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A HIGHLY SENSITIVE RADIOIMMUNOASSAY OF ATRIAL NATRIURETIC PEPTIDE (ANP) IN HUMAN PLASMA AND URINE

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A highly sensitive radioimmunoassay has been established for measurement of human plasma and urine concentrations of atrial natriuretic peptide (ANP) and requires no extraction or concentration process such as Sep-Pak C-18 cartridge treatment. An antiserum was prepared from rabbits immunized with α-human ANP (α-hANP) coupled with bovine-thyroglobulin. The sensitivity of this method was 0.3 pg/tube of synthetic α-hANP utilized as authentic standard. Recovery of α-hANP spiked to plasma and urine was 97.7 ± 15.4% and 97.1 ± 9.5% (mean ± SD), respectively. Plasma and urinary ANP concentrations versus assay data showed satisfactory linearity. In 124 healthy subjects, the plasma ANP concentration was 31.7 ± 12.0 pg/ml. Two different molecular forms of ANP in plasma and a single form in urine were found by gel permeation chromatography.

In 1981, DeBold et al. (1) reported a rapid and potent natriuretic response to intravenous injections of atrial myocardial extract in rats. The powerful natriuretic and vasorelaxant properties of this substance were soon confirmed by many other workers (2,3) and the complete amino acid sequence of α-hANP has recently been determined (4). Measurement of ANP concentration in plasma has been made by radioimmunoassay (RIA) using plasma extracts (5-8). This extraction was necessary since ANP in plasma is extremely low, usually less than 100 pg/ml and the antiserum sensitivity was not sufficiently high.

In the present study, we tried to establish a highly sensitive method for measuring ANP concentration in plasma and urine, and we have developed an RIA for measuring ANP concentrations in unextracted plasma and urine, using a highly sensitive anti-ANP serum under incubation conditions such as would allow maximal sensitivity.

MATERIALS AND METHODS

Immunizations A conjugate of α-hANP(1-28)(Peptide Institute, Inc.) with bovine-thyroglobulin (Sigma Chemical Co.), molar ratio of 60:1, was prepared using carbodiimide as the coupling agent (9). Outbred Japanese White rabbits, weighing 1.7 Kg, were immunized with the conjugate suspension in 0.9% NaCl

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mixed with an equal volume of Freund's complete adjuvant (Difco Lab.) by intramuscular injections. A total dose of 1.4 mg of α-hANP in the conjugate was administered to each animal over a period of 27 weeks at intervals of 5 to 28 days. They were bled 10 days after the last immunization.

Assay buffer The assay buffer was 0.01 M phosphate buffer containing 0.14 M NaCl, 0.01 M K_2EDTA, 0.02 M glycine, 0.01 M ε-aminocaproic acid, 0.001 M sodium azide, and 1.1 mg/ml inactivated human serum albumin, pH 7.4.

ANP standard Synthetic α-hANP(1-28) was used. A stock solution of 1 μg/ml in 0.01 M ammonium acetate buffer (pH 5.0) was fractionated into 1 ml aliquots and stored at -80 °C. The stock solution was diluted with the assay buffer to 2.5 - 313 pg/ml for each assay.

Specimens Five ml blood samples were deposited in ice-chilled tubes containing 6 mg K_2EDTA. The plasma was separated by centrifugation at 4 °C. The plasma and urine were assayed immediately or stored at -80 °C.

Radioimmunoassay procedure One hundred μl of the plasma or urine samples were mixed with 100 μl of the anti-ANP serum (1:80,000 diluted) and 100 μl of the assay buffer. After allowing the mixture to stand for 20 h at 4 °C, 50 μl of [^{125}I]α-hANP (Amersham, specific activity of 74 TBq/mmol, 120 pg/ml) were added followed by further incubation for 24 h at 4 °C. Free and bound fractions of radiolabeled α-hANP were separated by the addition of 500 μl of the assay buffer containing 10 μl of goat anti-rabbit γ-globulin, 1 μl of normal rabbit serum, and 5% polyethylene glycol (mean Mr, 7500)(10). The radioactivity in the precipitate centrifuged for 30 min at 3000 rpm was measured. The ANP amount in the sample was read from a standard curve drawn using the values obtained from the assays of α-hANP standard by the same procedure as above.

Gel permeation chromatography A sample (1 to 2 ml) was charged onto a Sephadex G-75 column (Pharmacia, fine, 2×45 cm) and eluted with 0.5 M acetic acid at flow rate of 5 ml/h at 4 °C. Two ml of each aliquot collected were assayed following evaporation of the acetic acid. Recovery was 85% when using [^{125}I]α-hANP.

RESULTS

Sensitivity and recovery analyses As shown in Fig. 1, the ID_{50} of cold α-hANP was 3.6 ± 0.3 pg, but 30 pg without our preincubation (not shown). This indicates that the sensitivity of non-equilibrium method considerably exceeds the equilibrium method when our antiserum was used. Table 1 shows the lowest level of ANP practically detected by our method to be about 0.3 pg, since 0.25 pg of α-hANP can be detected at 99% confidence. The coefficient of variance (CV) in within-assay, i.e., using the same reagents, for 10 replicates of a sample containing low (17.2 pg/ml), medium (31.7 pg/ml), or high (93.0 pg/ml) concentration of ANP was 7.8, 7.0, or 4.7%, respectively. In the between-assays, i.e., between the assays using separately prepared reagents, the values were 6.1, 6.2, and 6.2%, respectively, from 10 consecutive assays. When 19.5 or 39 pg of α-hANP were added to one ml of plasma containing 16 to 61 pg of endogenous ANP, 94.7 ± 7.5% or 96.7 ± 7.4% (mean ± SD), respectively, of the input α-hANP was recovered by 8 assays. For 8 urine samples (endogenous ANP, 10 to 50 pg/ml), the mean recoveries were 100.6 ± 11.3% and 99.7 ± 7.4%, respectively, for 9.8 and 19.5 pg of α-hANP. Without the antiserum, the binding ratio of [^{125}I]α-hANP was 2.7 ± 0.4% in plasma, 2.6 ± 0.3% in urine, and 3.6 ± 0.4% in

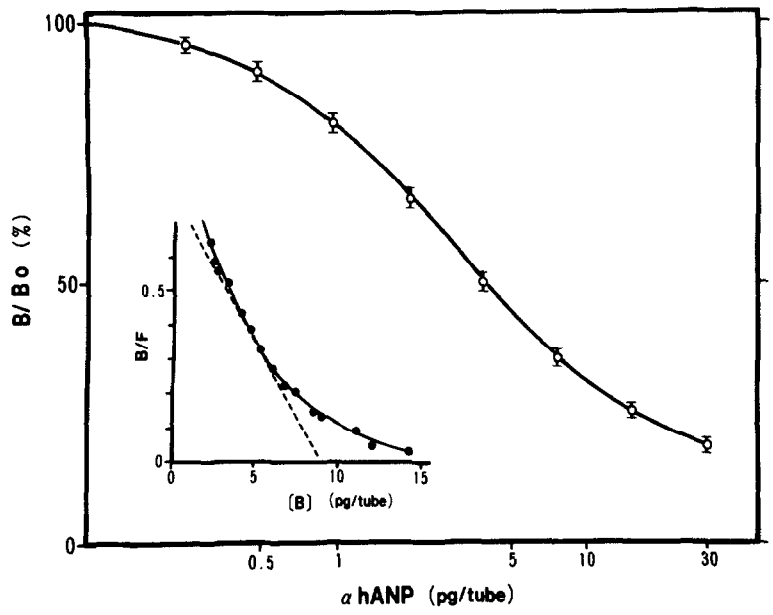

Fig. 1. Standard curve for radioimmunoassay of ANP. Mean (o) ± SD (vertical line) values of 10 assays are shown. (Inset) Scatchard plots in which the ratios of bound to free [^{125}I]α-hANP are plotted against the concentration of bound α-hANP.

the assay buffer, indicating interference by substances in plasma and urine not to be significant in this method. A linear correlation was observed on plotting the values of serially diluted samples versus the diluted ratios (not shown). By replacement experiments, the immunological cross-reactivity of the antiserum with α-rat ANP(1-28) and decomposed α-hANP(7-28) was 100%, while with ^{5}Val-angiotensin II (Peptide Institute, Inc.), ^{8}Arg-vasopressin (Sigma Chemical Co.), and bovine-thyroglobulin, it was less than 0.01% in molar ratio of these substances. Aprotinin (400 kallikrein inactivation units/ml) in the plasma did not serve to enhance chemical stability. Assayed values were not effected by storage of the plasma for 4 weeks at -40 °C nor by several cycled freeze-thawings.

Table 1. Binding of [^{125}I]α-hANP in the presence of cold α-hANP of low doses

Dose (pg/tube)	Bound % mean ± SD	p* n=5
0	32.4 ± 0.3	
0.08	31.7 ± 0.4	≦ 0.05
0.25	30.1 ± 0.2	≦ 0.01
0.5	28.1 ± 0.3	≦ 0.01

*p values in comparison with zero dose

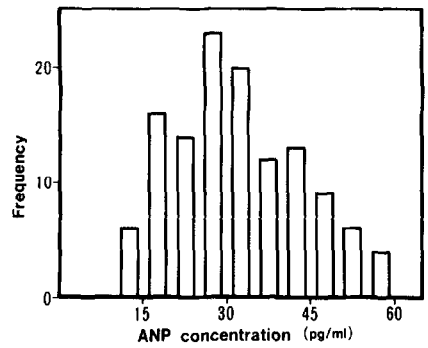

Fig. 2. Plasma ANP levels in healthy human subjects. A mean of 31.7 pg/ml with SD of 12.0 pg/ml was obtained in the assay of 124 subjects.

ANP concentration in healthy subjects Plasma ANP concentrations in 124 subjects (62 males and 62 females) who were normotensive, clinically having no illness, and whose serum Na^+ concentrations ranged from 136 to 145 mEq/l, were 31.7 ± 12.0 pg/ml (mean ± SD), ranging from 10 to 60 pg/ml, as shown in Fig. 2. There was no significant difference in plasma ANP levels between males and females. Urinary ANP concentrations in 10 healthy male subjects were 26.0 ± 12.6 pg/ml.

Molecular forms of ANP in plasma and urine Two major activity peaks were found in the gel permeation chromatography of healthy human plasma (Fig. 3). The higher peak is for the low molecular weight ANP since it corresponds to the radiolabeled α-hANP consisting of 28 amino acid residues. On the basis of apparent molecular size estimated by peak position, the lower peak was assumed to be formed by γ-hANP (11). But in contrast to plasma, only a peak corresponding to low molecular weight ANP was detected for urine.

DISCUSSION

In the present study, a direct and highly sensitive radioimmunoassay determination method for measuring plasma and urine ANP concentration was developed. A sensitive anti-ANP serum was produced with α-hANP(1-28) as the immunogen. A value as small as less than 0.08 pg/tube of α-hANP could be detected with 95% of confidence, and is lower than that of Gutkowska et al. reported in 1985 (12), i.e., 0.2 pg/tube with 95% of confidence. A detection limit of 0.3 pg/tube with 99% confidence was chosen in view of the very high reliability of our method. The dissociation constant, Kd, of the anti-ANP antibody in our serum with α-hANP was calculated as 1.1×10^{-11} M by a Scatchard plot analysis (13). This low Kd is considered responsible for the higher sensitivity in the present non-equilibrium method than the equilibrium method. It also results in a more

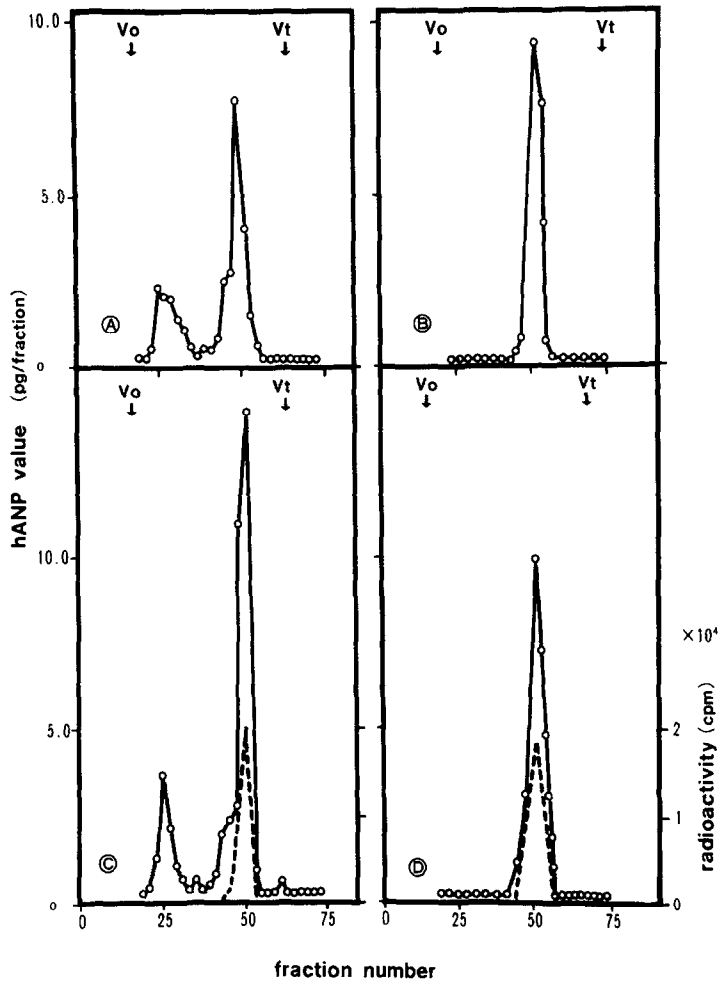

Fig. 3. Gel filtration profile of ANP in plasma and urine.
One and 2.0 ml of plasma (A and C), and 1.0 ml of urine (B and D) from two (upper and lower) healthy human subjects were charged onto a Sephadex G-75 column. Solid line, ANP activity; broken line, radioactivity of [^{125}I]α-hANP added to the samples.

efficient binding of the antibody to ANP as indicated by Zettner et al. (14). The ID_{50} for [^{125}I]α-hANP binding is 3.6 pg/tube, while that reported by Gutkowska et al. (12) was 11.5 pg/tube. The present method is thus shown to be more sensitive for measuring ANP concentrations in plasma and urine than any other so far reported using RIA.

The accuracy of measurements of unextracted plasma using RIA usually depends on how the antibody is separated from the tracer (15). The lowest nonspecific bound value (2.7%) was obtained when using double antibodies - polyethylene glycol techniques (10) for this purpose instead of polyethylene glycol, dextran coated charcoal, and double antibodies (not shown). The double antibodies - polyethylene glycol techniques was thus used in the present study.

Using our method, ANP concentrations in normal human plasma were determined using unextracted small samples (100 µl). The data (31.7 ± 12.0 pg/ml) obtained agree with those by other laboratories (7,12,16,17). Three peptides, such as α, β, and γ-hANP were found to be present in human atrial tissues (17). Miyata et al. (18) reported α-hANP to be the main component of plasma ANP when plasma was separated by reverse phase HPLC following treatment with a Sep-Pak C-18 cartridge. In our experience, ANP concentrations cannot be made stable by the conditions for measurement by a Sep-Pak C-18 cartridge treatment. By direct gel filtration, we obtained at least two peaks in a plasma sample, their ratio varied according to the sample. Consequently ANP circulation appears to have unique characteristics.

The existence of ANP in human urine is reported in this paper for the first time. Only one peak was noted in samples by gel filtration. α-hANP is thus filtered from glomerulus and reabsorbed or degenerated in part in the tubules.

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REFERENCES

1. DeBold, A.J., Borenstein, H.B., Veress, A.T., and Sonnenberg, H. (1981) Life Sci. 28, 89-94.
2. Trippodo, N.C., MacPhee, A.A., Cole, F.F., and Blakesley, H.D. (1982) Proc. Soc. Exp. Biol. Med. 170, 502-508.
3. Thibault, G., Garcia, R., Cantin, M., and Genest, J. (1983) Hypertension 5: suppl. 1:I, 75-80.
4. Kangawa, K., and Matsuo, H. (1984) Biochem. Biophys. Res. Commun. 118, 131-139.
5. Tanaka, I., Misono, K.S., and Inagami, T. (1984) Biochem. Biophys. Res. Commun. 124, 663-668.
6. Gutkowska, J., Horký, K., Thibault, G., Januszewicz, P., Cantin, N., and Genest, J. (1984) Biochem. Biophys. Res. Commun. 125, 315-323.
7. Sugawara, A., Nakao, K., Morii, N., Sakamoto, N., Suda, M., Shimokura, M., Kiso, Y., Kihara, M., Yamori, Y., Nishimura, K., Soneda, J., Ban, T., and Imura, H. (1985) Biochem. Biophys. Res. Commun. 129, 439-446.
8. Larose, F., Meloche, S., du Souich, P., Delean, A., and Ong, H. (1985) Biochem. Biophys. Res. Commun. 130, 553-558.
9. Skowsky, E.R., and Fisher. D.A. (1972) J. Lab. Clin. Med. 80, 134-144.
10. Wood, E.G., Stalla, G., Müllar, O.A., and Scriba, P.C. (1979) J. Clin. Chem. Clin. Biochem. 17, 111-114.
11. Kangawa, K., Fukuda, A., and Matsuo, H. (1985) Nature 313, 397-400.
12. Gutkowska, J., Bourassa, M., Roy, D., Thibault, G., Garcia, R., Cantin, H., and Genest, J. (1985) Biochem. Biophys. Res. Commun. 128, 1350-1357.
13. Scatchard, G. (1949) Ann. N.Y. Acad. Sci. 51, 660-672.
14. Zettner, A., and Duly, P.E. (1974) Clin. Chem. 20, 5-14.
15. Fyhrquist, F., Wallenius, M., and Hollemans, H.J.G. (1976) Scand. J. Clin. Lab. Invest. 36, 841-847.
16. Schiffrin, E.L., Gutkowska, J., Kuchel, O., Cantin, M., and Genest, J. (1985) N. Engl. J. Med. 313, 1196-1197.
17. Hartter, E., Weissel, M., Stummvoll, H.K., Woloszczuk, W., Punzengruber, C., and Ludvic, B. (1985) Lancet ii, 93-94.
18. Miyata, A., Kangawa, K., Yoshimori, T., Hatoh, T., and Matsuo, H. (1985) Biochem. Biophys. Res. Commun. 129, 248-255.