectively (Fig. 5). A smaller component in atrial extracts of both species was found to correspond to the elution volume of AIII. This suggests that the extracts contained the prohormone form(s) (9), and that these forms shared immunoreactive sites with AIII.

Elution of dog plasma from Sephadex G-100 showed that most of the immunoreactivity was eluted with the same elution volume (V_e) as that of AIII. An additional smaller peak eluted in void volume (V_o), and may represent the hormone precursor. Previous studies indicated that the prohormone is not released from the heart into circulation (9). However, coronary sinus plasma from the dog was used in our experiments. It is possible that small amounts of ANP precursor was present in the direct drainage of the heart. Gel filtration of rabbit plasma, on the other hand, showed only one iANP peak. The rabbit plasma for this experiment was from the femoral artery. The

elution of rabbit plasma iANP was retarded in comparison to AIII, suggesting that the predominant circulating form of iANP in rabbit is either of smaller molecular weight, or contains more aromatic residues than AIII.

The lack of stability in some, but not all, individual dog plasma samples (Table 3), introduces a source of error when samples from different experimental animals are stored for different intervals of time, and then compared in the same radioimmunoassay. In addition, the possibility exists that the degradation of iANP in plasma may continue during the RIA incubation period itself, affecting not only the material being measured, but also the iodinated tracer. This error was minimized in the present study in that all dog plasma samples were measured on the day following the collection of blood, and all rabbit samples within one week of collection. Storage at -70°C (Dog 8 and 9) for up to one month did not differ from -20°C storage in terms of iANP concentrations.

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Reprint requests to Nadine Wilson, Department of Physiology, Faculty of Medicine, University of B.C., Vancouver, B.C., Canada, V6T 1W5.

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