Journal of Chromatography, 490 (1989) 411–417
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4684

Note

Separation of synthetic cardiodilatin/atrial natriuretic factor and related peptides by reversed-phase high-performance liquid chromatography

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(First received October 17th, 1988; revised manuscript received January 16th, 1989)

Cardiodilatin (CDD)/atrial natriuretic peptide (ANP) represents a family of peptides, which are involved in the control of blood pressure and electrolyte balance [1,2]. The circulating CDD/ANP contains 28 amino acid residues [3] and comprises the N-terminal part of a 126-amino-acid prohormone stored in specific granules of the atrial myocytes [4,5]. In addition to its natriuretic and diuretic functions, CDD/ANP relaxes precontracted vascular smooth muscle strips [4,6,7] and inhibits aldosterone production [8]. The variety of functions of CDD/ANP suggests the existence of multiple, functionally distinct peptides.

In fact, a new natriuretic peptide (brain natriuretic peptide; BNP) has been isolated from porcine brain. The peptide consists of 26 amino acid residues and elicts a pharmacological spectrum very similar to that of CDD/ANP [9]. This peptide exhibits a remarkable homology to the known sequence of CDD/ANP [9]. More recently, BNP was detected in the porcine heart [10]. Moreover, urodilatin, another CDD/ANP-related peptide similar to CDD/ANP except that the N-terminus is extended by four amino acids [11], was isolated from human urine.

In addition to different circulating peptides, posttranslational modifications of CDD/ANP may occur and alter their exterior as well as biological activity. Phosphorylation of CDD/ANP in atria in situ at a serine residue in position 104 was reported [12], and cyclic AMP(cAMP)-dependent phosphorylation

0378-4347/89/$03.50

of this residue influences the vasorelaxing capacity [13,14], probably due to a decreased receptor binding of the peptide [14]. cAMP-dependent phosphorylation of CDD/ANP prevents specific proteolysis of the ring structure [15] by a specific peptidase (EC 3.4.24.11) [16] that cleaves the linkage between Cys-105 and Phe-106 [17–19]. These findings make it important to distinguish not only between CDD/ANP, urodilatin and BNP but also between the non-phosphorylated and phosphorylated CDD/ANP in routine chromatographic separation and identification procedures for plasma CDD/ANP and CDD/ANP in other body fluids. Therefore, using synthetic peptides, we wanted to demonstrate rapid chromatographic identification of the peptides of the CDD/ANP family according to their different properties.

EXPERIMENTAL

Synthetic cardiodilatin/α-hANP (CDD-99–126), urodilatin (CDD-95–126) and brain natriuretic peptide (BNP) were from Bissendorf Biochemicals (Hannover, F.R.G.). According to the manufacturer's description, the peptide purity is specified as $>97\%$. Hydrochloric acid, supra-pure grade, was from Merck (Darmstadt, F.R.G.) and acetonitrile from Rathburn (Walkerburn, U.K.). All other reagents were of the highest grade available and obtained from local suppliers.

Cyclic AMP-dependent phosphorylation of CDD/ANP

The preparation of phosphorylated CDD/ANP was performed as described earlier [13]. The peptide was incubated for 60 min at 30°C in 50 m*M* 3-(N-morpholino)propanesulphonic acid (MOPS) buffer (pH 6.8) containing 10 m*M* magnesium acetate and 0.34 m*M* ATP. The catalytic subunit of cAMP-dependent protein kinase (EC 2.7.1.37) was added in a final protein concentration of 20% of the peptide employed in order to ensure complete phosphorylation. For radioactive labelling studies, [γ-^{32}P]ATP (NEN, Dreieich, F.R.G.) was added (0.4 mCi/μmol ATP).

Purification of CDD/ANP and related peptides by cation-exchange high-performance liquid chromatography (HPLC)

Peptides were analyzed on a cation-exchange column (50 mm$\times$4 mm I.D.) using the HPLC instrument from LKB equipped with two pumps, a 2152 controller, a 2151 variable-wavelength monitor and a Rheodyne injector (1-ml loop). The column was eluted with a linear gradient of sodium chloride (0–1 *M*) in 10 m*M* Na_2HPO_4 (pH 5) at a flow-rate of 0.7 ml/min (absorbance 230 nm). The gradient slope, expressed by the increase of the salt concentration, was 13.3 m*M*/min. All steps were performed at room temperature. Radioactive labelling was detected by measuring incorporated ^{32}P. Fractions of 0.35 and 0.7 ml were collected and counted in 3 ml of water for 10 min in a liquid scintillation counter.

Separation of CDD/ANP and related peptides by reversed-phase HPLC

A reversed-phase column (HD-Gel-RP-7S-300, 125 mm×4 mm I.D., Orpegen, Heidelberg, F.R.G.) was used. The HPLC instrument was from Applied Biosystems and equipped with two pumps, a 1400 A solvent-delivery system, a Spectroflow 491 dynamic mixer–injector (0.2-ml loop) and a 1783A absorbance detector–controller. The column was equilibrated at 45 °C with buffer A containing 0.01 *M* hydrochloric acid and eluted by increasing the acetonitrile concentration (buffer B: 80% acetonitrile–0.01 *M* hydrochloric acid). The starting buffer was 20% buffer B. The column was eluted by an increase in the acetonitrile concentration of 0.5% per min. The flow-rate was 0.7 ml/min and the absorbance was determined at 230 nm.

Amino acid sequence analysis of the synthetic peptides after reversed-phase HPLC

The analysis was done with a gas phase sequencer 470 A from Applied Biosystems. The amino acids were identified by HPLC according to Lottspeich [20].

Identification of the phosphorylation site

Phosphorylation of the serine residue in position 104 by the catalytic subunit of cAMP-dependent protein kinase was identified as described by Meyer et al. [21].

Test of biological activity

For the tests, helical strips of aortae from New Zealand rabbits were incubated in organbath solution, as described earlier [4,13,19].

RESULTS AND DISCUSSION

A mixture of BNP (3.5 μg), phosphorylated (3 μg) and non-phosphorylated CDD/ANP (5 μg) and urodilatin (3.5 μg) was applied to the cation-exchange column. Peptides were eluted by a linear gradient of sodium chloride (Fig. 1, dashed line). As can be seen from the chromatographic profile of the absorbance (continuous line) in Fig. 1A, CDD/ANP and urodilatin are clearly separated, eluting at 320 and 350 m*M* sodium chloride, respectively. BNP and phosphorylated CDD/ANP, however, are coeluted at ca. 250 m*M* sodium chloride. The co-migration of phosphorylated CDD/ANP and BNP was identified from determination of ^{32}P incorporated into CDD/ANP after phosphorylation by the cAMP-dependent protein kinase. The lower part of Fig. 1A shows the radioactivity of the ^{32}P-labeled CDD/ANP. A maximum of incorporated radioactivity (filled bars) was found below the peak of BNP, which elutes at 250 m*M* sodium chloride. The altered cation-exchange binding capacity of phosphorylated CDD/ANP compared with non-phosphorylated peptide is due to

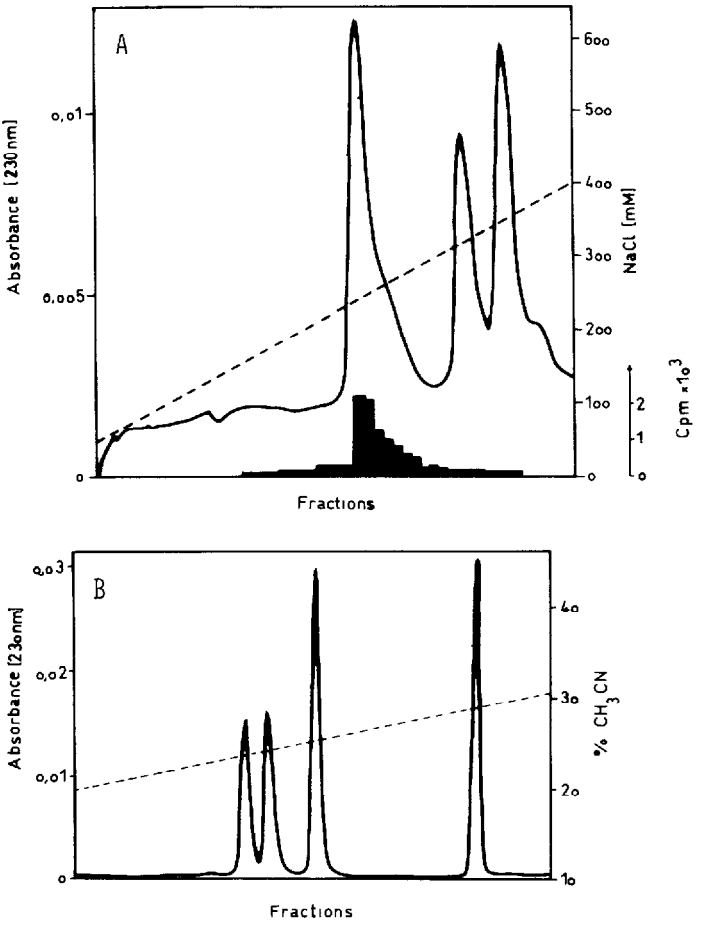

Fig. 1. HPLC profiles (continuous lines) of the separation of phosphorylated and non-phosphorylated CDD/ANP, urodilatin and BNP. (A) Separation by cation-exchange HPLC was achieved by use of a linear salt gradient (dashed line) from 0 to 1 *M* sodium chloride. BNP and phosphorylated CDD/ANP elute together at 250 m*M* sodium chloride in a single peak, followed by non-phosphorylated CDD/ANP and urodilatin. Determination of phosphorylated CDD/ANP was enabled by cAMP-dependent phosphorylation using [γ-^{32}P]ATP as co-substrate. The filled bars below the profile indicate the incorporated radioactivity (B) Separation by reversed-phase HPLC by an increasing acetonitrile gradient (dashed line) enables the investigated peptides to be separated in a single step. Urodilatin elutes first, at a concentration of 23.5% acetonitrile, followed by non-phosphorylated CDD/ANP (24.5%), phosphorylated CDD/ANP (26%) and BNP (29%).

the different charge of the molecule after the transfer of the phosphoryl group from ATP by the kinase reaction.

The amino acid sequence R-R-X-S represents a specific recognition site for the catalytic subunit of the cAMP-dependent protein kinase, which will phosphorylate the serine residue in the presence of ATP [22,23]. These conditions

hold true for the serine residue in position 104 in CDD/ANP. Tyrosine residues have not been shown to be sites of phosphorylation [22]. Olins et al. [14] showed that CDD/ANP, which lacks the two arginine residues, was not phosphorylated. Similarly, analogues of atrial peptides were not phosphorylated, if the recognition sequence was disrupted by deletion or substitution. The position of the phosphorylation site under our conditions was characterized as serine 104 by amino acid sequence analysis using the method of Meyer et al. [21]. According to this method, amino acid sequence analysis allows the identification of phosphorylated amino acid residues in CDD/ANP by conversion to S-ethylcysteine.

In order to discriminate between phosphorylated and non-phosphorylated CDD/ANP, urodilatin and BNP, the peptide mixture was separated by reversed-phase HPLC (Fig. 1B). A mixture of 2 μg of phosphorylated and 2 μg of non-phosphorylated CDD/ANP, 2 μg of urodilatin and 2 μg of BNP was loaded onto the column and eluted with a linear gradient of acetonitrile from 14 to 50% (dashed line). As indicated by the absorbance measurement (continuous line) in Fig. 1B, all peptides were eluted as distinct peaks. The first peak was urodilatin, followed by the non-phosphorylated CDD/ANP and the phosphorylated peptide. BNP eluted last.

The intactness of all the peptides was verified by biotesting. As can be seen in the Table I, in all peptides the two cysteine residues develop a disulphide bridge. Only this oxidized form of the CDD, urodilatin and BNP molecules exhibits biological activity, e.g. vasodilatation. Olins et al. [24] reported that a linear CDD molecule, which is equivalent to its reduced form, is inactive.

The positions of the synthetic peptides eluted by reversed-phase HPLC were identified by amino acid sequence analysis, and cAMP-dependent phosphorylated CDD/ANP by determination of incorporated radioactivity (data not shown). The amino acid sequence is given in Table I.

By use of chromatographic protocols and reversed-phase columns described earlier, urodilatin and CDD/ANP were either not separated and appeared as a single peak [19] or divided by only a small amount without baseline separation [11]. The chromatographic separation of CDD/ANP and related pep-

TABLE I

AMINO ACID SEQUENCE OF CDD/ANP, URODILATIN AND BNP

Peptide	Amino acid residues
CDD/α-hANP	S ^{100}LRRSS ^{105}CFGGR ^{110}MDRIG ^{115}AQSGL ^{120}GCNSF ^{125}RY
Urodilatin	TAPRS LRRSS CFGGR MDRIG AQSGL GCNSF RY
BNP	DSG CFGRR LDRIG SLSGL GCNVL RRY

tides by reversed-phase HPLC in this study enables a rapid and clear separation, and determination of the synthetic CDD/ANP in the phosphorylated and non-phosphorylated forms, as well as urodilatin and BNP. The separation of phosphorylated and non-phosphorylated CDD/ANP may be improved by cation-exchange chromatography showing high dissolution; however, the separation is impaired by the co-migration of BNP with phosphorylated CDD/ANP. Thus the one-step separation by reversed-phase HPLC may find use in the rapid determination of CDD/ANP and related peptides in clinical use.

ACKNOWLEDGEMENTS

This study was supported by Braunschweig Biotechnology Co., Braunkop 88. The authors thank Prof. Dr. V. Kinzel from the Cancer Research Institute (Heidelberg, F.R.G.) for the supply of the catalytic subunit of cAMP-dependent protein kinase.

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