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Adrenomedullin(1–52) measured in human plasma by radioimmunoassay: plasma concentration, adsorption, and storage

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We describe a specific and sensitive RIA for human adrenomedullin (AM)(1–52). The detection limit and the concentration required for 50% inhibition of binding were 0.1 and 1.2 fmol/tube, respectively. Cross-reactivities with AM(1–12), AM(13–52), calcitonin gene-related peptide, amylin, and other vasoactive hormones were negligible. AM immunoreactivity in normal subjects ranged from 2.7 to 10.1 pmol/L (n = 44). We investigated factors influencing the recovery and measurement of AM in the assay. Recovery of labeled AM (>80%) was markedly higher than that of unlabeled AM (56%). Immunoreactivity of exogenous AM added to plasma decreased up to 70% over four freeze–thaw cycles, whereas endogenous AM was stable. Alkali-treated casein (1 g/L) reduced adsorption of AM to surfaces and significantly increased assay precision compared with bovine serum albumin (P <0.0001). HPLC separation of extracted plasma verified the presence of AM(1–52). We suggest that considerable care is needed to ensure that accurate and reproducible results are obtained from studies quantifying this peptide.

Adrenomedullin (AM)[1] is a vasodilator peptide first isolated from human pheochromocytoma tissue in 1993 *[1]*. It has natriuretic and diuretic properties *[2, 3]* and may act in an autocrine or paracrine manner, although a physiological role for circulating AM remains possible *[4]*. Obtaining accurate measurements of the concentration of AM in plasma is therefore vital. In normal volunteers, mean plasma AM concentrations have been variably reported as 19 pmol/L *[1]*, 2–4 pmol/L *[5–8]*, or 8 pmol/L *[9]*, and urine concentrations were found to be six times higher than those in plasma *[8]*. Plasma concentrations are reportedly increased in various disease states, including congestive heart failure *[6, 10, 11]*, chronic renal failure *[8, 12]*, sepsis *[9]*, and acute myocardial infarction *[13]*. AM has structural homology with amylin, an extremely "sticky" peptide *[14]*, and thus may prove difficult to measure accurately in plasma. A number of assay systems for AM have been published in brief, but in our laboratory, most extraction methods gave low and inconsistent results. We address the difficulties associated with measuring AM in human plasma and present the steps we took to resolve them.

Materials and Methods

ASSAY REAGENTS

Human AM(1–52), AM(1–12), AM(13–52), Tyr^0-C-type natriuretic peptide 22 (Tyr^0-CNP-22), and calcitonin gene-related peptide were purchased from Peninsula Laboratories. Alkali-treated casein (ATC) was prepared according to the method of Livesey and Donald *[15]*. Bovine serum albumin (BSA), casein, aminopeptidase M, and carboxypeptidase A were obtained from Sigma Chemical Co.

The buffer (PATC) used in the assay to dilute standards and antibodies and to reconstitute extracts was 0.05 mol/L phosphate buffer, pH 7.4, with 1 g/L ATC, 1 mL/L Triton X-100, 0.1 g/L Na_2EDTA, and 0.2 g/L sodium azide.

Working standards of AM(1–52) were prepared by serial dilution of a stock standard with PATC buffer; aliquots were stored at −20 °C until the day of assay.

Antisera were prepared as follows. Human AM(1–52) (2 mg) was conjugated to BSA (4.4 mg) with use of 6.6 mg of carbodiimide *[16]* and then dialyzed and emulsified

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[1] Nonstandard abbreviations: AM, adrenomedullin; ATC, alkali-treated casein; PATC, ATC buffer (see text for composition); BSA, bovine serum albumin; TFA, trifluoroacetic acid; IC_{50}, median inhibiting concentration; irAM, immunoreactive adrenomedullin; and NSB, nonspecific binding.

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with an equal volume of Freund's complete adjuvant before being injected subcutaneously into each of four New Zealand White rabbits. Coupling efficiency, measured by incorporation of radiolabeled AM, was 35%. Each rabbit received 35 μg of AM per monthly injection for 5 months and was bled 14 days after injection. Antisera obtained on the fifth bleed from one rabbit was used in the assay at a final dilution of 1:9000.

IODINATION OF AM

We iodinated AM, using Chloramine-T. All solutions were prepared in 0.5 mol/L phosphate buffer (pH 7.5). AM (5 μL of a 1 g/L solution) was incubated with 2.5 mCi (2.5 μL) of $Na^{125}I$ and Chloramine-T (10 μL of a 2 g/L solution) at room temperature for 10 s. The reaction was stopped by the addition of cysteine (10 μL of a 10 g/L solution) followed by a mixture of 2.5 g/L BSA and 20 g/L potassium iodide (100 μL). The iodination products were immediately separated by HPLC, using a 35-min gradient of 230–330 mL/L acetonitrile in 49 mmol/L phosphate buffer, pH 2.9, on a 10-cm, 7-μm Brownlee RP300 cartridge (Applied Biosystems) with a flow rate of 1 mL/min. We collected fractions (0.5 mL) into an equal volume of PATC buffer. Each of the five iodination products (Fig. 1) was assessed for binding in the assay. Fraction 32 consistently yielded the highest zero binding; it was, therefore, diluted with PATC, aliquoted, and stored at −20 °C until used in the assay.

Carboxypeptidase and aminopeptidase digestions were used to determine whether the tyrosine moieties (three in human AM) in each of the iodination products were mono- or diiodinated. Fractions 28, 32, 34, 38, and 43 from the iodination profile (Fig. 1) were reextracted through Sep-Pak C_{18} cartridges (Waters) to remove buffer protein and then lyophilized. After reconstitution with Tris-HCl (400 μL), pH 8.0, and 1 g/L Triton X-100 (10 μL), the extracts were incubated with hydrolytic enzymes as follows. Carboxypeptidase A (6.3 g/L in 0.2 mol/L Tris-HCl, pH 8.0) was added to each of the five iodination products and incubated overnight at room temperature. The reaction was stopped with the addition of 200 mL/L acetonitrile–1 mL/L trifluoroacetic acid (TFA) (40 μL), and the solution was stored at −80 °C. The iodination products in fractions 32 and 38 and ^{125}I-labeled Tyr^{o}-CNP-22, were also incubated separately with aminopeptidase M (100 g/L in 0.2 mol/L Tris-HCl–5 mmol/L EDTA, pH 7.4) overnight at 37 °C. The reaction was stopped, and the sample was stored at −80 °C. For HPLC, the solutions were thawed, and the iodotyrosine residues were separated on a Brownlee RP300 (22.5 × 0.46 cm, 7 μm) column using a gradient of 120–420 mL/L acetonitrile in 1 mL/L TFA and a flow rate of 1 mL/min for 30 min. Products were detected by a UV spectrophotometer at 210 μm and identified by comparison with standards of mono- and diiodotyrosine.

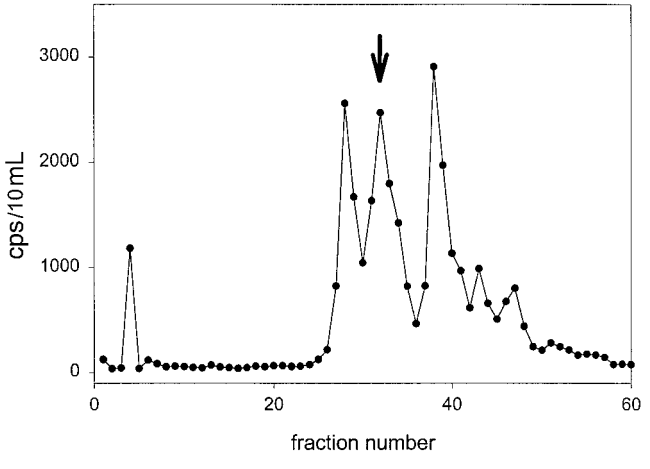

Fig. 1. Typical HPLC profile obtained from iodination of human AM.

Details of the iodination procedure and HPLC separation are given in the text. The *arrow* indicates the fraction (*32*) used as tracer in the assay.

SPECIFICITY

We measured the cross-reactivity of our AM antiserum by assaying serial dilutions (0.1 μmol/L to 1 pmol/L) of the following standards: AM(1–52), AM(13–52), AM(1–12), calcitonin gene-related peptide, and amylin. In addition, we assayed standards of other vasoactive hormones (human atrial natriuretic peptide, human brain natriuretic peptide, human C-type natriuretic peptide, ouabain, endothelin, angiotensin II, and angiotensin I) at 0.1 μmol/L.

SAMPLE COLLECTION

The protocols involving patients and healthy volunteers were approved by the Southern Regional Health Authority Ethics Committee (Canterbury, New Zealand). Fully informed consent was obtained from all subjects before blood collection or intravenous AM infusion. We collected venous blood samples into EDTA (1.5 g/L) on ice and centrifuged them (1500*g* for 15 min at 4 °C) within 30 min. Plasma was separated and stored at −80 °C.

EFFECT OF HEMOLYSIS

To assess the effect of hemolysis on apparent plasma AM concentrations, erythrocyte lysate prepared from packed erythrocytes lysed by freezing and thawing was added to plasma samples (2.5–320 μL/mL of plasma) before the final extraction and assay.

FINAL EXTRACTION METHOD

Plasma samples (routinely 1 mL) were mixed with an equal volume of PATC buffer and loaded onto Sep-Pak C_{18} cartridges that had been prewashed with methanol and saline (9 g/L). Plasma was applied under gravity to the cartridge, which was subsequently washed with saline (NaCl 9 g/L) under reduced pressure (<17 kPa). AM was eluted with 2 mL of 800 mL/L isopropanol–0.013 mol/L HCl under reduced pressure (<17 kPa). Triton X-100 (10 mL/L, 10 μL) was added to each extract, which was then dried under air (37 °C). Extracts were stored at −20 °C until the day of assay.

Extraction efficiency was measured by the addition of labeled or unlabeled AM to plasma or buffer. Recovery was calculated from the amount of AM measured in the extract compared with a control of buffer with the same standard added and assayed or counted without extraction.

FINAL RIA METHOD

Before they were assayed, extracts were reconstituted with 250 μL of PATC buffer and 20 μL of 0.75 mol/L NaOH, vortex-mixed, and centrifuged (1500*g* for 5 min at 4 °C). Standards and extracts (50 μL) were mixed with 50 μL of antiserum (1:3000 working dilution) on ice. After a 3-h preincubation at 4 °C, ^{125}I-labeled AM (2000 counts/min per 50 μL) was added to each tube, and the tubes were incubated at 4 °C for 16–24 h. Bound counts were separated from free counts by using goat anti-rabbit γ-globulin and normal rabbit serum (Peninsula Laboratories). After centrifugation, the pellet was counted for radioactivity (Gammamaster; LKB Wallac). Results were interpolated from a computer-fitted standard curve, using a four parameter logistic fit function *[17]*, or from segmentally linearized standard curves *[18]*. All studies in this paper used the above assay format. However, subsequent experimentation has shown that omitting the 3-h preincubation and substituting a 24-h incubation of sample, antiserum, and tracer reduced the zero binding by only 2% and minimally increased the 50% inhibition concentration (IC_{50}) from 1.2 to 1.6 fmol/tube. Because of its relative simplicity, we currently use the latter format.

PHARMACOKINETICS AND HPLC

Human AM(1–52) was diluted in Hemeaccel (Behring) before incremental intravenous infusion into a forearm vein of a healthy human subject at 8, 16, and 32 $ng \cdot kg^{-1} \cdot min^{-1}$ each for 60 min. Blood samples were taken from a forearm vein in the opposite arm every 30 min during the infusion, with rapid serial sampling immediately after the cessation of the high dose, as described previously *[19]*. These samples were extracted and assayed as detailed above.

Additional plasma samples (3 mL) obtained on a control day (Hemeaccel alone infused) and during the high-dose AM infusion (2 mL) were extracted by using the final extraction method and then reconstituted with 240 mL/L acetonitrile in 1 g/L TFA before being loaded onto a Brownlee RP18 reversed-phase column (22.5 × 0.46 cm, 7 μm, 40 °C; flow rate, 1 mL/min). Extracted peptides were separated by using a 30-min gradient of 240–540 mL/L acetonitrile in 1 g/L TFA. Fractions (0.5 mL) were collected for the initial 30 min of the HPLC run. Triton X-100 (10 mL/L; 10 μL/fraction) was added before fractions were dried under air at 37 °C and stored at −20 °C. On the day of assay, fractions were reconstituted with 250 μL of PATC buffer. Standard AM, buffer (5 mL), and extracts (obtained from 5 mL of plasma) from patients with congestive heart failure and normal human volunteers were also analyzed by HPLC.

STATISTICS

Differences between methods were assessed for statistical significance by using Student's *t*-test. Differences between standard curves were assessed by ANOVA. Results are given as mean ± SE.

Results

A typical calibration curve for AM, obtained by using the final RIA protocol described in *Materials and Methods*, is shown in Fig. 2. Human plasma drawn from normal subjects, patients with congestive heart failure, and normal subjects during infusion of human AM(1–52) diluted in parallel with the standard curve. Our assay detection limit (calculated as 2 SD from zero binding) 0.1 fmol/tube (corresponding to an in vivo plasma concentration of 0.5 pmol/L when 1 mL of plasma was extracted), and our IC_{50} was 1.2 fmol/tube (6 pmol/L when 1 mL of plasma was extracted).

AM(1–12), AM(13–52), calcitonin gene-related peptide, amylin, and other vasoactive compounds listed in *Materials and Methods* did not cross-react with our antibody.

HPLC analysis of carboxypeptidase-digested iodination products revealed that they all contained a monoiodotyrosine on the C terminus and at least one other labeled tyrosine. We subsequently incubated fractions 32 and 38 with aminopeptidase. Confirmation of the activity of our aminopeptidase preparation was shown by the release of monoiodotyrosine after incubation of ^{125}I-labeled Tyr^{o}-CNP-22 with this enzyme. No new radioactive products were released after incubation of fractions 32 and 38. We concluded that the N-terminal tyrosine of the iodination products was not iodinated, that the C-

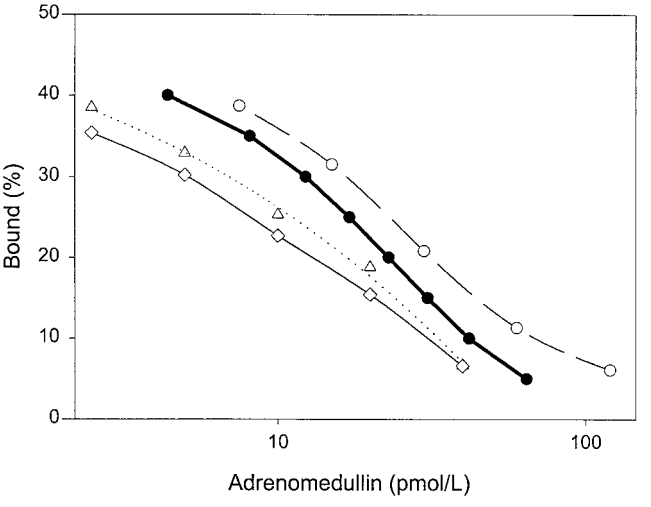

Fig. 2. Typical calibration curve for human AM(1–52) showing parallelism with serially diluted human plasma samples.

(●), calibration curve; (○), plasma from a patient with congestive heart failure; (△), a healthy volunteer; (◇), a healthy volunteer infused with human AM (1–52).

terminal tyrosine was monoiodinated, and that at least one other monoiodinated tyrosine was present in each product, presumably the internal tyrosine.

We assessed assay precision by repeated extraction and assay of three human plasma samples, each measured at least 24 times in duplicate. A nonextracted buffer sample supplemented with unlabeled AM was also assessed to determine assay variation in the absence of extraction variation. Mean (± SE) results were 3.7 ± 0.1, 11.5 ± 0.6, and 23.7 ± 0.7 pmol/L for the plasma samples and 10.7 ± 0.2 pmol/L for the nonextracted buffer sample. Corresponding intraassay CVs for extracted plasma samples were 6.2%, 5.7%, and 8.2%, and interassay CVs were 18.5%, 26.7%, and 17.0%, respectively. The intraassay CV for the supplemented buffer sample (5.8%) was similar to extracted plasma samples, whereas the interassay CV was lower (8%), indicating a degree of batch-to-batch variability in the extraction process.

Recovery of unlabeled AM added to human plasma was 56% ± 1.9% (range, 40–75%; n = 25 assays) when the final extraction method described in *Materials and Methods* was used, whereas recovery of ^{125}I-labeled AM was 92% ± 2.9% (range, 80–100%; n = 10). Linearity between the volume of plasma extracted (0.5–2.0 mL) and the amount of irAM measured was observed; the slope of the relationship between measured and expected irAM was 0.98, and the correlation coefficient was 0.995.

Addition of up to 40 μL of erythrocyte lysate per milliliter of plasma, although it produced a dark red sample, had no effect on the measured AM concentration. Addition of higher concentrations of cell lysate (80 μL or 320 μL/mL plasma) decreased AM immunoreactivity up to 20% and 75%, respectively.

OPTIMIZATION OF THE EXTRACTION TECHNIQUE

Nonspecific binding (NSB) of AM to extraction tubes. We assessed NSB of labeled AM to polystyrene, polypropylene, and glass test tubes by adding ^{125}I-labeled AM in assay buffer with or without 1 mL/L Triton X-100 and containing 1 g/L ATC, 1 g/L BSA, 5 g/L BSA, or no protein. After 1 or 21 h at room temperature, the buffer was removed from the tubes and the remaining radio activity was counted. NSB of AM to polystyrene or polypropylene tubes was <3%, irrespective of the protein composition of the buffer. NSB increased when glass tubes were used, with 1 g/L ATC being the protein additive most effective in preventing NSB (23%), compared with 1 g/L BSA, 5 g/L BSA, or no protein (NSB of 78%, 84%, and 79%, respectively). In the absence of Triton X-100, 5 g/L BSA and 1 g/L ATC were equally effective at preventing binding of labeled AM to tubes, although in all cases NSB was >30%.

When 1 g/L BSA (n = 9 assays) or 5 g/L BSA (n = 18 assays) was used (both with 1 mL/L Triton X-100), we observed unacceptably high CVs for points on the standard curve. In contrast, ATC (1 g/L), in conjunction with 1 mL/L Triton X-100 (n = 16 assays), significantly decreased this variability (P <0.0001). In addition to its effect on precision, we found that ATC also improved the assay NSB (3.4% ± 0.23%) when compared with 1 g/L BSA and 5 g/L BSA (12.1% ± 0.7% and 4.7% ± 0.4%, respectively). For these reasons, we used PATC buffer in all subsequent assays.

Some authors have used high concentrations of Triton X-100 in their AM assays *[5, 7]*, presumably to overcome NSB and precision difficulties. However, we found that increasing the Triton X-100 concentration in the buffer from 1 mL/L to 5 mL/L caused loosening of the pellet after centrifugation in the second antibody separation phase of the assay, and this tended to reduce assay precision. As a consequence, we routinely used 1 mL/L Triton X-100 in the assay buffer.

Adsorption to extraction cartridges. Various groups have published extraction methods where saline/HCl *[7]* or 1 mL/L TFA *[8]* solutions were mixed with plasma before extraction. We compared the losses of ^{125}I-labeled AM due to adsorption to sample tubes and plasma precipitates after acidification or dilution of plasma. NSB of ^{125}I-labeled AM to polystyrene sample tubes was greater (9–12%) when plasma was acidified before extraction than when it was diluted with PATC buffer or 1 mL/L Triton X-100 (1%). Centrifugation (1500*g*, 5 min, 4 °C) of the plasma solution increased NSB to 18% in samples that had been acidified and to 1.5% and 2.6% for samples mixed with PATC buffer and 1 mL/L Triton X-100, respectively. Presumably, this increased NSB is due to sedimentation of precipitates containing adsorbed ^{125}I-labeled AM. Adding an equal volume of buffer to the sample before extraction also improved recovery of ^{125}I-labeled AM from 66% to 93% (n = 3).

Cartridge washes. Saline, TFA, water, and other solvents have been used to wash cartridges during the extraction of AM *[7, 8, 20]*. We did not detect any difference in the recovery of unlabeled AM between these wash methods when they were used in extractions, as described in the final extraction method in *Materials and Methods*, although TFA or low concentrations of acetonitrile (100–200 mL/L) tended to increase the elution of ^{125}I-labeled AM.

Eluting solvents. Acetonitrile/TFA *[7, 8]* and methanol/TFA *[6]* combinations have been used to elute AM from extraction cartridges. Under the final extraction method conditions described above, recoveries of ^{125}I-labeled AM were consistently >80% when 800 mL/L isopropanol–0.013 mol/L HCl (2 mL), 600 mL/L acetonitrile–1 mL/L TFA (4 mL), or 950 mL/L methanol–10 mL/L TFA was used as the eluting solvent. Recovery of unlabeled AM, although lower than that of labeled AM (40–65%), was also similar with all three solvent combinations.

Storage of AM in plasma. Plasma irAM concentrations, whether endogenous or added, did not change for up to 6 months at either −20 °C or −80 °C. Similarly, loss of

immunoreactivity was not detected after storage at room temperature or 4 °C for 3 h, although up to 20% irAM was lost after 24 h in both cases.

Endogenous AM immunoreactivity in plasma was stable for at least four freeze–thaw cycles, but when unlabeled AM was added to plasma, immunoreactivity decreased up to 70% after a single freeze–thaw cycle and up to 90% after subsequent freeze–thaw cycles.

In contrast, AM in plasma extracts remained stable for at least two freeze–thaw cycles, and there was no substantial loss of irAM at −20 °C for extracts that were stored dry or reconstituted in PATC buffer (n = 6/treatment).

Separation of bound from free AM. We assessed various methods for separating antibody-bound AM from free AM, including the use of a second-antibody precipitation (goat anti-rabbit immunoglobulin/normal rabbit serum), solid-phase second antibody (SacCell; Wellcome Diagnostics), and dextran-coated charcoal. SacCell was unsatisfactory, giving NSB values of up to 23%. Charcoal separation tended to strip the tracer from the antisera, even at low charcoal (0.6 mg/tube) concentrations. These problems were not encountered with goat anti-rabbit immunoglobulin/normal rabbit serum, which was therefore preferred.

ENDOGENOUS AND EXOGENOUS PLASMA AM CONCENTRATIONS

The mean plasma AM concentration in normal volunteers (n = 44; ages 23–62 years; on no medication) was 6.09 ± 0.3 pmol/L, with a range of 2.7–10.1 pmol/L. Infusion of human AM(1–52) into a normal volunteer caused a dose-dependent increase in plasma AM immunoreactivity (Fig. 3). This was followed by a sharp decline in immunoreactivity immediately after the cessation of infusion.

HPLC analysis of AM in human plasma. Reversed-phase HPLC analysis showed that standard AM eluted at fractions 27–30. Immunoreactivity eluting at the same time as standard AM was observed in fractions obtained from plasma extracts from a healthy volunteer, a patient with heart failure (Fig. 4a), and from a volunteer infused with AM (Fig. 4b). No AM was observed after a control injection of extracted buffer. The recovery of injected AM through HPLC followed by RIA was 70–90%. A late-eluting peak of unknown identity was periodically detected at fractions 44–48.

Discussion

Improved understanding of the biochemistry and physiology of AM has occurred since this 52-amino acid peptide was discovered in 1993. The need for accurate measurement of AM in plasma will grow as studies on its production in human and animals and its role in various disease states progress. Although others have published methods for the measurement of AM in human and animal plasma, none have been in detail and with substantial validation, nor have they drawn attention to the

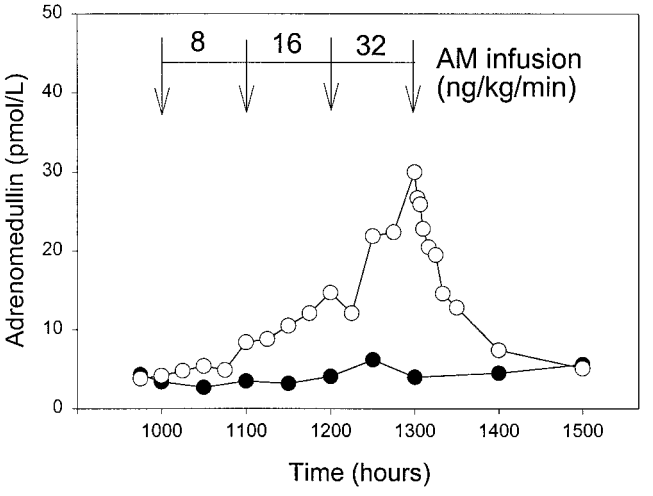

Fig. 3. AM concentrations obtained in extracts of human plasma during infusion of AM(1–52) into a normal human volunteer at rates of 8, 16, and 32 ng · kg^{-1} · min^{-1} for 1 h each.
(●), control infusion; (○), active infusion.

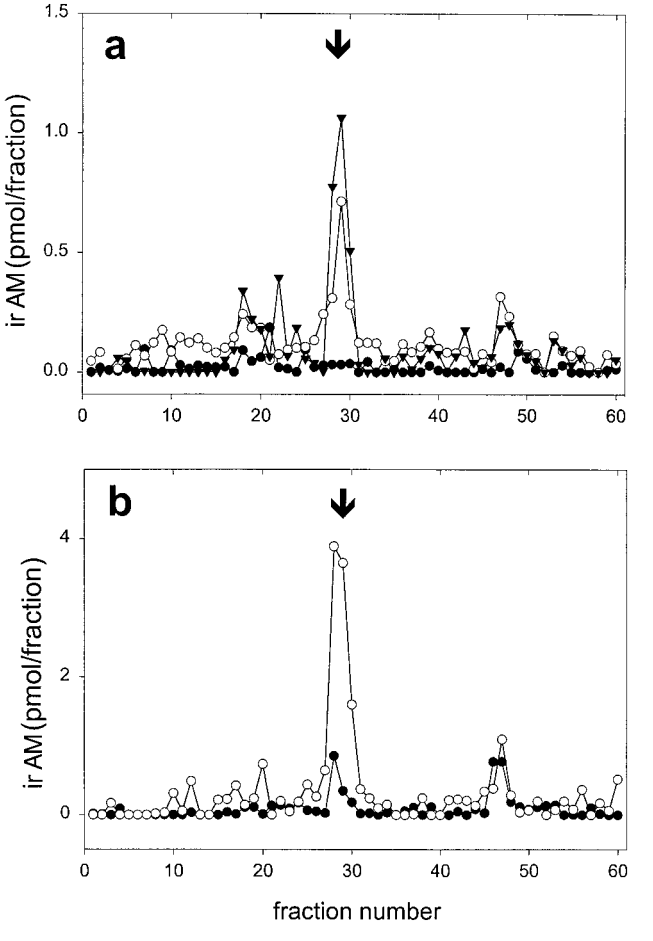

Fig. 4. HPLC profiles of plasma extracts.
(*a*) (○), normal human volunteer; (▼), volunteer with congestive heart failure; (●), buffer injection. (*b*) Volunteer infused with 8 ng · kg^{-1} h^{-1} AM: (●), preinfusion; (○), during infusion. The elution time of standard human AM(1–52) is indicated by the *arrows*.

numerous pitfalls associated with measuring this peptide. Furthermore, there has been no systematic investigation of factors affecting the performance of AM assays. The importance of understanding and controlling assay variables is demonstrated by the wide variation in plasma AM concentrations reported for normal subjects (ranging from 2.1 ± 0.7 pmol/L *[8]* to 19 ± 5.4 pmol/L *[1]*) as well as those reported between different laboratories using the same method *[8, 9]*.

In our final assay format, our assay detection limit was low (0.1 fmol/tube), as was our IC_{50} (1.2 fmol/tube). Intraassay CVs ranged from 5.7% to 8.2% but, as discussed below, the interassay CVs were somewhat higher (8.0–26.7%) due to batch-to-batch variations in the extraction process. We established a normal range of 2.7–10.1 pmol/L, with a mean ± SE of 6.09 ± 0.3 pmol/L.

Like the related peptide amylin, AM has a tendency to adhere nonspecifically to surfaces, a property that can markedly affect recovery and NSB in the assay. We assessed AM adsorption to surfaces commonly used in RIA and found that 1 mL/L Triton X-100 was adequate to prevent ^{125}I-labeled AM adsorption to polystyrene or polypropylene tubes but not glass. Inclusion of up to 5 g/L BSA in addition to Triton X-100 did not further decrease NSB. However, inclusion of 1 g/L ATC, a protein shown previously to be more effective than BSA at preventing the NSB of other peptide hormones *[15]*, substantially decreased the amount of ^{125}I-labeled AM binding to glass tubes. Our results suggest that glass tubes should be avoided in assays of AM.

After these early studies, we investigated factors affecting the recovery of AM from plasma extracted on Sep-Pak C_{18} cartridges. Recovery of AM can be decreased by adsorption to the sample tube before extraction, poor adsorption to the cartridge, elution off the cartridge by the wash solvent, or by inadequate elution off the cartridge by the eluting solvent. We investigated these conditions, using (in most cases) both labeled and unlabeled AM for measurement. Losses of AM by adsorption from plasma onto the sample container was <2% with human plasma mixed with PATC buffer or with 1 mL/L Triton X-100 alone but was >5% with saline/HCl or TFA. We routinely add PATC buffer to all plasma samples before extraction. One of the disadvantages of using ATC in the assay buffer is that it precipitates at pH 4. Because we used an acidic eluting solvent, it was necessary to add alkali to reconstituted extracts to bring the pH to neutral and to redissolve a precipitate of ATC. We are currently investigating the use of Triton X-100 alone.

After loading the plasma onto the cartridge, a variety of wash steps might be used. In our laboratory, TFA or low concentrations (100–200 mL/L) of acetonitrile caused ^{125}I-labeled AM to elute from the cartridge at a higher rate than did saline washes. In contrast, recoveries of plasma supplemented with cold AM were not altered by the composition of the wash solvent. We decided to use saline as a wash solvent primarily because of its low cost and absence of toxicity. Elution of ^{125}I-labeled AM from Sep-Pak cartridges was similar with isopropanol/HCl, acetonitrile/TFA, and methanol/TFA. We chose the isopropanol reagent because it avoided the toxicity problems associated with acetonitrile and methanol.

In the RIA phase, we encountered unacceptably high NSB and poor precision in our assay duplicates when 1 g/L BSA plus 1 mL/L Triton X-100 was used to prevent nonspecific adsorption of AM. These characteristics were improved by increasing the BSA concentration in the buffer to 5 g/L. Other groups *[5, 7]* routinely use 5 mL/L Triton X-100 in their assay buffer; however, we found that increased Triton X-100 destabilized the pellet obtained during the assay separation, resulting in little or no improvement in precision. Inclusion of 1 g/L ATC and 1 mL/L Triton X-100 markedly improved assay precision and also reduced NSB. Because we had already shown that 1 mL/L Triton X-100 was adequate to prevent NSB to polystyrene assay tubes, it is most likely that ATC acts at the assay separation phase. The second-antibody separation method generates a precipitate with a large surface area, to which we presume free AM adsorbs. ATC probably reduces adsorption to the precipitate, improving the NSB and precision (duplicates) simultaneously. Similar effects are apt to occur in other second-antibody separation methods. We obtained high NSB with SacCell separation also, although we did not examine the effect of ATC on precision with this method. Adsorption of AM to precipitates is likely to affect many aspects of AM RIA and should be considered in their design. For example, we found a much greater loss of labeled AM (up to 12%) in plasma samples acidified/diluted with TFA or saline/HCl compared with PATC buffer. These losses probably occur through adsorption of AM to the proteins precipitated during the acidification process.

Recovery of ^{125}I-labeled AM was 80–100% when it was added to plasma and was closer to 90% when it was added to buffer. However, when unlabeled AM was added to plasma and extracted, the recovery decreased to 56%. The higher recovery of ^{125}I-labeled AM (up to 30% higher than recovery of unlabeled AM) indicates that iodinated AM has physical properties and activities different from those of the noniodinated form. This highlights the importance of determining the recovery of unlabeled hormones from plasma, at least in the case of AM.

We have shown that recovery of endogenous AM from plasma is not altered by up to four freeze–thaw cycles on plasma or two cycles on extracts stored at −20 or −80 °C. However, recovery of exogenous AM is markedly reduced after a single freeze–thaw cycle. Exogenous AM was added to plasma samples at 4 °C, with mixing, for 30 min before samples were aliquoted and stored frozen. A protein may be present in circulation that can bind AM with low affinity, aiding in its stability in plasma; 30 min may not be sufficient for exogenously added AM and this binding protein to attain equilibrium. Preliminary data from our laboratory suggest that the AM concentration of

plasma samples obtained from individuals after infusion of AM does not change with a single freeze–thaw cycle.

Endogenous AM in plasma from normal subjects, patients with heart failure, and from a healthy volunteer infused with synthetic AM had elution times identical to standard human AM(1–52) when analyzed by reversed-phase HPLC coupled with RIA (Fig. 4). Another, later-eluting immunoreactive peak was also detected in some cases (fractions 44–50), the identity of which is currently under investigation.

In summary, we have developed a sensitive and specific method for measuring AM in human plasma. In our laboratory, assay precision was markedly improved by using ATC as the protein component of the RIA buffer. We have shown that AM will readily adhere to surfaces and that endogenous AM appears more stable than exogenous AM in plasma, factors that should be considered when handling this peptide. Although we have highlighted some of the difficulties associated with measuring this peptide, many questions remain to be answered in pursuit of the "perfect" assay. For example, why do endogenous and exogenous AMs in plasma have different storage characteristics? How can adsorption of AM to surfaces be minimized? What effect will these factors have on results obtained from infusion of AM into volunteers, particularly with respect to calculations of metabolic clearance rates? Clarification of these issues by providing improved assays for plasma AM will increase understanding of the dynamics of this hormone in circulation.

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