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Radioimmunological Determination of Arginine Vasopressin and Human Atrial Natriuretic Peptide after Simultaneous Extraction from Plasma

By *E. Hartter*

II. Medizinische Universitätsklinik Wien and

W. Woloszczuk

Ludwig Boltzmann Institut für Klinische Endokrinologie, Wien

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Summary: Human atrial natriuretic peptide and arginine vasopressin were coextracted from EDTA-plasma with Sep-Pak C18 cartridges, and the hormones were coeluted from the cartridges by a solution containing 900 ml/l of methanol, 5 ml/l of trifluoroacetic acid and 95 ml/l of water. After volume reduction under nitrogen the eluates were lyophilized, dissolved in buffer and aliquots were used for radioimmunological determination of both hormones. Both procedures used delayed addition of [^{125}I]-labelled tracer to enhance sensitivity. Recoveries were 95 ± 2% for human atrial natriuretic peptide and 96 ± 3% vor arginine vasopressin (mean ± SD). Minimal detectable doses were 2 pg/tube of human atrial natriuretic peptide and 0.5 pg/tube of arginine vasopressin (B_0 − 3 SD). Intra-assay variabilities were ± 8% for both tests. Simultaneous extraction of both hormones with high recovery was achieved, thus increasing sensitivity and specificity, at the same time reducing sample volume requirements and technician time.

Radioimmunologische Bestimmung von Arginin-Vasopressin und humanem atrialen natriuretischen Peptid nach gemeinsamer Extraktion aus Plasma

Zusammenfassung: Humanes atriales natriuretisches Peptid und Arginin-Vasopressin wurden mittels Sep-Pak C18 Säulen aus EDTA-Plasma gemeinsam extrahiert, mit einer Lösung, bestehend aus 900 ml/l Methanol, 5 ml/l Trifluoressigsäure und 95 ml/l Wasser coeluiert und nach Volumenreduktion im Stickstoffstrom lyophilisiert. In Puffer gelöste Aliquote des Lyophilisats wurden zur radioimmunologischen Bestimmung beider Hormone verwendet. Bei beiden Prozeduren wurde zur Erhöhung der Sensitivität der [^{125}I]-markierte Tracer verzögert zugegeben. Die Wiederfindung betrug 95 ± 2% für humanes atriales natriuretisches Peptid und 96 ± 3% für Arginin-Vasopressin (Mittelwert ± Standardabweichung). Die Nachweisgrenze war bei 2 pg/Röhrchen für humanes atriales natriuretisches Peptid und bei 0,5 pg/Röhrchen für Arginin-Vasopressin (B_0 − 3 Standardabweichungen), in beiden Fällen ergab sich eine Intraassay-Variabilität von ± 8%. Die Vorteile der Methode sind die gleichzeitige Extraktion der beiden Hormone, die hohe Wiederfindung und damit erhöhte Sensitivität und Spezifität sowie die Verringerung des nötigen Probenvolumens und des Laboraufwandes.

Introduction

Specific granules of mammalian cardial atriocytes (1, 2) contain structurally closely related peptides (3–9), which, after processing (9) are released into the circulation (10–18) and are important mediators of renal and cardiovascular homoeostasis.

In humans the so called alpha-form of human atrial natriuretic peptide is released into circulating blood (14, 16, 19). α-Human atrial natriuretic peptide consists of 28 aminoacids: NH-Ser-Leu-Arg-Arg-Ser-Ser-CysS-Phe-Gly-Gly-Arg-Met-Asp-Arg-Ile-Gly-Ala-Gln-Ser-Gly-Leu-Gly-CysS-Asn-Ser-Phe-Arg-Tyr-COOH. The intrachain disulphide bridge is essential for biological activity. α-Human atrial natriuretic peptide is cleaved off from the carboxy-terminus of its precursor γ-human atrial natriuretic peptide, 126 amino acids in length. β-Human atrial natriuretic peptide, whose biological role is quite unclear, most probably is an antiparallel dimer of α-human atrial natriuretic peptide (7, 8; 9).

Human atrial natriuretic peptide induces natriuresis/diuresis, relaxes smooth muscle and suppresses aldosterone production in the adrenal cortex ((25−30); see l. c. (9, 20−23) for recent reviews). Close regulatory association with the renin-angiotensin-aldosterone system has been reported (23, 24, 29, 30). Furthermore human atrial natriuretic peptide probably antagonizes arginine vasopressin-excretion (31, 32). Elucidation of physiological functions of human atrial natriuretic peptide therefore implicates measurements of plasma levels of the whole set of these hormones.

In this paper we present a method for simultaneous extraction by Sep-Pak C 18 cartridges of human atrial natriuretic peptide as well as arginine vasopressin from the same blood plasma sample. Aliquots of purified and concentrated plasma extracts are used for human atrial natriuretic peptide and arginine vasopressin RIA. A significant simplification together with high recovery, high sensitivity and increased specificity are the advantages of the method.

Materials and Methods

Blood sampling

Blood samples were collected into EDTA-coated plastic-tubes (Greiner, Vienna, Austria) kept on ice. Centrifugation gave EDTA-plasma which was frozen quickly with liquid nitrogen and stored at −70 °C until used (although we did not find any significant decay of human atrial natriuretic peptide if the samples were stored at −25 °C for 1 month).

Extraction of the hormones from EDTA-plasma samples

Sep-Pak C 18 cartridges (Waters/Millipore) were adapted to the lid of a manifold extraction apparatus built according to our instructions (Fa. Augmüller, Vienna, Austria). A 5 ml plastic syringe without plunger was then stuck onto each cartridge, to serve as sample/eluent recipient. Liquid placed into the syringes could be forced to flow through the cartridges by applying a moderate negative pressure (vacuum-pump). The cartridges were first prewashed with 4 ml of methanol. Additionally three sequential pretreatment steps were performed with 4 ml each of solutions containing methanol/trifluoroacetic acid/water in the following volume amounts per liter: 50 ml/10 ml/940 ml, 100 ml/5 ml/895 ml, and 900 ml/5 ml/95 ml. Finally, prior to adsorption of sample, they were reequilibrated with the solution used in the first step of the pretreatment cycle. Both hormones were coextracted from EDTA-plasma samples (usually 5 ml) by loading the samples onto the Sep Pak-syringe combination, followed by vacuum-aided slow passage of the samples through the cartridge (about 1 min duration). Non-adsorbed material was washed away by 4 ml of the solution used in the second step of pretreatment. Both hormones were finally eluted simultaneously by 4 ml (2 × 2 ml) of the above solution used for the third pretreatment step. After volume reduction to about 0.5 ml by a nitrogen stream at 37 °C, samples were lyophilized and stored at −25 °C until assayed. The lyophilized samples were dissolved in 1 ml RIA buffer and aliquots were used for hormone assay. Sep-Pak cartridges could be used at least three times without any detectable differences in results, when identical samples were applied.

Human atrial natriuretic peptide RIA components

Rabbit anti-α-human atrial natriuretic peptide as well as synthetic α-human atrial natriuretic peptide were purchased from NOVAbiochem AG (Läufelfingen, Switzerland). [^{125}I]iodotyrosyl-28-α-human atrial natriuretic peptide was obtained from Amersham International (Amersham, UK). Immunoprecipitating reagent (sheep anti-rabbit/polyethyleneglycol complex) from SORIN Biomedica (Saluggia, Italy) was used as second antibody. For human atrial natriuretic peptide RIA standards, radiolabelled α-human atrial natriuretic peptide and antibody to α-human atrial natriuretic peptide were dissolved in RIA buffer consisting of: 0.05 mol/l sodium phosphate, pH = 8.1, 1 mmol/l EDTA, 3 g/l bovine serum albumin (RIA grade, fraction V, Sigma, St. Louis, USA), 1 ml/l Triton X 100 (Serva Feinbiochemikalien, Heidelberg, FRG), 0.1 g/l sodium azide. Antibody from one vial was dissolved in 24 ml RIA buffer and kept on ice until used (freezing destroys antibody). Serial dilutions of synthetic α-human atrial natriuretic peptide in RIA buffer were used to construct the standard curve. Aliquots of lyophilized EDTA-plasma extracts dissolved in RIA buffer were used as samples of unknown human atrial natriuretic peptide content.

Arginine vasopressin components

The components of the arginine vasopressin RIA kit were from either Immuno Nuclear Corporation (Stillwater, Minnesota) or Bühlmann Laboratories AG (Basel, Switzerland), except for the sample buffers and second antibodies.

Performance of the assays

Aliquots of the lyophilized extracts dissolved in human atrial natriuretic peptide RIA buffer were used for assay of both hormones. Both radioimmunoassays were done as assays with delayed addition of tracer and incubation at 4 °C. Incubation conditions for arginine vasopressin RIA were modified and adapted to those used for RIA of human atrial natriuretic peptide: sample or standard (human atrial natriuretic peptide: 0.1 ml; arginine vasopressin: 0.2 ml) were added to 0.1 ml of antibody solution. After 24 hours incubation, 0.1 ml of the respective [^{125}I]-labelled tracer was added and incubation continued for a further 24 h ([^{125}I]α-human atrial natriuretic peptide approx. 20 000 counts/min; [^{125}I]arginine vasopressin 6000 to 15 000 counts/min). Bound antigen was separated from unbound by incubation with second antibody-immunoprecipitating reagent for 30 min at room temperature, followed by addition of 1 ml RIA buffer and centrifugation at 4000 *g* for 20 min at 4 °C. Supernatants were removed by aspiration, and the radioactivity in the pellet was determined in a gamma-counter. Data evaluation was computer-aided, using a linear logit/log fit.

haANP [^{125}I] RIA

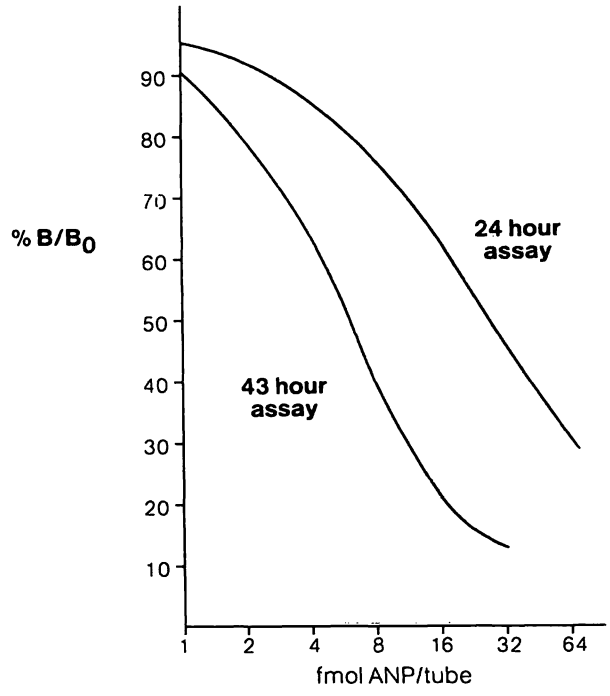
o High specificity

For example, cross-reactivity with atriopeptin I, atriopeptin II and atriopeptin III is less than 1%.

o Convenient separation

Utilizes Amersham's Amerlex-M* magnetic separation system. Conventional centrifugation can be used if preferred.

o High sensitivity, wide range



o Flexible

Alternative protocol allows results to be obtained in 24 hours with little loss of sensitivity.

o Adaptable

High cross reactivity (70%) with rat αANP allows assays to be carried out in this species.

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Results

Characteristics of adsorption and elution of the hormones from Sep-Pak C 18 cartridges

Adsorption and elution were evaluated by spiking EDTA plasma samples with either [^{125}I]α-human atrial natriuretic peptide or [^{125}I]arginine vasopressin, followed by adsorption onto Sep-Pak C 18 cartridges as described above, and step-wise elution with diluted trifluoroacetic acid (5 ml/l) containing increasing amounts of methanol. The adsorption/desorption-conditions for human atrial natriuretic peptide and arginine vasopressin determined in these experiments are shown in figure 1. As the peptides cannot be separated satisfactorily from each other because of overlapping peaks, we chose elution with 900 ml/l methanol in 5 ml/l of trifluoroacetic acid to desorb both hormones together from the Sep-Pak-cartridge.

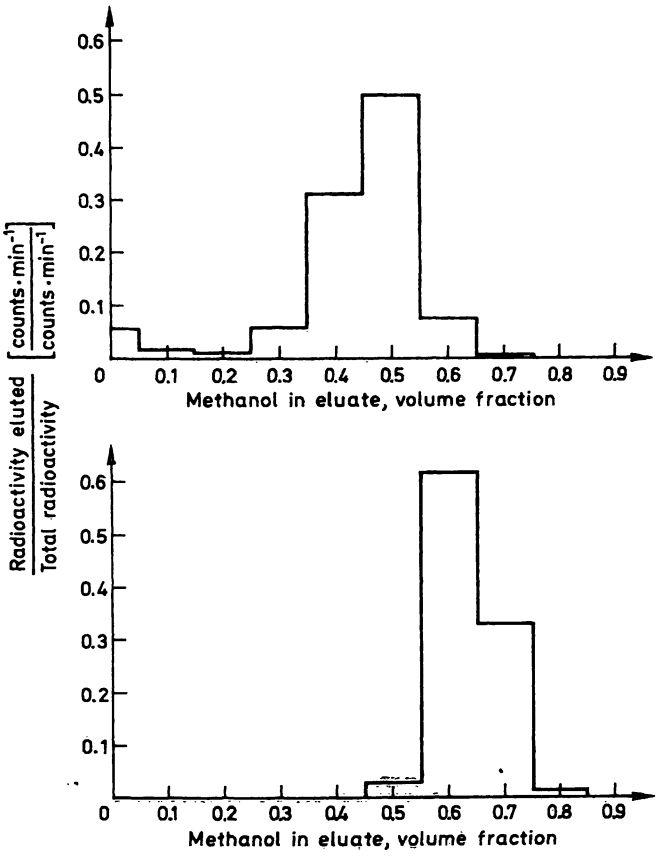

Fig. 1. Elution profile of
a) [^{125}I]-labelled arginine vasopressin and
b) α-human atrial natriuretic peptide adsorbed onto Sep-Pak C 18 cartridges.
To 5 ml EDTA-plasma, either [^{125}I]arginine vasopressin (148 000 counts/min) or [^{125}I]α-human atrial natriuretic peptide (530 000 counts/min) were added. After adsorption onto separate Sep-Pak cartridges as described in the text, the hormones were eluted by a stepwise gradient (4 ml/fraction) of methanol in diluted trifluoroacetic acid (5 ml/l).

Recovery

Aliquots of EDTA-plasma were spiked with synthetic hormone preparations simultaneously in a series of concentration steps according to table 1. After extraction of the hormones as described above, they were determined by specific RIA. The amounts found were compared with those calculated, and the results are summarized in table 1 a for human atrial natriuretic peptide and in table 1 b for arginine vasopressin. Typical standard curves are shown in figure 2.

Tab. 1. Recoveries of both hormones from plasma samples using synthetic preparations.

a

EDTA-plasma sample No.	α-Human atrial natriuretic peptide			Recovery
	added (ng/l)	found mean ± SD (ng/l)	expected (ng/l)	(%)
1	0	53 ± 1.8	–	–
2	12.5	63 ± 4.9	65.5	97
3	25.0	73 ± 2.4	78.0	94
4	50.0	95 ± 2.5	103.0	92
5	100.0	145 ± 6.4	153.0	95

b

EDTA-plasma sample No.	Arginine vasopressin			Recovery
	added (ng/l)	found mean ± SD (ng/l)	expected (ng/l)	(%)
1	0	3.1 ± 0.3	–	–
2	2.8	5.5 ± 0.3	5.9	93
3	5.6	8.3 ± 0.7	8.7	95
4	11.3	14.6 ± 0.4	14.4	101
5	22.5	24.6 ± 2.0	25.6	96

In the assay procedure originally developed in our laboratory for determination of human atrial natriuretic peptide (33) the peptide was eluted from the Sep-Pak cartridge after an additional washing step with methanol/trifluoroacetic acid/water (400 ml/5 ml/595 ml) which would elute part of arginine vasopressin. Control experiments did not reveal any significant difference in human atrial natriuretic peptide recovery when human atrial natriuretic peptide was eluted as described above.

Discussion

It is about one year since the discovery of human atrial natriuretic peptide and the first reports of its determination in human plasma by several groups (10–19). Blood sampling conditions and procedures for concentrating human atrial natriuretic peptide

Fig. 2a

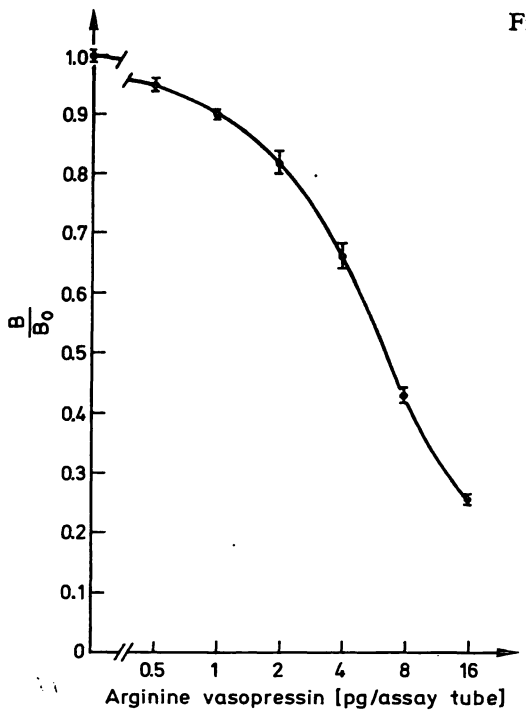

Fig. 2b

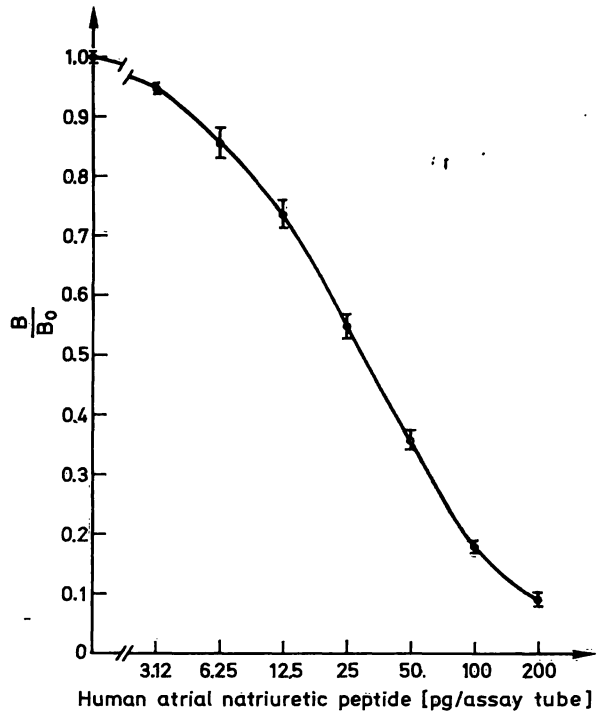

Fig. 2. RIA-calibration curves for arginine vasopressin (a) and human atrial natriuretic peptide (b) B_0 = [^{125}I]-labelled tracer bound at zero dose, B = [^{125}I]-labelled tracer bound at dose shown on the abscissa; B_0 and B values are corrected for non-specific binding (NSB). Results shown are mean ± SD of triplicates.
Fig. 2a: NSB = 3.5%, B_0 = 44%; maximal coefficient of variation in the useful range (between B/B_0 = 85% and B/B_0 = 15%) was ± 8%; minimal detectable amount of peptide (B_0 − 3 SD) was 0.5 pg/tube.
Fig. 2b: NSB = 4.2%, B_0 = 32%; maximal coefficient of variation in the useful range (between B/B_0 = 85% and B/B_0 = 15%) was ± 8%; minimal detectable amount of peptide (B_0 − 3 SD) was 2 pg/tube.

had to be elaborated and antibodies with sufficient affinity and specificity had to be raised. In our hands human atrial natriuretic peptide proved to be stable in ice-cooled EDTA-plasma for at least 2 hours (recovery of added [^{125}I]-labelled α-human atrial natriuretic peptide not lower than 95%, recovery of authentic endogenous human atrial natriuretic peptide 92% without any significant change when compared to samples processed immediately).

There is some controversy concerning normal levels of human atrial natriuretic peptide. This might be due to assay-dependent variations in human atrial natriuretic peptide-recoveries, insufficient sensitivity of the assay, plasma interference with direct assays, variation in the salt/water status of the „normal persons" due to lack of control of water balance, the possibility of circadian variations in human atrial natriuretic peptide-levels, orthostasis-dependent variations (16) not taken into account at blood sampling, etc.

To circumvent recovery problems due to proteolytic degradation or intrinsic sample variables, we chose extraction-enrichment and purification of the hormones prior to determination. Interfering sample constituents were thereby eliminated and a higher accuracy of the assay at low concentrations was achieved. At this point our somewhat sophisticated precycling procedure of the Sep-Pak catridges should be substantiated: When screening for variabilities in recoveries we suspected that during the adsorption-elution procedure small particles of column material might bleed out from the Sep-Pak column. Such particles, if eluted together with the peptides, could bind part of these when the lyophilised extracts were dissolved, thereby rendering considerable amounts of peptides undetectable by RIA. Although we did not test this possibility in detail, observation of the eluate showed that it sometimes contained granular material. Recoveries were better and results more reproducible when we pretreated the cartridges identically to the adsorption-washing-desorption procedure for the peptides.

In our opinion, the increased laboratory effort is largely compensated by coextraction of both hormones and by the high accuracies and recoveries achieved in comparison with other methods. This assay procedure therefore might be useful to those interested in investigation of the physiological role of human atrial natriuretic peptide and its regulatory association with the other systems of salt and water homoeostasis in man.

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DDr. Engelbert Hartter
II. Medizin. Univ.-Klinik Wien
Garnisongasse 13
A-1090 Wien