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Purification of three rat atrial natriuretic factors and their amino acid composition

G. Thibault, R. Garcia, N.G. Seidah, C. Lazure, M. Cantin, M. Chrétien and J. Genest

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

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A natriuretic factor has been described in the specific granules of rat atria. We have purified three factors which seem to be low-M_r peptides. They have been purified by means of acid extraction, octadecyl Sep-Pak cartridges, and chromatography on Bio-Gel P-10, CM Bio-Gel A, Mono S and reverse-phase high-performance liquid chromatography columns. The factors contain 26, 31 and 33 amino acids and may have been partially degraded during isolation. They are all 3 biologically active and the shorter one is the most active with a specific activity of 450000 units/mg.

Atrial natriuretic factor *Natriuresis* *Diuresis* *Peptide purification*

1. INTRODUCTION

A rat atrial natriuretic factor (ANF) was first described by authors in [1], who found that the injection of atrial homogenates induced marked diuresis and natriuresis in the rat. Subsequent work has shown that the ANF is localized in the granules of the atrial cardiocytes [2,3]. Purification of ANF and its partial characterization indicated that it consists chiefly of a basic peptide of molecular mass about 5000 [4–6]. However, heterogeneity was observed and some studies have indicated the presence of larger molecules of active material [4,6–9]. We report the complete purification of 3 biologically active atrial natriuretic factors and their amino acid composition.

2. MATERIALS AND METHODS

2.1. *Purification of ANF*

Atria from female or male Sprague-Dawley rats were homogenized (1 g/10 ml) in 1 M acetic acid containing 1 mM EDTA, 1 mM phenylmethyl sulfonyl fluoride and 12.5 μM pepstatin. After centrifugation at 30000 × *g* for 20 min, the supernatant was frozen at −20°C overnight, thawed and centrifuged at 100000 × *g* for 30 min. The final supernatant was passed through octadecyl Sep-Pak cartridges (1 cartridge/2 g of atria) and the active material was deposited on a Bio-Gel P-10 column (2.5 × 90 cm) and eluted with 0.1 M acetic acid. The active material from this column was clearly separated into two active regions of different molecular masses (4000–6000 and 6000–15000). Each pooled region was further purified separately. The active material was next applied to a CM Bio-Gel A column (1.5 × 30 cm) and eluted with a linear gradient of 450 ml of 0.01–1.0 M ammonium acetate, pH 5.0. ANF was then purified on a Mono S HR5/5 column (Pharmacia Fine Chemicals) adapted to be used on a liquid chromatograph (Varian Model 5060). A gradient of 0.02–1.0 M triethylamine acetate (pH 6.5) was used. Two-minute fractions were collected. With the low M_r material the most active component was eluted with 0.9 M buffer, but the most active of the high-M_r factors was eluted with about 0.8 M buffer. Low-M_r natriuretic factor was subsequently purified by reverse-phase high performance liquid chromatography (HPLC) in the following sequence: CN μBondapak (Waters Associates) (0.39 × 30 cm) 0.1% (v/v) trifluoroacetic acid in CH_3CN at 0°C; C_{18} μBondapak (Waters Associates) (0.39 × 30 cm) 0.1%

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(v/v) trifluoroacetic acid in CH_3CN at room temperature; C_{18} μBondapak (Waters Associates) (0.39 × 30 cm) 0.13% (v/v) heptafluorobutyric acid in CH_3CN at room temperature. This active material was designated as ANF-I.

The high-M_r natriuretic factor was passed through a Bio-Sil TSK IEX-530 CM column (Bio-Rad Laboratories) (0.4 × 30 cm) and eluted with a linear gradient of 0.02 M–0.4 M ammonium formate (pH 5.0) containing 15% CH_3CN with a slope of 0.008 M/min and a flow rate of 0.7 ml/min. The active peak was finally purified by reverse-phase HPLC on a CN μBondapak column as described above. Two major active peaks were found and designated ANF-II and ANF-III.

When possible, all steps were carried out at 4°C. At the end of each chromatographic step the material was lyophilized. For small volumes (<6 ml) lyophilization was carried out in polystyrene tubes (1.5 × 7 cm) in a Savant Speed Vac concentrator. Larger volumes in the first steps were lyophilized in glass flasks. Samples were dissolved in 1–3 ml of 0.01 M ammonium acetate, pH 5.0. Aliquots were taken for the biological assay [2,4], which was done in duplicate. Proteins were measured by the Bradford assay [10] with bovine albumin as a standard, except in the last step where the amount of peptide was estimated from the amino acid analyzer.

2.2. *Carboxymethylation of ANF*

The cysteine residues were reduced with dithiothreitol as in [11]. Reduced cysteines were then alkylated with iodoacetic acid. The peptide was desalted on a C_{18} μBondapak column, using 0.1% trifluoroacetic acid in CH_3CN.

2.3. *Amino acid analysis*

Amino acid analysis was performed on 5 μg of each carboxymethylated peptide after 22 h of hydrolysis at 105°C in 5.7 N HCl and 0.1% mercaptoethanol. The amino acids were separated in a modified 120C-Beckman amino acid analyzer using a Beckman W3 or Dionex DC5A column [12].

3. RESULTS AND DISCUSSION

For this study, 27 g atria (320 rats) were used. The initial homogenate contained an activity equivalent to 840000 μmol of Na^+ excreted per 20 min (here called 840000 units), as measured by bioassay, with a specific activity of 360 units/mg protein. At the Bio-Gel P-10 step about half of the active material corresponded to a low-M_r (4000–6000) natriuretic substance. The elution pattern for the low-M_r natriuretic factor on the Mono S is shown in fig.1. Although active material was found in almost all fractions, indicating the heterogeneity of the product, most of the activity was found in the 40- to 44-min fractions. At this step, the yield is less than 5%, and ANF-I has a specific activity of 131000 units/mg. These results are similar to those published earlier [4]. However, to achieve further purification we used reverse-phase HPLC. Fig.2 illustrates the elution of the ANF-I on the CN μBondapak column. Although we were able to identify a peak with natriuretic activity, the specific activity of the material recovered was much lower than in the preceding step (84000 units/mg), which indicates partial inactivation of the peptide. The peptide ANF-I was

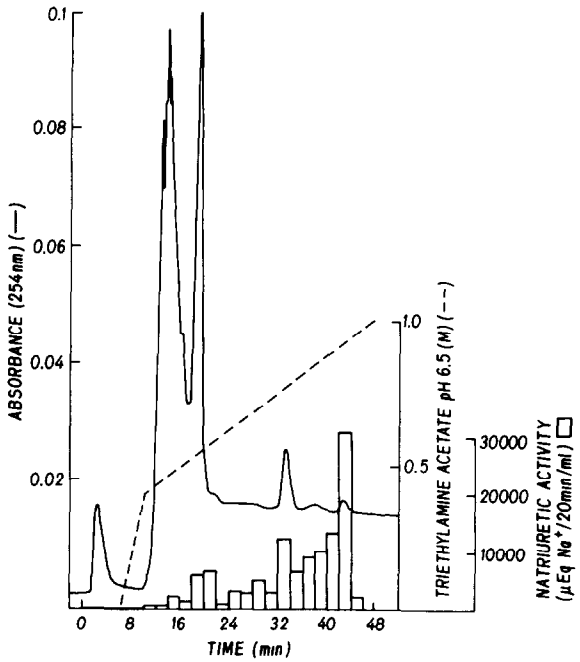

Fig.1. Elution of the low-M_r active material from the CM Bio-Gel A column off a Mono S HR5/5 column with a concentration gradient of triethylamine acetate, pH 6.5. Flow rate, 1 ml/min: 2-min fractions collected. The slowest fractions, collected at 40–44 min, were together designated ANF-I.

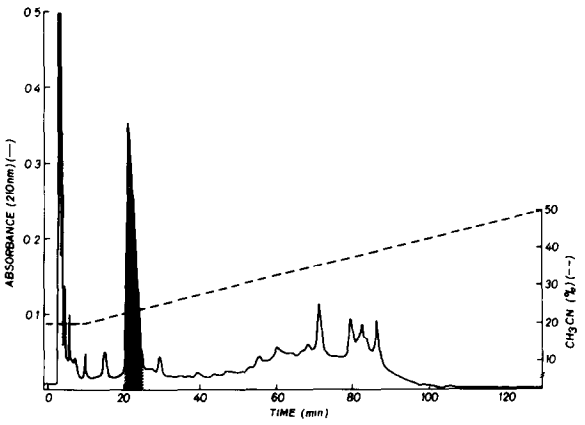

Fig.2. Chromatography of ANF-I on CN μBondapak, eluted with acetonitrile 20–50% in 0.1% trifluoroacetic acid at a gradient of 0.25%/min and a flow rate of 1 ml/min. The shaded area indicates the peak with natriuretic activity.

then applied to a C_{18} μBondapak column eluted with a gradient of 0.1% trifluoroacetic acid in acetonitrile (0.2%/min, 1 ml/min) and finally to the same column, but with 0.13% heptafluorobutyric acid instead of 0.1% trifluoroacetic acid (fig.3). In the two last steps the peptide appeared as a single peak. The final yield of ANF-I was about 120 μg.

The high-M_r natriuretic factor was eluted on the Mono S with 0.8 M buffer. The elution pattern, as for the low-M_r factor, indicated considerable heterogeneity. Only the purest fractions were pooled. On the Bio-Sil column the material was eluted with 0.3 M ammonium formate. In the last step (fig.4), the CN μBondapak column, two major peaks were seen and denoted ANF-II and ANF-III with a final yield of 30 and 20 μg, and specific activities of 450000 and 297000 units/mg, respectively.

The amino acid compositions of carboxymethylated ANF-I, ANF-II and ANF-III are given in table 1. These data suggest that the 3 peptides are very similar and contain mostly hydrophobic and basic amino acids. Similar results were reported in [6], but the peptide purified there seems to be longer (49 amino acids).

ANF-I and ANF-II are probably shorter peptides. The presence of these 3 peptides and the heterogeneity encountered after the gel filtration and the Mono S column may be due to proteolytic

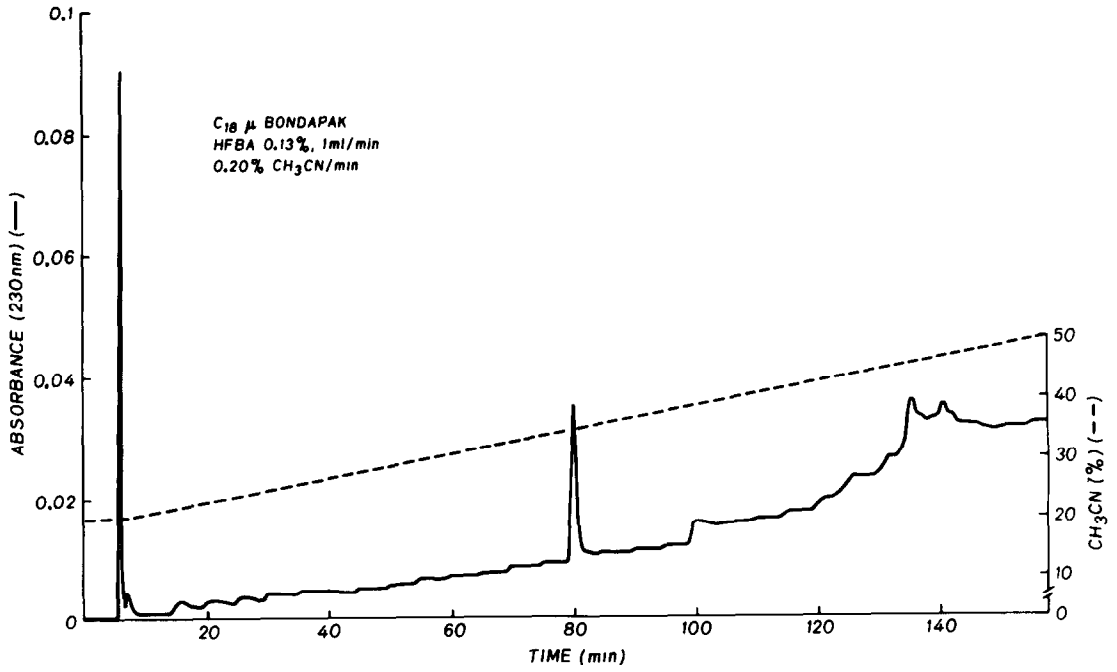

Fig.3. Chromatography of the purified peptide ANF-I on CN_{18} μBondapak, eluted with acetonitrile 20–50% in 0.13% heptafluorobutyric acid at a gradient of 0.2%/min and a flow rate of 1 ml/min.

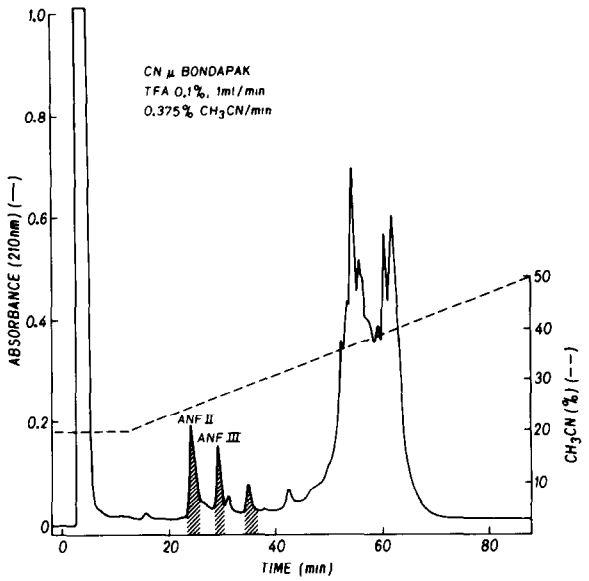

Fig.4. Chromatography of the high-M_r material on CN μBondapak, eluted with acetonitrile 20–50% in 0.1% trifluoroacetic acid at a gradient of 0.375%/min and a flow rate of 1 ml/min. The shaded areas indicate the peaks with natriuretic activity. The major peaks were designated ANF-II and ANF-III.

digestion in the homogenate. Moreover the fact that ANF-II and ANF-III, purified from the high-M_r region, nevertheless have about the same M_r-value as ANF-I from the low-M_r fraction, may indicate artefact formation due to adsorption during gel filtration.

The 3 purified peptides were biologically active after the CN μBondapak column. A dose range of 0.15–0.75 nmol, depending on the peptide, will induce a 10-fold increase in urine output and a 20-fold increase in sodium excretion when injected intravenously into a rat. ANF-II, the shortest one, was the most active, which suggests that some parts of the amino acid sequence of ANF-I and ANF-III may not be essential for the activity.

In conclusion we have isolated 3 pure peptides which cause natriuresis and diuresis in the rat. Further studies on the amino acid sequence will indicate relationships between the structures of these peptides and their biological activities.

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Table 1

Amino acid compositions of ANF peptides[a]

Amino acid residue	ANF-I	ANF-II	ANF-III
Asx	2.17 (2)[b]	2.06 (2)	2.13 (2)
Ser	4.61 (5)	3.57 (4)	4.53 (5)
Glx	1.26 (1)	1.29 (1)	1.38 (1)
Pro	1.08 (1)	–	1.02 (1)
Gly	6.00 (6)	4.97 (5)	6.16 (6)
Ala	1.14 (1)	1.14 (1)	2.07 (2)
Ile	1.97 (2)	1.93 (2)	1.91 (2)
Leu	2.15 (2)	1.19 (1)	3.17 (3)
Tyr	0.69 (1)	0.98 (1)	1.00 (1)
Phe	2.22 (2)	2.03 (2)	2.06 (2)
Arg	6.19 (6)	4.83 (5)	5.86 (6)
Cys[c]	1.86 (2)	2.00 (2)	1.71 (2)
Trp[d]	ND	ND	ND
Total	31	26	33

[a] Amino acid analysis was performed on reduced and alkylated ANF-I, ANF-II and ANF-III
[b] Numbers in parentheses give the nearest integer
[c] Cys was quantitated as the sum of Cys, carboxymethyl-Cys and cysteic acid
[d] ND, not determined

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