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DEVELOPMENT OF A SOLID-PHASE EXTRACTION TECHNIQUE FOR α-HUMAN ATRIAL NATRIURETIC PEPTIDE IN HUMAN PLASMA

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SUMMARY

A reliable extraction method was developed for α-human atrial natriuretic peptide (α-hANP) using Bond Elut C_8 columns in tandem. This involved activation of the columns using methanol followed by a water wash to remove the excess methanol. Plasma (1 ml) was then added and a vacuum applied until all was drawn through. Excess protein and other endogenous compounds were removed by washing the columns with water and elution of the α-hANP was achieved with 0.75 ml acetonitrile–water–trifluoroacetic acid (80:19.8:0.2, v/v/v). Samples were evaporated under nitrogen and reconstituted in radioimmunoassay buffer ready for analysis. The recovery of α-hANP from plasma using this method was found to be 90% ± 0.6% [mean ± standard error of the mean (S.E.M.); coefficient of variation (C.V.) = 1.5%] which will allow more precise measurement of the peptide than is presently available. With this high precision of analysis available, having a limit of detection of 0.4 fmol/ml and a range of 0 to 32 fmol/ml, a low-dose infusion of α-hANP was conducted and the changes in plasma concentration were followed.

INTRODUCTION

The recently discovered peptide, α-human atrial natriuretic peptide (α-hANP) is released from the heart atrium. It contains 28 amino acids and is formed by peptidase cleavage of a much larger pro-peptide. It has many physiological effects including natriuresis, diuresis, kaliuresis, hypotensive effects, inhibition of adenylate cyclase[1], inhibition of aldosterone production[2], vasodilation[3], inhibition of angiotensin II release[4], noradrenaline and potassium-induced vasoconstriction and modulation of renal gluconeogenesis[5]. The many physiological effects of α-hANP indicate that it is important in the regulation of fluid retention, therefore precise quantitation of small changes in circulating levels of α-hANP and monitoring of physiological effects will give a more accurate indication of this peptides role. Many methods have been published on the extraction of α-hANP using Sep-Pack C_{18} reversed-phase extraction cartridges. Richards *et al.*[6] quote recoveries of unlabelled peptide from plasma of 65 ± 5% [mean ± standard deviation (S.D.)] which could be increased to 83 ± 5% (mean ± S.D.) using an acidification step. Morice *et al.*[7] quote recoveries of between

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80 and 90%, however plasma α-hANP levels were not corrected for recovery. Since all other groups had concentrated on Sep-Pak extractions and considering that Analytichem produce a wider range of reversed-phase columns we decided to screen these columns and choose the best on which to base our extraction.

Morice *et al.*[7] have attempted to demonstrate a change in circulating α-hANP levels during infusions of increasing concentrations of α-hANP (0.4, 2.0 and 10.0 pmol min^{-1} kg^{-1}) using their extraction procedure. Since our method is more precise, we decided to repeat their work using an infusion rate of 1.2 pmol min^{-1} kg^{-1} to test the effectiveness of our method by monitoring plasma levels of α-hANP.

EXPERIMENTAL

Reagents and materials

All tubes used in the method were polypropylene and were obtained from Sarstedt (Leicester, U.K.). Methanol and water, both high-performance liquid chromatographic (HPLC) grade, were obtained from Rathburn Chemicals (Walkerburn, U.K.). Disodium EDTA (reagent grade) was obtained from Sigma (Poole, U.K.). Trasylol was obtained from Bayer (Newbury, U.K.). Bond Elut columns C_2 (ethyl bonded silica, catalogue number: 603101), C_8 (octyl bonded silica, catalogue number: 606101), C_{18} (octadecyl bonded silica, catalogue number: 607101), PH (phenyl bonded silica, catalogue number: 608101), CN (cyanopropyl bonded silica, catalogue number: 613101) and CH (cyclohexyl bonded silica, catalogue number: 610101) were obtained from Jones Chromatography (Llanbradach, U.K.). The radioimmunoassay (RIA) kit (RPA 512) used was from Amersham International (Amersham, U.K.) and included the [^{125}I]α-hANP used to develop the extraction. The α-hANP used in the infusion was obtained from Peninsula Labs. (Merseyside, U.K.).

Apparatus

Extraction was carried out with a 10-channel Vac Elut device (Jones Chromatography). Gamma counting was carried out using a Clinigamma 1272 counter (LKB/Pharmacia; Milton Keynes, U.K.).

Sample collection and storage

Blood samples were collected into chilled polypropylene tubes containing 1 mg/ml disodium EDTA and sufficient Trasylol to give a final concentration of 1000 kallikrein inhibitor units per ml. These were centrifuged at 2000 *g* for 20 min at 4°C. The plasma was then separated into two 1.5-ml aliquots and stored under liquid nitrogen until analysed.

Plasma pool preparation

A plasma pool was prepared by taking 20 ml of blood from six normal human volunteers via an antecubital vein. This was centrifuged at 2000 *g* for 10 min at 4°C. The resultant plasma was bulked and stirred on ice for 2 min, then aliquots were taken. The aliquots were frozen in dry ice, then stored under liquid nitrogen until analysis.

Radioactivity measurements

All gamma counting was carried out using a 4-well γ-counter which was fully automated with a sample capacity of 500. Since the amount of [^{125}I]α-hANP added to each plasma was approximately 2000 cpm/ml, a counting time of 30 min was required to achieve a good precision of counting ($\chi^2 < 1\%$). This amount of radioactivity (2000 cpm/ml) is equivalent to 2 pg of unlabelled α-hANP which is within the normal range found in humans.

Selection of the column to be used in the extraction

Pool plasma was thawed and centrifuged at 2000 *g* for 10 min at 4°C. The supernatant was transferred to another tube. Known amounts of [^{125}I]α-hANP were added. This was then extracted on six different reversed-phase Bond Elut columns, *viz.* C_2, C_8, C_{18}, PH, CN and CH columns. Following activation with methanol then water, a 1-ml aliquot of plasma was applied to each column. The extracted plasma was collected and retained for γ-counting as were the wash cycles and the final eluates containing radioactive α-hANP.

Extraction procedure

Extractions were carried out using a Vac Elut device with a sample capacity of 10. The columns were first activated with 1 ml HPLC grade methanol and applying a vacuum of 35–50 kPa. The excess methanol was removed by applying 1 ml of water, again a vacuum of 35–50 kPa was applied. Plasma samples were thawed in a stream of air at room temperature (18°C) to prevent rapid thawing and were then centrifuged at 2000 *g* for 10 min at 4°C to ensure the isolation of platelet free plasma. Aliquots (1 ml) were then applied to a column and a vacumn of 85 kPa applied until all the plasma had been drawn through. Unretained proteins and endogenous material were removed by two 1-ml water washes using a vacuum of approximately 35–50 kPa. The α-hANP was eluted into conical polypropylene tubes using 0.5 ml of acetonitrile–water–trifluoroacetic acid (80:19.8:0.2, v/v/v), followed by a further 0.25 ml to ensure no α-hANP remained in the column and apparatus. To increase the precision of the extraction two Bond-Elut C_8 columns were connected together, this required increasing the activation volumes to 1.5 ml but the elution volume remained unchanged. Samples were then dried down under nitrogen and reconstituted in 250 μl RIA buffer (0.025 mol/l phosphate, pH 7.8). The tubes were vigorously mixed for 3 min and then assayed by RIA.

Radioimmunoassay

The RIA was carried out using the commercially available Amersham α-hANP kit. Standards used in the RIA were prepared by serial dilution of a supplied stock solution of α-hANP (640 fmol/ml). These ranged from 0 to 32 fmol/ml, thus samples may require dilution so that they lie within this range. The antibody was 100% crossreactive with α-hANP and was less than 0.3% crossreactive with atriopeptins 1, 2 and 3. For better sensitivity a disequilibrium technique was used which is only applicable to peptides with the approximate molecular weight of α-hANP or greater (3100 M_r). This method was carried out by adding antibody to reconstituted samples and prepared standards, then incubated at 4°C for up to 24 h. Next the tracer was added to all tubes and again incubated for up to 24 h, then the phase separation was

carried out. In this RIA care was also taken in the phase separation to prevent "stripping" of the antibody bound peptide by the use of a second antibody to the antibody–antigen complex. This antibody (Amerlex second antibody) was coated with magnetisable polymer particles and allowed the phase separation to be carried out without the use of a centrifuge but with an Amerlex-M-accessory (Amersham International). The supernatant was discarded and then γ-counted.

Recovery of α-hANP

Pool plasma was thawed and centrifuged at 2000 *g* for 10 min at 4°C. The supernatant was transferred to another tube to which sufficient ^{125}I was added to give 2000 cpm/ml. This was stirred on ice for 2 min and extracted and γ-counted as described above.

Optimisation of plasma volume

Pool plasma was thawed, centrifuged at 2000 *g* for 10 min at 4°C. The supernatant was divided into three different aliquots which were kept stirring on ice. [^{125}I]α-hANP was added to each aliquot to give final concentrations of 2000, 4000 and 8000 cpm/ml. These were extracted ($n = 6$ in each case) and counted for 30 min.

Volunteer study

Volunteers were recruited as part of a study involving low dose infusions of α-hANP. This had Medical Ethics Committee approval from the Royal Postgraduate Medical School and Hammersmith Hospital. This application shows the results from one male subject (age 29, weight 75 kg), who was infused for 3 h on two separate occasions with either α-hANP (1.2 pmol kg^{-1} min^{-1}) or 44 ml h^{-1} of 0.9% saline as vehicle. The subject was on an unrestricted salt diet, nil by mouth from 9 pm the day before being investigated. On the first day of the study the subject was seated and a cannula was inserted into an antecubital vein in both arms. One line was used for saline infusion, the other for *p*-aminohippuric acid (PAH), inulin infusion and blood sampling. On the second day of the study α-hANP was infused. The method, instrumentation and calculations used are as published in ref. 8.

RESULTS AND DISCUSSION

The impetus for carrying out this work was to devise a precise method for the extraction of α-hANP. There are currently many methods being employed[6,7]; however, the variation in the results is large and bearing in mind that we intended to carry out a low dose infusion of α-hANP we decided to develop this method.

In the first instance the manufacturer of the reversed-phase columns had to be chosen. Since Analytichem produce an Applications Kit which includes C_2 and C_8 columns not produced by Waters Assoc. this was decided upon. The results of the initial experiments to decide which of the six columns we would use are shown in Table I. These results indicate that C_8 columns would be the most appropriate as the loss when plasma is loaded is least, while the recovery from the elution step is the highest. Another contributory factor in the choice of the C_8 column was the consistently unhindered flow. Since all samples were centrifuged at 2000 *g* for 10 min at 4°C prior to extraction to remove any precipitated fibrins flow should not have been

TABLE I

RESULTS OF PHASE SCREENING

Single Bond Elut columns were used.

Column used	*Recovery*				
	loaded plasma (%)	*1 ml water wash (%)*	*1 ml water wash (%)*	*1 ml water wash (%)*	*elution (%)*
C_8	6.0	3.8	2.8	3.0	84.4
C_{18}	11.0	3.6	2.0	2.9	80.3
C_2	10.7	4.9	3.4	2.0	79.2
CN	11.9	3.1	6.3	3.8	75.0
PH	12.8	5.0	5.5	4.4	72.2
CH	15.8	4.0	5.7	6.9	67.5

obstructed. This may be due to the fact that Bond Elut columns are dry packed while Sep-Pak are pressure packed and exhibit better flow characteristics. The specification of the packing used is as follows; Bond Elut columns contain 40-μm silica particle size with a porosity of 60 Å, while Sep-Pak cartidges contain 10-μm silica particles with a porosity of 120 Å. This indicates the advantage of using Bond Elut columns as the inter-particle flow maybe increased due to larger particle size, thus high-molecular-weight proteins pass easily through the column. The lower porosity indicates a higher selectivity, as only small peptides would enter the pores, while higher porosity would facilitate entry of more contaminating peptides. For these reasons, in this methods development 100-mg Bond Elut columns were used throughout. Waters Assoc. recommend the use of positive pressure to carry out extractions using their Sep-Pak columns. As with other peptides, processing time must be kept to a minimum to avoid the introduction of error a multichannel vacuum device was employed. Since some α-hANP was lost when the plasma was applied to the activated C_8 column (Table I) we decided to connect two columns together, so that what was lost on the first would

TABLE II

RESULTS OF 1.0 ml PLASMA EXTRACTED ON C_8 COLUMNS IN TANDEM

Mean ± S.E.M. = 90.24 ± 0.61%.

Recovery				
loaded plasma (%)	*1 ml water wash (%)*	*1 ml water wash (%)*	*1 ml water wash (%)*	*elution (%)*
3.76	1.88	1.64	1.45	88.43
3.77	2.07	1.64	1.43	89.37
3.50	1.53	1.38	1.00	91.22
3.56	1.49	1.69	0.90	91.79
3.44	1.56	1.50	1.06	90.42
3.50	1.76	1.50	1.36	90.24

TABLE III

RESULTS OF 1.0 ml, 0.5 ml AND 0.25 ml PLASMA EXTRACTED ON BOND ELUT C_8 COLUMNS IN TANDEM

$n = 6$.

Plasma volume (ml)	*C.V. (%)*	*Recovery (mean ± S.E.M.) (%)*
1.00	1.50	90.24 ± 0.61
0.50	1.78	94.89 ± 0.69
0.25	2.59	89.26 ± 0.95

be retained on the second. The results of this tandem extraction of 1 ml of plasma ($n = 6$) containing 2000 cpm/ml are shown in Table II. These results showed that the method had been improved by this step, primarily because the recovery from the columns was higher, 90 ± 0.6% (mean ± S.E.M.) secondly, the precision of analysis had been increased (C.V. = 1.5%) and finally, the loss from the plasma loading step had been significantly reduced. The next task in the development was to decide the volume to be used and to accurately determine the recovery. Three volumes were tested, the results of which are shown in Table III. It was concluded that the optimum volume to be used in the assay was 1 ml of plasma at this gave the lowest C.V. (1.5%).

The increased precision of this method for extraction of α-hANP is primarily due to the use of Bond Elut C_8 columns in tandem. It gives basal values (1.5–6 pg/ml for normal volunteers remaining supine for 30 min prior to determination, $n = 25$) which are significantly lower than levels published[6,7] suggesting our method removes contaminating immunoreactive material not excluded by the previously available methods. The volume of plasma required to carry out an assay was also reduced compared to other methods which is particularly advantageous when blood loss to the subject could be detrimental as in neonates or laboratory animals, especially mice and rats.

To demonstrate the effectiveness of the extraction an infusion of α-hANP was carried out (1.2 pmol min^{-1} kg^{-1}). The results of which are shown in Tables IV and V and graphically in Figs. 1 and 2.

TABLE IV

RESULTS OF SALINE INFUSION

Na_{ex}/GFR = Sodium excretion per unit glomerular filtration rate (GFR). Cl_{Li}/GFR = Lithium clearance per unit GFR.

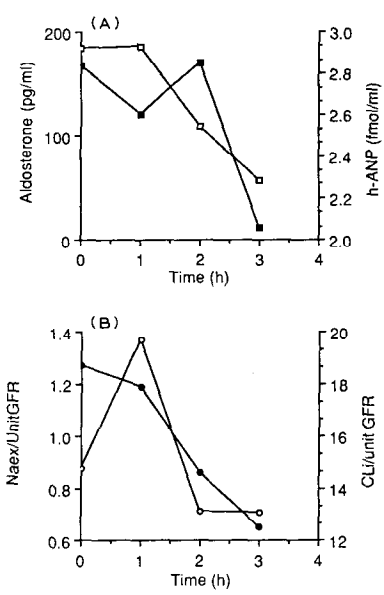
Time (h)	*Aldosterone (pg/ml)*	*α-hANP (fmol/ml)*	*Na_{ex}/GFR (μmol/mol)*	*C_{Li}/GFR (%)*
0	184	2.84	1.27	14.75
1	186	2.60	1.19	19.69
2	109	2.85	0.86	13.14
3	56	2.06	0.65	13.07

TABLE V

RESULTS OF α-hANP INFUSION

Na_{ex}/GFR = Sodium excretion per unit glomerular rate (GFR). Cl_{Li}/GFR = Lithium clearance per unit GFR.

Time (h)	*Aldosterone (pg/ml)*	*α-hANP (fmol/ml)*	*Na_{ex}/GFR (μmol/mol)*	*C_{Li}/GFR (%)*
0	266	3.90	1.46	12.39
1	158	20.58	1.51	45.81
2	96	41.08	1.98	31.02
3	62	57.76	2.20	43.94



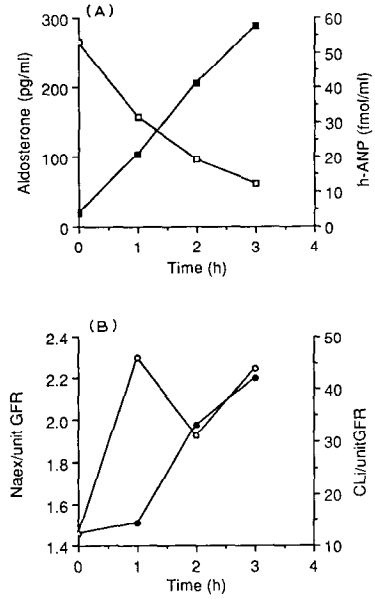


Fig. 1. (A) Graph showing the response of plasma α-hANP and aldosterone levels during an infusion of saline, (■) α-hANP and (□) aldosterone. (B) Graph showing the excretion of sodium (Na_{ex}), expressed as a fraction of the glomerular filtration rate (GFR) and lithium clearance (Cl_{Li}) again expressed as fraction of GFR, (●) Na_{ex}/GFR and (○) Cl_{Li}/GFR, respectively, during an infusion of saline.

Fig. 2. (A) Graph showing the response of α-hANP and aldosterone during an infusion of α-hANP (1.2 pmol kg^{-1} min^{-1}), (■) α-hANP and (□) aldosterone. (B) Graph showing the excretion of sodium (Na_{ex}), expressed as a fraction of the glomerular filtration rate (GFR) and lithium clearance (Cl_{Li}) expressed as a fraction of GFR, (●) Na_{ex}/GFR and (○) Cl_{Li}/GFR, respectively, during an infusion of α-hANP.

CONCLUSIONS

A reliable extraction method was developed for α-hANP in human plasma using Bond Elut bonded-phase C_8 columns. This approach gave excellent recoveries (90%) and good precision of analysis (1.5% intra assay C.V.) which can be coupled with RIA and should be useful in detecting small changes in α-hANP levels with a higher degree of confidence. Sample handling is kept to a minimum and the volume of plasma required for analysis is 1 ml although this can be reduced to 0.25 ml if repeated sampling is necessary, *i.e.* in animals with a lower blood volume than man, however the analysis would not be as precise.

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