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ATRIAL NATRIURETIC FACTOR IN HUMAN PLASMA

Jolanta Gutkowska*, Raoul Bonan**, Denis Roy**, Martial Bourassa**,
Raul Garcia, Gaétan Thibault, Jacques Genest, and Marc Cantin

Clinical Research Institute of Montreal
110 Pine Avenue West
Montreal, Quebec, CANADA
H2W 1R7

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SUMMARY. A reproducible and sensitive radioimmunoassay (RIA) was developed to measure ANF in human plasma. Immunoreactive ANF was extracted from plasma with Sep-Pak cartridges, using 0.2% ammonium acetate (pH 4) with acetonitrile. The sensitivity of the assay was 3.9 pg/ml. The coefficient of variance for inter-assay and intra-assay was 16.8% and 6.8%, respectively. In normal healthy subjects (n=67), ANF content was 11.9 ± 1.3 pg/ml (mean ± SEM). Significantly-higher ANF concentrations were found in proximal coronary sinus blood, being 6 to 37 times greater than in the peripheral circulation. Comparison of the prior extraction method with direct RIA revealed a good correlation (r=91) in samples containing higher than 100 pg/ml ANF. No correlation was observed with lower values. The elution profiles of reverse-phase HPLC of peripheral and coronary sinus plasma extracts were similar but somewhat complex, with the main immunoreactive peak corresponding to a low-molecular-weight peptide.

Atrial natriuretic factor (ANF), family of potent biologically-active peptides, isolated from mammalian cardiocytes (1, 2), has been detected in specific granules anatomically similar to the secretory granules of endocrine tissues. These peptides, which are secreted by cardiocytes, originate from a larger precursor molecule (3). ANF has been classified as a new hormone with demonstrable effects in various tissue (4, 5, 6). Because of its possible physiological importance, techniques have been developed for its measurement. ANF quantification was first attempted by bioassay (7, 8) but this method was not sensitive enough to measure immunoreactive ANF (IR-ANF) in plasma. Several radioimmunoassay (RIA) techniques designed recently gene-

* To whom correspondence should be addressed.

** Montreal Heart Institute, 5000 Bélanger Street E.,
Montreal, Quebec, CANADA H1T 1C8.

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rated a great variability (0 - 300 pg/ml) of results for ANF in human plasma (9-17).

In this study, we describe a RIA of ANF, accomplished via prior extraction of ANF in Sep-Pak cartridges and comparison with direct RIA. We also report the values of ANF concentrations in peripheral and proximal coronary sinus blood.

MATERIALS AND METHODS

Preparation of plasma samples

Blood (6 ml) was drawn in plastic syringes and immediately transferred to chilled (iced) tubes containing 6 mg EDTA, 60 µl PMSF (10^{-3} M) and 60 µl pepstatin A (0.5 mM). Plasma was separated within a short time (15-20 min), extracted immediately or stored at -70 °C until assayed.

ANF extraction

The frozen plasma samples were extracted within 3-4 days (not longer than one week). ANF extraction was performed with Sep-Pak cartridges (Waters Associates, Milford, MA), which were activated by washing with 8 to 10 ml acetonitrile, then washed with 8 to 10 ml ammonium acetate (0.2% at pH 4.0). The plasma samples (2 ml or more) were then applied on the cartridges, washed with 5 ml ammonium acetate (0.2%, pH 4.0), and the absorbed ANF was eluted with 3 ml acetonitrile (60%) in ammonium acetate (0.2% pH 4.0). The organic solvent was evaporated under a nitrogen stream (about 50-60% of volume) and then dried in a Speed-Vac. The residue was taken up in 500 l RIA buffer containing 0.1% trifluoroacetic acid.

RIA buffer

The phosphate buffer (0.1 M, pH 7.4), which was prepared every two weeks, contained 0.02% sodium azide, 0.1% bovine serum albumin and 0.1% Triton.

Standards

Synthetic α-human ANF (Ser 99 - Tyr 126) was purchased (lot 007131) from Peninsula Laboratories Inc., Belmont, CA. Its purity was verified by hydrolysis, followed by amino acid determination. A stock solution (1 mg/ml) in 0.1 M acetic acid, prepared after taking into consideration the content of the pure peptide, was fractionated into 50- µl aliquots and stored at -70°C for at least 2 months. It was then diluted to 1 µg/50 µl in 0.1 M acetic acid, and this solution was used to chart the standard curves. A new solution of 1 µg/50 µl was prepared every two weeks. Serial 1:2 dilutions were made in RIA buffer just before the assay to obtain a range from 1.9 to 488 pg/ml.

Iodination procedure

The iodinated tracer was prepared with Ser 99 - Tyr 126 h-ANF (Peninsula Laboratories), as described elsewhere (9), using Chloramine-T as oxydant or lactoperoxydase. Five µl of highly-purified ANF (5 µg peptide) dissolved in 0.1 M acetic acid, was placed in a small test tube (10 X 75 mm) with 25 µl 0.05 M phosphate buffer (pH 7.4) followed by 40 µl $Na^{125}I$ (1 mCi), 5 µl lactoperoxydase and 10 µl diluted hydrogen peroxide (20 µg/ml). Another

10 µl aliquot of hydrogen peroxide was added after 5 and 10 min. The mixture, kept at room temperature, was purified by reverse-phase HPLC on a C_{18} µ Bondapak column. The first immunoreactive peak was used in the RIA.

RIA

Plasma extracts (50 and 100 µl) were incubated overnight in duplicate with antibody (50 µl) and RIA buffer in a total volume of 200 µl at 4°C. I^{125} ANF (100 µl, 6000 cpm) was then added and incubation continued overnight at 4°C. Separation of free from antibody-bound iodinated hormone was achieved with a second antibody precipitation, by adding 100 µl of goat antirabbit gamma globulin (1:50) and 100 µl of normal rabbit serum (1:35). After 2 hours of incubation at room temperature, we added 1 ml of a 6.25% solution of polyethylene glycol (PEG) 8000 in water. The tubes were centrifuged for 20 min at 4000 rpm at 4°C. The supernatant was then discarded and the pellet counted in a LKB gamma counter. The following controls were employed for each series of unknown samples: RIA buffer, standard ANF in RIA buffer (34.5 and 69 pg/ml), pooled plasma, and the same plasma pool with standard ANF added (34.5 and 69 pg/ml). As an additional recovery control, the plasma pool (1 ml) containing I^{125} ANF (≃ 15000 cpm) was extracted.

Direct RIA

A direct RIA was performed as described elsewhere (9).

Reverse-phase HPLC

Reverse-phase HPLC of plasma extracts was undertaken for 70 min with a LKB system, using a semi-preparative C_{18} µ Bondapak column (7.8 mm X 30 cm) and a linear gradient (15-50%) of acetonitrile. The flow rate was 2 ml/min, and each 2-ml fraction collected was assayed for ANF.

RESULTS

Characteristics of antiserum

The crossreactivity of antiserum (lot 009325) was determined by Peninsula Laboratories. This antiserum was directed toward the C-terminal portion of the molecule. The working 50- µl antiserum dilution (2 times greater dilution than that proposed by Peninsula) binds 40-42% of freshly-prepared tracer.

Standard curve

The typical standard curve is shown in Fig. 1. Linearity was observed between 3.9 and 488 pg/ml. This standard curve was used for all the assays and precision studies. The plasma samples were concentrated 4 times during the extraction procedures. As illustrated in Fig. 1, a Logit-log plot of serial dilutions of plasma extracts perfectly paralleled the standard curve.

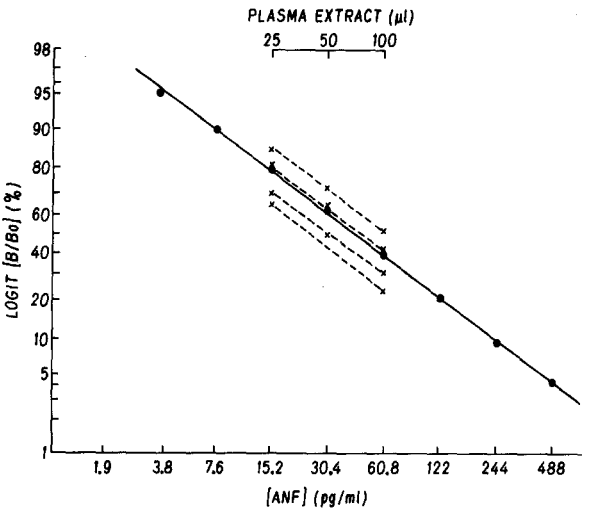

Figure 1. Standard curve of α-human ANF (Ser 99 - Tyr 126) with two-fold serial dilutions from 488 to 1.9 pg/ml. Serial dilutions of plasma extracts: 25 µl, 50 µl, 100 µl.

The non-specific binding value of freshly-prepared tracer (counts in pellet after precipitation with the second antibody and PEG) was less than 4% of the total radioactivity added to the tube.

^{125}I labeling of ANF

The labeling procedures with Chloramine-T or lactoperoxydase resulted in isolation by reverse-phase HPLC of two immunoreactive peaks of which the first was a monoiodinated form used in the RIA. The specific activity of this peak varied between 500 and 800 µCi/µg, calculated by comparison of the self-displacement ability of increasing amounts of iodinated tracer with the standard curve, as described by Morris (18). The purified radiolabeled ANF could be stored for 6 weeks with a very low decrease in binding (from about 40% to 35%).

Accuracy

In analytical recovery studies with two different quantities of ANF added to plasma, recovery ranged from 67.5% to 100% with a mean (± SE) of 77.1% ± 2.4 (n=35). Slightly higher recoveries (86.4% ± 7.2) were obtained with Sep-Pak extraction of I^{125} ANF added to plasma (n=35).

The plasma pool assayed as control with each batch of samples over a four-month period gave a mean value (± SE) of 25.9 ± 4.4 (n=64), with a

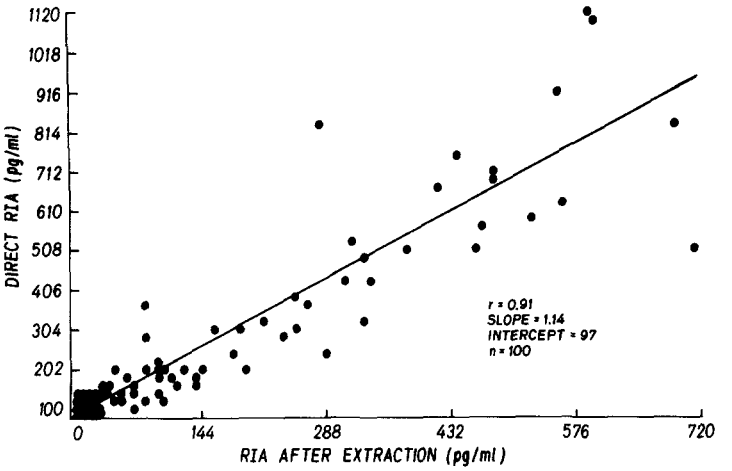

Figure 2. Correlation of ANF measured by direct RIA (y) and prior extraction on Sep-Pak (x).

16.8% coefficient of variance. The inter-assay coefficient of variations was 6.8% (n=18).

Comparison of direct assay with prior extraction on Sep-Pak

Comparison of both assays was done with a linear regression curve on 177 plasma samples. No correlation was observed in plasma values below 100 pg/ml, as determined by direct RIA. However, a significant correlation (r=0.91) was noted between both assays in plasma with more than 100 pg/ml ANF (Fig. 2).

Reference values

For the determination of normal values in our laboratory, plasma samples were collected from 67 healthy volunteers of both sexes and of different ages. The mean (± SEM) ANF value was 11.9 ± 1.3 pg/ml. Blood samples were also collected from the proximal coronary sinus during routine cardiac catheterization. Significantly greater values (6 to 37 times higher) were recorded for plasma obtained from this source (Table I).

Chromatographic pattern

Fig. 3 depicts the reverse-phase HPLC of peripheral and coronary sinus plasma extracts. Both revealed a similar but complex elution pattern. The predominant peak emerged at a position suggesting a low-molecular-weight C-terminal fragment of the prohormone.

TABLE 1

IR-ANF (PG/ML) IN PLASMA AFTER EXTRACTION WITH SEP-PAK

	PERIPHERAL	PROXIMAL CORONARY SINUS	DIAGNOSIS	RATIO *
1.	49.6	675.5	Severe coronary atherosclerosis	14
2.	18.9	698.7	Mild to modorate coronary atherosclerosis	37
3.	10.3	325.3	Mild coronary disease, mild aortic stenosis	32
4.	14.9	179.6	Idiopathic cardio-myopathy	12
5.	14.9	88.5	Moderate mitral stenosis, mild coronary atherosclerosis	6
6.	17.6	392.2	Severe atherosclerosis, moderate aortic disease	22
7.	79.3	1372.4	Coronary atherosclerosis	17

* Ratio represents IR-ANF content in proximal coronary sinus versus peripheral plasma.

DISCUSSION

There has been much activity in the last two years to develop a reliable method for the quantification of ANF in plasma and tissues. The difficulties encountered in the RIA of ANF are reflected by markedly-variable

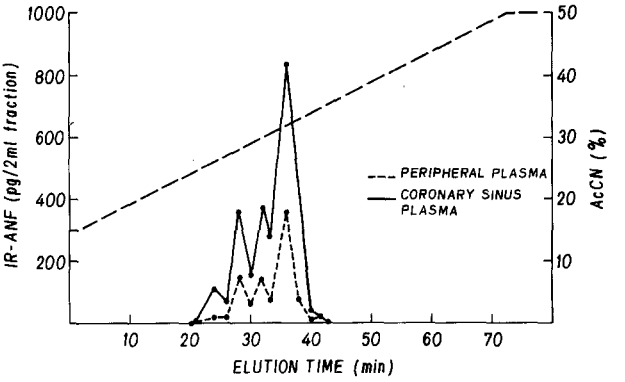

Figure 3. Reverse-phase HPLC on semipreparative C_{18} μ Bondapak column (7.8 mm X 30 cm) of human plasma following extraction with Sep-Pak cartridges. Reverse-phase HPLC elution pattern of peripheral plasma (- - -) (of 6-ml extract of high ANF-containing plasma pool). Pattern of proximal coronary sinus plasma (——) (pool of 6 ml).

basal values reported in the literature, ranging from undetectable in a majority of samples determined by direct RIA (13) to about 300 pg/ml with prior extraction on Sep-Pak (10). This variability of results may be due in part to the effectiveness of proteolytic enzyme inhibition during storage of plasma, different methods of ANF extraction and recovery, purity of standards, and specificity of antibodies employed.

However, the direct RIA described earlier (9) fulfilled some of the criteria of a valid method, such as the parallelism between serial dilutions of plasma and the standard and recovery curves of ANF added to plasma. But a large portion of measurable immunoreactivity was found to be non-dialysable. Plasma protein(s) evidently interfere in the direct RIA. Nevertheless, linear regression analysis has demonstrated a significant correlation between direct RIA and assay with prior extraction in plasma in which ANF was higher than 100 pg/ml, but no correlation was obtained for lower plasma ANF values. It therefore, appears that extraction is an essential step for the valid determination of ANF particularly when plasma ANF levels are normal or low. The normal ANF basal values are in the same range as those reported by other authors (11, 12, 15, 17). The method described is simple and reproducible. Our data show that inhibitors used for neutralization of plasma proteases give stability during at least four-months of storage of plasma at -70°C.

Comparison of ANF values for peripheral plasma and proximal coronary sinus plasma reveals a much higher content in the latter (6 to 37 times greater). These results suggest that ANF is released from the heart into the circulation. We have studied 7 patients: 6 males and 1 female. Patients' age ranged from 41 to 66 years. Two of them had mild coronary disease (#2 and #3, Table I) with normal or nearly normal ventricular function. Two had moderate to severe coronary atherosclerosis (#1 and #7) with impaired ventricular function with an ejection fraction equal to 35%. One patient had compensated congestive cardiomyopathy (#4), probably secondary to alcohol intake. One had moderate mitral stenosis (#5) with mild coronary atheroscle-

rosis and normal ventricular function (#5). One had moderate aortic disease with severe atherosclerosis (#6) and normal ventricular function. Although the population of patients was heterogenous, five (#1, #4, #5, #6, and #7) out of seven had in common an impaired left ventricular compliance, as an elevated left atrial pressure. It is interesting to note that when the stimulus is only an increase in left atrial pressure without impairment of ventricular function or compliance (#5), the ANF level at the proximal coronary sinus is only 6 times higher as compared to the level in peripheral circulation. When ventricular compliance is impaired, however, because of hypertrophy or ischemia, the ratio between ANF content in coronary sinus versus periphery is markedly increased. This preliminary observation requires confirmation on a larger group of patients.

Analysis of peripheral and coronary sinus plasma extracted by reverse-phase HPLC revealed a very similar pattern, demonstrating that in both cases the main circulating form is a low-molecular-weight (about 3000) fragment of the 126-amino-acid prohormone. The presence of a low-molecular-weight peptide in coronary sinus plasma indicates that the prohormone stored in granules may be processed before exocytosis occurs or that the secreted prohormone is immediately cleaved. The latter possibility is unlikely, as the disappearance of the purified 126-amino-acid rat prohormone added to rat plasma is not instantaneous (G. Thibault, personal communication). Further work is needed to elucidate this problem.

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