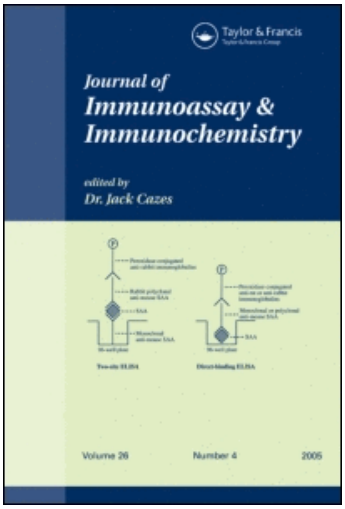

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A NEW RADIOIMMUNOASSAY FOR HUMAN ALPHA ATRIAL NATRIURETIC PEPTIDE AND ITS PHYSIOLOGICAL VALIDATION

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ABSTRACT

A new sensitive, specific radioimmunoassay for human alpha atrial natriuretic peptide (hANP) and a novel extraction method for hANP have been developed. Antiserum to hANP (Keq = 1.923 x 10^{11} l/mol) showed no cross-reactivity with related analogues. Displacement of 50% bound ^{125}I-hANP occurred at 4.7 ± 0.1 fmol/tube (n=15). The limit of detection of plasma hANP after extraction of 2ml plasma with Florisil was 1.2 pmol hANP/litre plasma. The recovery of synthetic hANP from plasma over the range 6.5-162.5 pmol/l was 71.2 ± 1.9%. Inter- and intra-assay coefficients of variation were 13.1% and 10.1% respectively. Extracted plasma was stored at -80°C without loss in immunoreactivity. The radioimmunoassay was physiologically validated in man by measuring plasma hANP following central volume expansion -
(a) plasma hANP rose from 2.5 ±0.5 to 4.1 ±0.6 pmol/l in 9 normal volunteers after postural change from seated to lying
(b) infusion of normal saline (2 litre/2 hour) increased hANP from 1.6 to 4.3 pmol/l (n=2).
Infusion of hANP (2pmol/kg/min) increased plasma hANP to 19.9 ±3.4 pmol/l (n=6).

INTRODUCTION

In 1981, de Bold [1] demonstrated that the bolus injection of an extract of cardiac atrial tissue induced potent natriuretic and diuretic responses in rats.

Three peptides with natriuretic activity have been isolated from human atrial extracts [2], the most potent of which is alpha human atrial natriuretic peptide (hANP), a 28 amino acid residue polypeptide. These peptides are secreted from the myocardium into the systemic circulation in response to increased atrial pressure and have a wide range of haemodynamic, renal and endocrine actions which contribute to the regulation of circulatory volume [3,4].

In this paper, we report a new method for the measurement of hANP in human plasma, and demonstrate that the assay is sensitive enough to detect physiological changes in hANP.

MATERIALS AND METHODS

^{125}I-hANP was purchased from Amersham, Bucks. and had specific activity of 66Bq/mmol; synthetic hANP (99-126) for human studies was purchased from Shire Pharmaceuticals Ltd, Andover, Hants., and synthetic hANP for radioimmunassay use and the ANP analogues from Peninsula Laboratories. Trasylol was purchased from Bayer UK,Newbury,Berks., Florisil from Sigma, Poole, Dorset and second antibody (goat anti-rabbit IgG) was donated by Dr J Bradwell (The Binding Site Ltd.,Birmingham University). All other reagents were AnalaR grade. Assay buffer was made up as follows: 50mM disodium hydrogen phosphate (Na_2HPO_4) 7.098g, 10mM disodium ethylenediametetraacetic acid (Na_2 EDTA) 3.722g, 0.2% (w/v) bovine serum albumin (BSA) 2.0g, 0.1% (v/v) 1ml Triton X-100 made up to 1 litre with distilled water, pH 7.4 and stored at 4°C. Second antibody separation buffer was made up from 0.1M Na_2EDTA 18.6g, 2.5ml 0.5% (v/v) normal rabbit serum (NRS) in 500ml of assay buffer,

pH7.4 and stored at 4°C. Column eluant was made up as 40mM glacial acetic acid (2.5ml), 3mM sodium azide (NaN_3) 0.2g, 0.125% (w/v) BSA 1.25g in 1 litre of distilled water, pH7.4 and stored at 4°C. The column used for chromatography was a C-16/40 column from Pharmacia, Uppsala, Sweden.

Antiserum production

Antisera to hANP was raised in rabbits using a similar method to that of Skowksy and Fisher [5] i.e., hANP conjugated to bovine thyroglobulin by the carbodiimide reaction. hANP 1mg was dissolved in 300µl distilled water (d/w) to which 10mg bovine thyroglobulin was added. Carbodiimide (CDI) 10mg was added to 250µl d/w, and 50µl of the CDI solution was added to the original solution. After incubation for 24h at 4°C, a further 50µl of CDI was added to the hANP and bovine thyroglobulin mixture, and incubated for 3h at room temperature. The total volume was made up to 2ml, and 0.5ml aliquots (250µg ANP) were injected with Freunds' incomplete adjuvant into New Zealand White rabbits by the multisite intradermal injection technique at 2-4 week intervals for 14 months. Cross-reactivity of the antiserum was investigated using fragments of hANP (1-11, 7-28, 13-28) and the hormones, aldosterone, oxytocin, arginine vasopressin, angiotensin I and II, and adrenocorticotrophin. Standard curves were set up containing serial dilutions of these peptides over a wide range of concentrations. Cross-reactivity was calculated as the ratio of the peptide under study required to produce 50% inhibition of binding of ^{125}I-hANP to antiserum, to the amount of hANP standard required to produce the same inhibition in binding, expressed in molar terms.

Radioimmunoassay for hANP

In order to determine the antiserum titre required for maximum assay sensitivity and precision, aliquots of antisera were serially diluted from 1:1,000-

1:100,000 and binding tubes containing 100µl of each dilution were incubated with 100µl of ^{125}I-hANP and 200µl buffer. At dilution 1:100,000, 30% binding of ^{125}I-hANP occurred and this dilution was used for further studies.

To determine the incubation time for the reaction between first antibody and ligand, binding tubes were set up and incubated for varying periods of time before the reaction was stopped by adding second antibody. Binding of ^{125}I-hANP with antiserum plateaued after 32h incubation with a specific binding of 29% (non-specific binding 2.1%), and this incubation time was used in further studies. To determine the optimum time of incubation for second antibody, tubes incubated for 32h with first antibody were incubated with second antibody at differing concentrations in the presence of varying amounts of normal rabbit serum, at 4°C for varying time intervals before being separated by centrifugation and aspiration. Equilibrium was reached at 24h.

To assess whether sensitivity would increase if disequilibrium conditions were employed, preincubation studies were performed; antibody and standards were incubated at 4°C for 24h before ^{125}I-hANP was added for varying time intervals.

After addition of second antibody, antibody bound tracer was separated from the free, and standard curves plotted. Pre-incubation resulted in low zero-binding (<20%) and no curve showed a significant increase in sensitivity at 50% displacement of tracer; the assay was therefore carried out under equilibrium conditions.

Standard curves were set up with polystyrene binding tubes containing 200µl hANP standard in concentrations from 0.1-100 fmol/tube and incubated with 100µl of antiserum to hANP (final dilution 1:400,000) and 100µl of ^{125}I-hANP. Zero (initial) binding tubes contained 200µl buffer, 100µl antiserum and

100µl ^{125}I-hANP, and non-specific-binding tubes contained 300µl buffer and 100µl ^{125}I-hANP. All tubes were assayed in triplicate (total volume 400µl). After incubation at 4°C for 32h, separation of anti-serum bound from free ligand was achieved using goat anti- rabbit-IgG (1:20, 0.5% (v/v) normal rabbit serum) incubated for 24h at 4°C.

Extraction of hANP from plasma

Venous blood (5ml) samples were transferred into chilled tubes containing K_2EDTA (~16µmol) and Trasylol (1000KIU) and centrifuged at 4°C for 20 minutes and the plasma separated and stored at -20°C until extraction. For extraction, 2ml of plasma was dispensed into glass tubes and 20 mg of heat-activated Florisil [6] added. Tubes were rotated for 20 min, plasma aspirated and the Florisil washed with distilled water and then 2M HCl. Then, 500 µl of 90% aqueous acetone was added and mixed for 10 min and the eluate transferred to a conical glass tube. The previous step was repeated and the pooled eluates (1ml) blown to dryness under a stream of nitrogen at 37°C in a water bath. The dried extracts were reconstituted with 800 µl of assay buffer and mixed vigorously.

To assess extraction recovery, synthetic peptide hANP was added to fresh plasma over a concentration range 6.5-162.5 pmol/l, extracted and assayed.

Characterisation of immunoreactive hANP

In order to characterise the immunoreactive material in plasma, 22ml of blood was withdrawn from one normal subject at the end of a supine hypertonic saline infusion [7] into chilled K_2 EDTA/Trasylol tubes, and the plasma immediately separated and stored at -20°C until extraction. The extract was resuspended in 1ml of column eluant and chromatographed on a Sephadex G-25

column. Fractions (2ml) were collected, freeze-dried and resuspended in assay buffer for measurement of hANP. The elution profile of synthetic hANP was determined separately.

Validation experiments

All studies were approved by the local Ethical Committee and subjects gave informed consent. All volunteers were on an unrestricted salt diet, and none was taking medication. Volunteers were asked to attend at 0900h having fasted and abstained from alcohol and cigarettes from 2400h the previous evening; on arrival they were seated (studies 1,3) or lay supine (study 2). For study 1, an intravenous cannula was placed in the antecubital fossa for blood sampling and for studies 2 & 3 an intravenous cannula was placed in the antecubital fossa of each forearm for blood sampling or infusion.

Study 1: Response of plasma hANP to change in posture

Nine healthy male subjects were studied after an overnight fast. After sitting for 30 min, subjects changed to the supine position and raised their legs to 30° to the horizontal. Blood samples for hANP were collected at 0 min (seated), and after 15 and 30 min in the supine position.

Study 2: Response of plasma hANP to isotonic volume expansion

Two male subjects were studied after an overnight fast. After lying supine for 30 min, two litres of normal saline were infused over 2 hours and blood samples for haematocrit and hANP collected at 30 min intervals.

Study 3: Infusion of synthetic hANP

Following an overnight fast, healthy male volunteers rested for 30 min in the seated position before a 90 min infusion of hANP at a rate of 2pmol/kg/min was started (n=6). Blood samples were collected for measurement of hANP at 15 minute intervals during the hANP infusion, and for a further 45 minutes after the infusion had stopped.

Statistics

Results are expressed as means ±SEM and the statistical significance of the changes in plasma hANP concentrations estimated using Student's paired t-test. Undetectable hANP samples were given a value of 50% the detection limit (i.e. 0.6 pmol/l) for the purpose of statistical analysis.

Change in blood volume was estimated using the formula $(H_1-H_2) / H_1 \times 100\%$ where H_1 represents the initial haematocrit and H_2 the final haematocrit [8].

RESULTS

Antibody

All rabbits produced antisera to hANP and the antiserum with the highest avidity was selected for use in these studies. Under equilibrium assay conditions, Scatchard analysis gave an equilibrium constant of 1.923×10^{11} l/mol. Cross-reactivity of the antiserum with fragments of hANP and various other hormones is shown in Table 1.

Assay characteristics

The standard curve (n=15) and precision profile are shown in Figure 1; the specific binding was 23.3 ± 1.1%, the non-specific binding was 3 ±0.3% and

TABLE 1

Results of cross-reactivity studies of hANP antiserum with fragments of hANP and related analogues.

HORMONE	RELATIVE MOLAR DISPLACEMENT
hANP fragment 1-28	100%
hANP fragment 1-11	0.0001%
hANP fragment 7-28	1700%
hANP fragment 13-28	5%
Oxytocin	0.0001%
Arginine vasopressin	0.0001%
Angiotensin I	0.0001%
Angiotensin II	0.0001%
Aldosterone	0.0001%
Adrenocorticotrophin	0.0001%

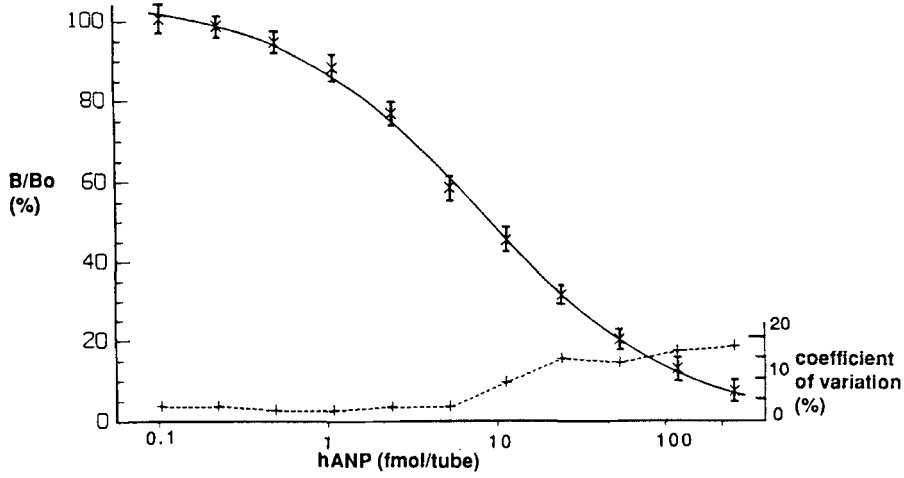

FIGURE 1: Representative standard curve for hANP (n=15) and percentage coefficient of variation for each point (---). Standard curves were set up with polystyrene tubes containing 200µl of hANP standard in concentrations from 0.1-100fmol/tube and incubated with 100µl of antiserum to hANP (final dilution 1:400,000) and 100µl of ^{125}I-hANP. All tubes were assayed in triplicate (final volume 400µl) and after incubation at 4°C for 32h, separation of anti-serum bound from free ligand was achieved using goat anti-rabbit-IgG incubated for 24h at 4°C.

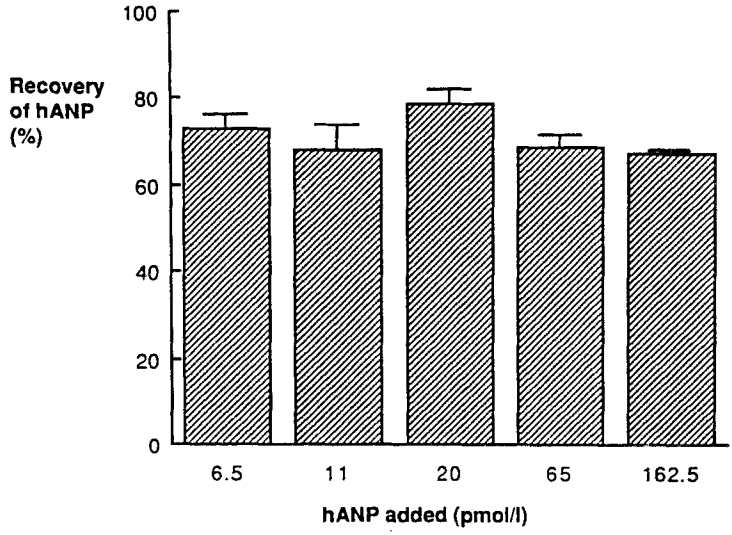

FIGURE 2: Shows the percentage recovery of varying amounts of synthetic hANP added to 2ml aliquots of plasma and recovered using the Florisil method. The linear correlation between hANP added and that recovered was r=0.96 (p<0.001).

displacement of 50% bound ^{125}I-hANP occurred at 4.7 ± 0.1 fmol/tube. The limit of detection of the assay has been defined as depression from initial binding by 2 standard deviations (8.6%) and was 0.6 fmol hANP/tube.

Extraction recoveries

Extraction of assay buffer, and assay buffer/Trasylol/K_2 EDTA did not depress binding in the immunoassay. The recovery of synthetic hANP added to 2ml aliquots plasma over the range 6.5- 162.5 pmol/l is shown in Figure 2 ; recovery was linear (r=0.96, p<0.001) and averaged 71.2 ± 1.9% after Florisil extraction. A total of 39 samples were analysed in a series of 5 separate studies.

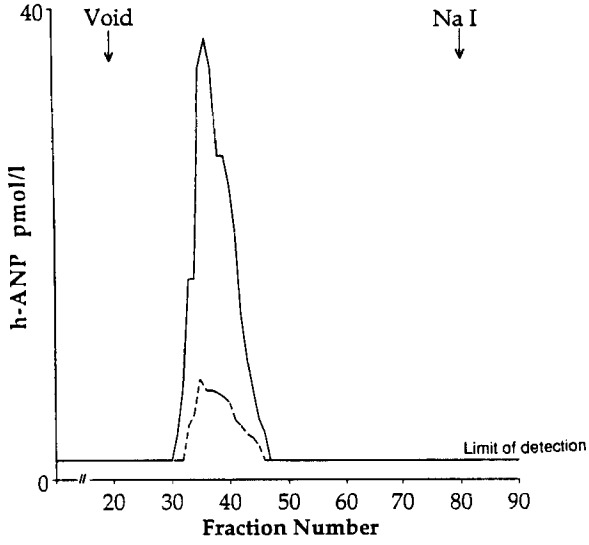

FIGURE 3: Elution profile of synthetic hANP (—) and pooled venous plasma extract (---) from a Sephadex G-25 fine column. hANP was determined in each fraction by radioimmunoassay.

All samples from each study were estimated in a single assay, and the limit of detection was 1.2 pmol hANP/litre plasma. The results of hANP concentrations were not corrected for extraction losses. Serial dilution of extracts ran parallel to the standard curve. The intra-assay coefficient of variation obtained from 10 extractions of one sample was 10.1%. The inter-assay coefficient of variation was 13.1%

Characterisation of immunoreactive hANP

Figure 3 demonstrates the elution profile of extracted ANP and that of synthetic hANP from a G-25 Sephadex column. It can be seen that the peak of

extracted plasma hANP coincides with that of synthetic hANP, consistent with the fact that the immunoreactive material from extracted human venous samples was hANP.

Stability of hANP in plasma and plasma extracts

There was a loss in immunoreactive hANP in plasma samples stored at -20°C or -80°C for more than one week; the loss in immunoreactivity was ~1% per day (n=20) during the first week but increased with time ; after storage of plasma for 6 weeks there was less than 30% recovery of immunoreactive hANP. There was no loss in immunoreactive hANP following storage of the plasma extracts at -80°C for six weeks.

Physiological validation

Study 1: Response of plasma hANP to change in posture

The result of changing posture on plasma ANP levels is shown in Figure 4. Plasma hANP rose from a mean of 2.5 ± 0.5 pmol/l at 0 minutes, to 4.0 ± 0.6 pmol/l at 15 min ($p< 0.001$) and to 4.1 ± 0.6 pmol/l at 30 min in the supine position ($p<0.001$).

Study 2: Response of plasma hANP to isotonic volume expansion

Isotonic volume expansion caused a fall in haematocrit from 42 to 38% in both subjects, indicating an estimated rise in blood volume of 9.5%. Plasma hANP rose from a basal undetectable concentration to 2.5 pmol/l in one subject, and from 2.7 to 6 pmol/l in the other.

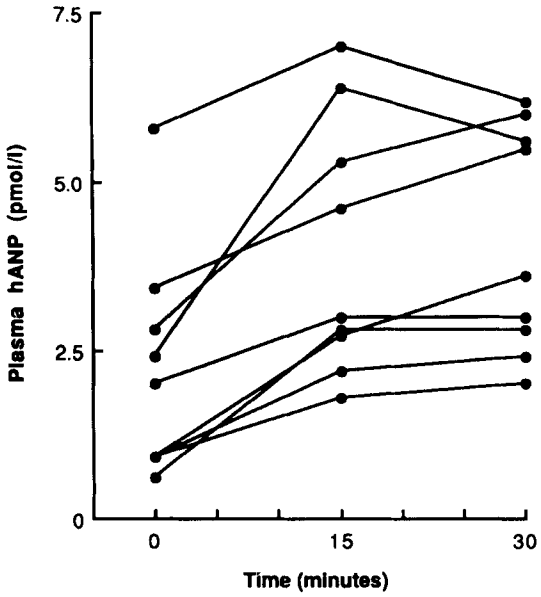

FIGURE 4: Plasma hANP concentrations in 9 subjects undergoing change from seated (0min) to supine (15,30min) position. Plasma hANP rose from 2.5 ±0.5pmol/l at 0 min, to 4.0 ±0.6pmol/l at 15 min and to 4.1 ±0.6pmol/l at 30 min (mean±SEM).

Study 3: Infusion of synthetic hANP

Figure 5 illustrates the change in plasma hANP concentrations which occurred during the infusion of hANP in six male volunteers. Plasma hANP rose from 2.1 ±0.2 to 19.9 ± 3.4 pmol/l by the end of the infusion, and fell rapidly to basal concentrations after the infusion was stopped.

DISCUSSION

We report a new specific RIA for plasma hANP which is also extremely sensitive and allows physiological changes in plasma hANP to be measured.

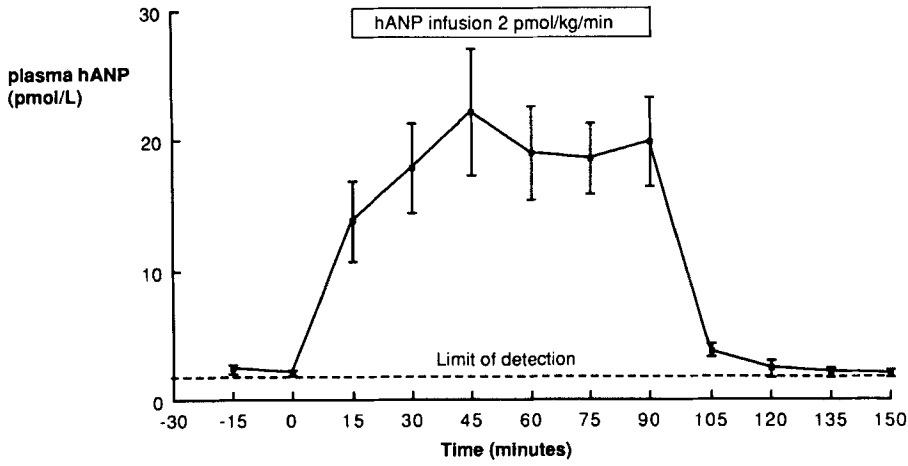

FIGURE 5: Plasma hANP concentrations in extracted venous samples before, during and after the intravenous infusion of synthetic hANP in seated healthy volunteers. Each point represents mean ±SEM.

The antiserum has a high affinity and no cross-reactivity with other peptides important in the control of salt and water balance. The cross-reactivity data regarding hANP fragments suggests that the antiserum recognises a site in the 11-13 region of the hANP molecule, ie. within the ring structure.

The standard curve of the radioimmunoassay is reproducible and the precision profile satisfactory. We have developed a new extraction method for hANP based on the Florisil adsorption technique which gave an average recovery of 71.2% over a wide range of hANP concentrations and is therefore comparable with the other extraction methods in use. Anderson in his excellent review [3] reported that the recovery from the extraction step can vary from 43% [9] to 98% [10], with an average recovery of 70-80%. Similarly the mean basal venous plasma hANP concentrations quoted in normal adults on an unrestricted diet

range from 5-20 pmol/l [10,11,12]; the poor agreement between laboratories in the absolute value of hANP concentrations was again illustrated in an international collaborative study involving 23 centres [13].

Using 2ml of plasma, we found a basal range for hANP in normal male subjects of 1.2-5 pmol/l (n=20), which is in accordance with basal concentrations reported by Anderson [12] and Woolf [14].

During the infusion of synthetic hANP, plasma hANP concentrations rose rapidly to a steady state level and fell promptly to normal levels on stopping the infusion, a finding in agreement with other workers [15].

We found that storage of the plasma samples beyond one week at -20°C led to a loss of immunoreactive hANP, possibly due to breakage of the ring and subsequent altered binding of the antisera. Results on the storage of plasma hANP extracts at -80°C indicate that hANP remains intact under these conditions. There are scant details regarding either the storage of plasma or extracted samples of hANP in the literature. Tikkanen [16] noticed no reduction in hANP levels after storage for one month but did not mention whether the samples were extracted prior to storage; Yandle [11] found no significant difference in hANP levels of extracted samples stored for 16 weeks at -20°C, but only 6 samples were studied. The elution profile of synthetic hANP was similar to that of extracted peripheral venous blood, consistent with the fact that we are measuring an immunoreactive substance in human plasma identical to synthetic hANP.

Lang [17] reported six-fold increases in plasma ANP concentrations in the rat following volume expansion, and it has also been shown that intravenous infusion of saline produces plasma release of hANP in man [11,13,18]. The effect of increasing venous return [19,20] by either changing position to head-

down or by leg-raising to 60° to the horizontal has been shown to stimulate release of hANP in man. The results of our studies on the effect of posture on release of hANP are in agreement with other workers' findings, and taken together with the work of Anderson [21] suggest that the secretion of hANP is regulated physiologically by changes in atrial distension.

In conclusion, we report a novel radioimmunoassay for hANP and a new extraction method for hANP which is safe, simple and suitable for batch-processing. The Florisil method has the added advantage of being considerably cheaper than some extraction methods in use. We also present evidence of physiological validation in man.

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