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Assay for the Measurement of Copeptin, a Stable Peptide Derived from the Precursor of Vasopressin

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Background: **Arginine vasopressin (AVP) is a key regulator of water balance, but its instability makes reliable measurement difficult and precludes routine use. We present a method for quantifying AVP release by use of copeptin, a glycopeptide comprising the C-terminal part of the AVP prohormone.**

Methods: **We measured copeptin in 50-μL serum and plasma samples from healthy individuals and from critically ill patients with sepsis. Our sandwich immunoluminometric assay used 2 polyclonal antibodies to amino acids 132–164 of pre-provasopressin.**

Results: **The assay yielded results within 3 h. The analytical detection limit was 1.7 pmol/L, and the interlaboratory CV was <20% for values >2.25 pmol/L. The assay was linear on dilution of the analyte. Ex vivo copeptin stability (<20% loss of analyte) for at least 7 days at room temperature and 14 days at 4 °C was shown for serum and EDTA-, heparin-, and citrate plasma. Copeptin (median, 4.2 pmol/L; range, 1–13.8 pmol/L) was detectable in 97.5% of 359 healthy individuals and was not associated with age. Median concentrations were considerably higher in men than women, increased significantly after exercise, and were influenced by fasting and water load. Copeptin was significantly (P <0.001) increased in 60 critically ill patients with sepsis (median, 79.5 pmol/L; range, 10.6–228.0 pmol/L). The correlation between copeptin and AVP for 110 samples was r = 0.78 (P <0.0001).**

Conclusions: **Copeptin is stable for days after blood withdrawal and can be quickly and easily measured. The copeptin assay may be a useful alternative to direct measurement of AVP concentration.**

Arginine vasopressin (AVP), also termed antidiuretic hormone, is a nonapeptide produced in the hypothalamus. AVP is released from the neurohypophysis into the blood to induce water conservation by the kidney, contributing to the regulation of osmotic and cardiovascular homeostasis *(1, 2)*. AVP is derived from a larger precursor peptide (pre-provasopressin) along with 2 other peptides, neurophysin II and copeptin (Fig. 1) *(3)*. Neurophysin II has a complex structure with many putative intramolecular disulfide bonds, and it may be associated with AVP during maturation and transport *(4)*. Copeptin, the C-terminal portion of provasopressin, is a 39-amino acid glycopeptide of unknown function *(5, 6)*. Copeptin may have a role during the intracellular processing of provasopressin, possibly contributing to correct structural formation of the precursor, which leads to efficient proteolytic maturation *(7)*.

We recently characterized the structure of circulating copeptin *(8)*. With size-exclusion chromatography, we demonstrated the molecular mass to be ~5 kDa, which is in accordance with the theoretical prediction for processed copeptin (4021 Da for the amino acid constituents plus a sugar moiety). Lectin chromatography revealed that serum copeptin is indeed glycosylated and contains a sugar moiety *(8)*.

The diagnostic use of AVP in certain endocrine disorders has been described *(9–11)*. The molecule has recently gained additional interest as a supplementary vasopressor agent in the treatment of septic shock *(12, 13)*, in which endogenous concentrations of AVP apparently decrease during the progression of shock *(14)*. The measurement of endogenous AVP in plasma is a useful tool to guide therapy in pathologies in which osmotic and cardiovascular homeostasis are disturbed *(9)*. Serious concerns exist, however, about the methodologic reliability of plasma AVP measurements because AVP is known to be

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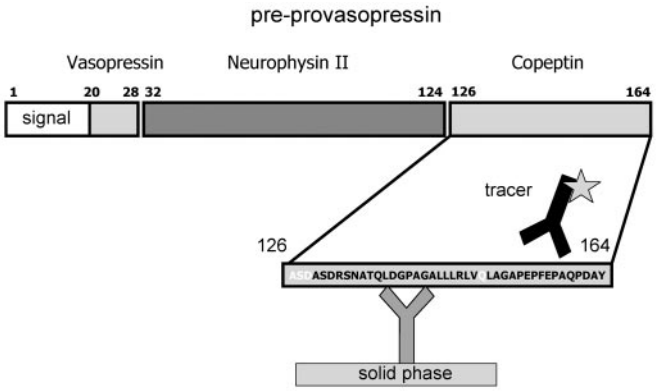

Fig. 1. Sequence of pre-provasopressin.

Numbers indicate amino acids. *Signal*, signal peptide. The assay principle for copeptin is also shown. *Tracer*, labeled antibody; *solid phase*, antibody coated on tubes. Single-letter amino acid sequence for copeptin is shown.

unstable, largely attached to platelets, and rapidly cleared *(15, 16)*. The complex preanalytical requirements and lack of readily available and fast AVP assays have limited the clinical use of AVP measurements. Because of its stoichiometric generation, copeptin should represent the release of AVP, a situation similar to that of C-peptide and insulin; we therefore investigated the immunometric quantification of copeptin in human serum and plasma as an alternative method for AVP measurement.

Materials and Methods

PEPTIDES

Three peptides related to copeptin were chemically synthesized, purified, and quality-controlled by JERINI AG. The peptides were PATV17 (sequence CATQLDGPAGALLLRLV, representing positions 132–147 of pre-provasopressin plus an N-terminal cysteine residue), PLAY17 (sequence CLAGAPEPFEPAQPDAY, representing positions 149–164 of pre-provasopressin plus an N-terminal cysteine residue), and PAY33 (sequence ATQLDGPAGALLLRLVQLAGAPEPFEPAQPDAY, representing positions 132–164 of pre-provasopressin).

ANTIBODIES

Sheep antisera containing antibodies directed against peptides PATV17 and PLAY17, and generated according to standard procedures, were purchased from Micropharm Ltd. Briefly, peptides were conjugated via *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester to keyhole limpet hemocyanin, and sheep were immunized with 100 μg of peptide, initially in its conjugate form, and with 50 μg in 4-week intervals thereafter. Antisera were obtained starting 3 months after initial immunization. For the purification of peptide-specific antibodies, 5 mg each of peptides PATV17 and PLAY17 were immobilized on SulfoLink gel (Pierce Biotechnology) according to the manufacturer's instructions. Affinity purification was performed as follows: 50 mL of antiserum was diluted with 50 mL of binding buffer [100 mmol/L potassium phosphate, 1 mL/L Tween 20 (pH 6.8), 1 tablet of Complete Protease Inhibitor (Roche) per 50 mL] and incubated according to the manufacturer's instructions. The gel was washed with 300 mL of binding buffer. Bound antibodies were eluted with 50 mmol/L citric acid (pH 2.2) and neutralized with 50 mmol/L potassium phosphate (pH 7.4) and NAP size-exclusion chromatography (Amersham), according to the instructions of the manufacturer. The homogeneity of the antibody preparations was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and the protein concentrations were measured by the bicinchoninic acid method (Pierce).

IMMUNOASSAY

We set up a chemiluminescence sandwich immunoassay with coated tubes, as follows: Purified anti-PLAY17 antibody (1 g/L) was labeled by incubation with a 1:2 molar ratio of MACN-Akridinium-NHS-Ester (1 g/L; InVent GmbH, Germany) for 15 min at room temperature. The reaction was stopped by addition of 1/10 volume of 1 mol/L Tris (pH 7.8), and labeled antibodies were separated from label-free antibodies by size-exclusion chromatography with a Protein-Pak SW300 HPLC column (Waters). Tracer was produced by diluting the labeled antibody into assay buffer (300 mmol/L potassium phosphate, 50 mmol/L NaCl, 10 mmol/L sodium EDTA, 1 g/L bovine serum albumin, 1 g/L nonspecific sheep IgG, 1 g/L nonspecific bovine IgG, 0.9 g/L sodium azide, pH 7.4) to achieve a concentration of 1 000 000 relative light units per 200 μL, determined by a LB952T luminometer (Berthold). Polystyrene tubes (Greiner) were coated with anti-PATV17 antibody (per tube, 2 μg/0.3 mL of 100 mmol/L Tris, 50 mmol/L NaCl, pH 7.8) overnight at room temperature, and tubes were blocked with 10 mmol/L sodium phosphate, 30 g/L Karion FP, 3 g/L bovine serum albumin (pH 6.5), and then lyophilized. Dilutions of peptide PAY33 in normal horse serum (Sigma) served as calibrators. The immunoassay was performed by incubating 50 μL of samples/calibrators and 200 μL of tracer in coated tubes under agitation (170–300 rpm) for 2 h at room temperature (18–24 °C). Tubes were washed 4 times with 1 mL of LUMItest wash solution (B.R.A.H.M.S AG), and bound chemiluminescence was measured for 1 s per tube with a LB952T luminometer (Berthold).

AVP ASSAY

AVP was measured with a commercial RIA (DRG Diagnostics), according to the manufacturer's instructions. Briefly, all samples were measured as a batch in 1 assay run. From each patient sample, 1 mL of EDTA plasma was obtained by centrifugation immediately after blood withdrawal. After ethanol extraction and evaporation, the sample was reconstituted in assay buffer (phosphate-buffered saline containing protease inhibitors) and incubated with AVP antiserum overnight at 4 °C. After addition of ^{125}I-labeled AVP, samples were incubated for 1 h at

4 °C, and then the antigen–antibody complex was precipitated by centrifugation with a goat anti-rabbit antibody coupled to a solid-phase suspension. After the supernatant was discarded, the precipitated complex was counted in a gamma counter for 2 min. The range of the calibration curve was 1.9–60 pmol/L. According to the manufacturer, the analytical detection limit (counts/min of zero calibrator plus 3 SD) is 0.5 pmol/L, and the intraassay CV is 6.5% at 4.1 pmol/L and 4.9% at 20.2 pmol/L. Data below the analytical detection limit were set to 0.5 pmol/L. All AVP measurements were performed in the laboratory of Professor Siegfried Schwarz, Institute of Pathophysiology, Innsbruck Medical University (Innsbruck, Austria).

PATIENTS AND CONTROLS

Serum and plasma samples (EDTA, citrate, and heparin) from healthy individuals were collected from the members of a local health club. Participants had to be without clinical evidence of acute disease or a history of chronic illness. Of ~900 regular members, 326 showed interest in participation; 62 of these had to be excluded because of a history of cardiovascular disease, diabetes, autoimmune disease, cancer, or infections within the last 3 months. Written consent was obtained from the remaining 264 participants. We used the same exclusion criteria to recruit another 95 healthy individuals from the employees of a local biotechnology center. Six men and 6 women volunteer blood donors performed a bicycle exercise test, starting with 50 W and increasing in 50-W steps every 3 min until physical exhaustion or maximum exercise activity. Another 6 participants fasted overnight, consumed a 1-L water load the next morning, and underwent subsequent measurements for the remainder of the working day. All blood samples were obtained with peripheral venipuncture and, within 1 h of collection, were centrifuged and frozen in aliquots at −20 °C.

Samples from intensive care unit patients with sepsis, severe sepsis, or septic shock [as defined by the American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference *(17)*] were collected in accordance with ethics guidelines and were stored at −20 °C until further use.

STATISTICAL ANALYSIS

We performed all statistical analyses with GraphPad Prism 4.0. Distribution was tested with the Kolmogorov–Smirnov test. We performed comparisons of parametric data with an unpaired *t*-test or ANOVA and nonparametric data with the Mann–Whitney *U*-test, Wilcoxon signed-rank test, or Kruskal–Wallis test. Correlation was done by Spearman rank correlation. P values <0.05 were considered significant.

Results

TECHNICAL CHARACTERISTICS OF COPEPTIN ASSAY

Calibration. For the highest calibrator (S6), chemically synthesized PAY33 (amino acids 132–164 of pre-provasopressin) peptide was added to horse serum at a concentration of 1215 pmol/L and then diluted to prepare calibrators (S1 to S5) with final concentrations of 5, 15, 45, 135, and 405 pmol/L. As controls, pools of human sera containing 10 pmol/L (control I) and 70 pmol/L (control II) copeptin were added at the beginning and end of each assay run. A typical calibration curve is shown in Fig. 2A.

Measuring range and precision. The lower detection limit of 1.7 pmol/L was determined with horse serum (mean relative light units of 10 determinations plus 2 SD). The total assay imprecision was determined by measuring, in duplicate, 22 human serum samples with concentrations of 2.1–246.7 pmol/L. These data were generated by 6 different operators in 12 assay runs, with 2 different lots of reagents in 2 different laboratories. The interlaboratory CV was <20% for all samples >2.25 pmol/L and <15% in 20 of 22 samples with values >4.0 pmol/L (Fig. 2B).

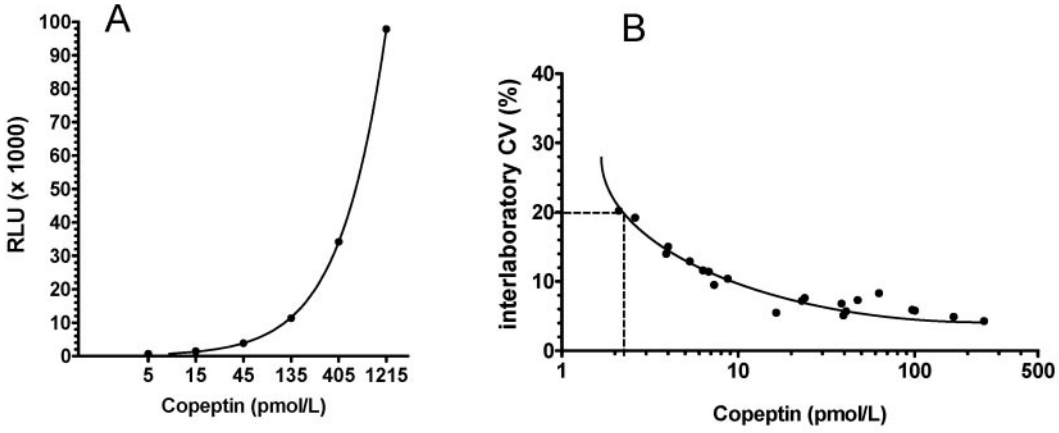

Fig. 2. Measurement range and precision of copeptin assay.

(*A*), representative calibration curve of copeptin assay. *RLU*, relative light units (luminometer). (*B*), total assay imprecision determined with 22 serum samples measured on 12 different days by 6 different operators using 2 different luminometers with 2 different lots of reagents. The interlaboratory CV of 20% is indicated by the *dashed line*.

Alternatively, following Clinical and Laboratory Standards Institute (formerly NCCLS) guidelines, 2 operators determined total imprecision in 20 assay runs on 20 days with 2 lots of reagents in duplicate. All CVs were <15% in 12 serum samples containing 1.5–420 pmol/L copeptin. The within-run imprecision (CV) of the same samples was <10%.

DILUTION STUDY

Linear dilutions (up to 1:32) were tested in 10 serum samples (Fig. 3A). Measured concentrations were multiplied by the dilution factor and compared with the original undiluted concentrations. During dilution, none of the 10 samples showed a deviation >20% of the original value.

STABILITY OF ANALYTE IN SERUM AND EDTA-, HEPARIN-, AND CITRATE PLASMA

We tested the stability of the analyte at room temperature in serum and citrate-, EDTA-, and heparin-plasma samples from 5 different patients. In all 4 matrices, the analyte was stable for at least 7 days at room temperature. Mean values (as a percentage of the initial value for the samples) were 93.3%–102.7% for serum, 96.5%–110.7% for EDTA plasma, 90.9%–98.7% for heparin plasma, and 96.6%–102.9% for citrate plasma (Fig. 3B). After 14 days at room temperature, the mean measured value was still 97.6% of the original value for EDTA plasma, but had decreased to 78.1%, 78.6%, and 80.2% for serum, heparin plasma, and citrate plasma, respectively.

For the same samples, stability at 4 °C was tested for up to 14 days. After 14 days of storage, the mean measured copeptin value was still 103.7% of the original value for serum, 93.2% for EDTA plasma, 104.7% for heparin plasma, and 109.9% for citrate plasma (Fig. 3C).

In 15 samples subjected to 4 cycles of freezing and thawing, no change in the analyte was detected. The mean measured values after the fourth thawing were 102.2% (range, 92%–116%) of the original values.

COPEPTIN IN HEALTHY INDIVIDUALS

In 359 healthy individuals (153 men and 206 women), median copeptin values were 4.2 pmol/L [range, 1–13.8 pmol/L; 95% confidence interval (CI), 4.0–4.4 pmol/L]. The 99th percentile of the healthy population was 13.5 pmol/L, the 97.5th percentile was 11.25 pmol/L, and the

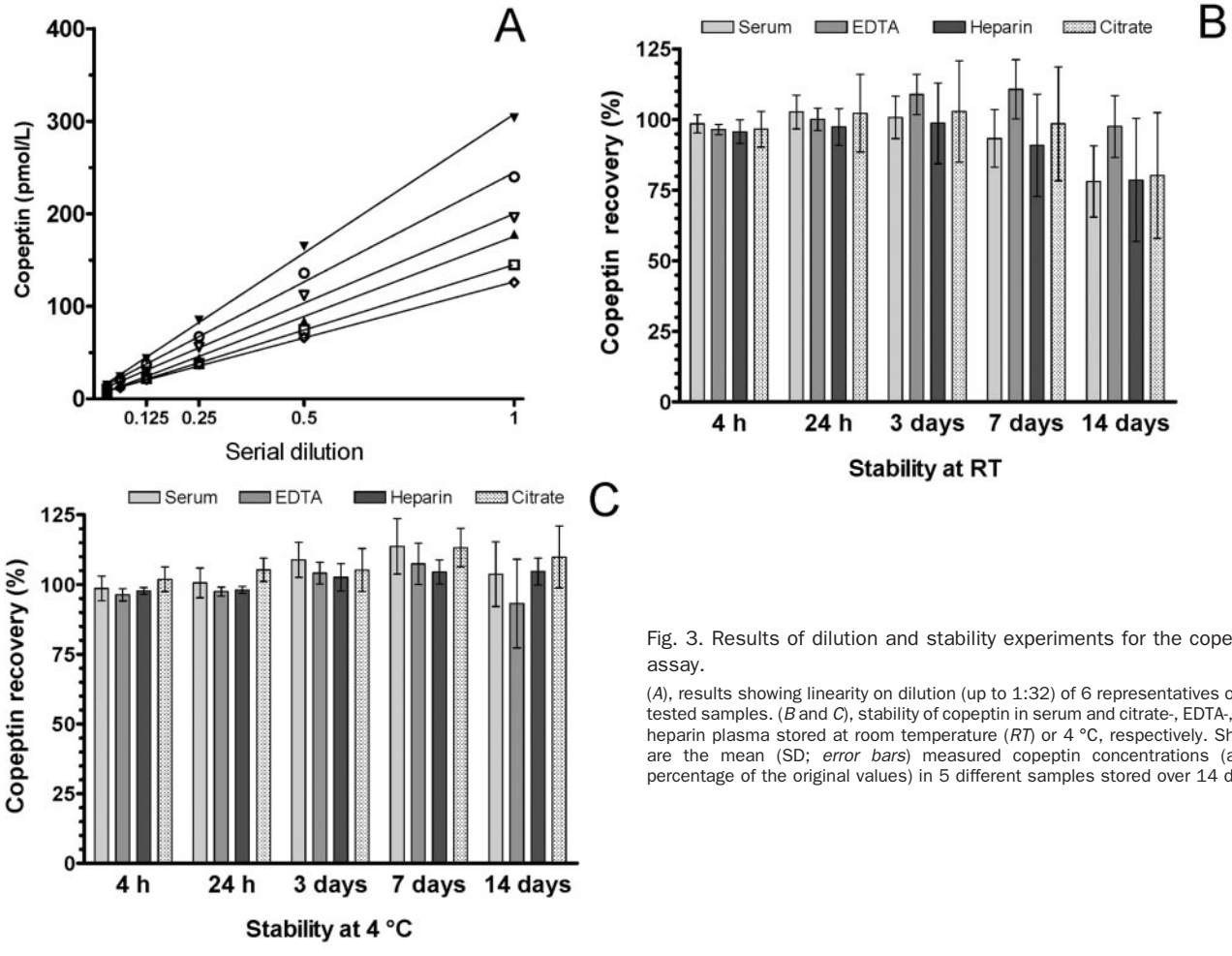

Fig. 3. Results of dilution and stability experiments for the copeptin assay.

(*A*), results showing linearity on dilution (up to 1:32) of 6 representatives of 10 tested samples. (*B* and *C*), stability of copeptin in serum and citrate-, EDTA-, and heparin plasma stored at room temperature (*RT*) or 4 °C, respectively. Shown are the mean (SD; *error bars*) measured copeptin concentrations (as a percentage of the original values) in 5 different samples stored over 14 days.

2.5th percentile was 1.7 pmol/L (based on the nonparametric percentile method). Of all 359 tested individuals, 9 (2.5%) had copeptin values below the analytical detection limit of 1.7 pmol/L. Those values were defined as 1 pmol/L.

Median copeptin values differed significantly ($P < 0.0001$) between men and women (men, 5.2 pmol/L; women, 3.7 pmol/L). The male population showed a gaussian distribution with a mean (SD) of 5.5 (2.4) pmol/L, but the female population did not (Fig. 4A).

There was no major difference in median copeptin concentrations after stratification according to age groups (Table 1). In addition, correlation analysis revealed no significant correlation of copeptin concentration and age ($r = 0.02$).

A direct comparison of copeptin values in matched citrate-, EDTA-, and heparin-plasma and serum samples from 50 individuals randomly selected from the 359 healthy participants showed no significant differences, and results were similarly distributed in all matrices. Median copeptin values for citrate-, EDTA-, and heparin plasma and serum were 4.3, 4.0, 4.3, and 4.1 pmol/L, respectively.

To determine intraday variations in healthy individuals, we monitored 6 participants (3 men and 3 women) from 0800 to 1700 h and measured copeptin concentrations in 15 consecutive samples collected during this period. The first blood samples were drawn from a male and a female participant, both of whom had been without water and food for 14 h. Initial copeptin values differed between these individuals, showing 2 extremes of the distribution of copeptin values in healthy individuals. The individual curves for these 2 participants are shown in Fig. 4B. After the initial high value of the postfasting sample, copeptin decreased rapidly in the man after he drank 1 L of water and increased again later during the day and after food intake (standardized lunch of ~1200 kcal), whereas the values in the woman were constantly

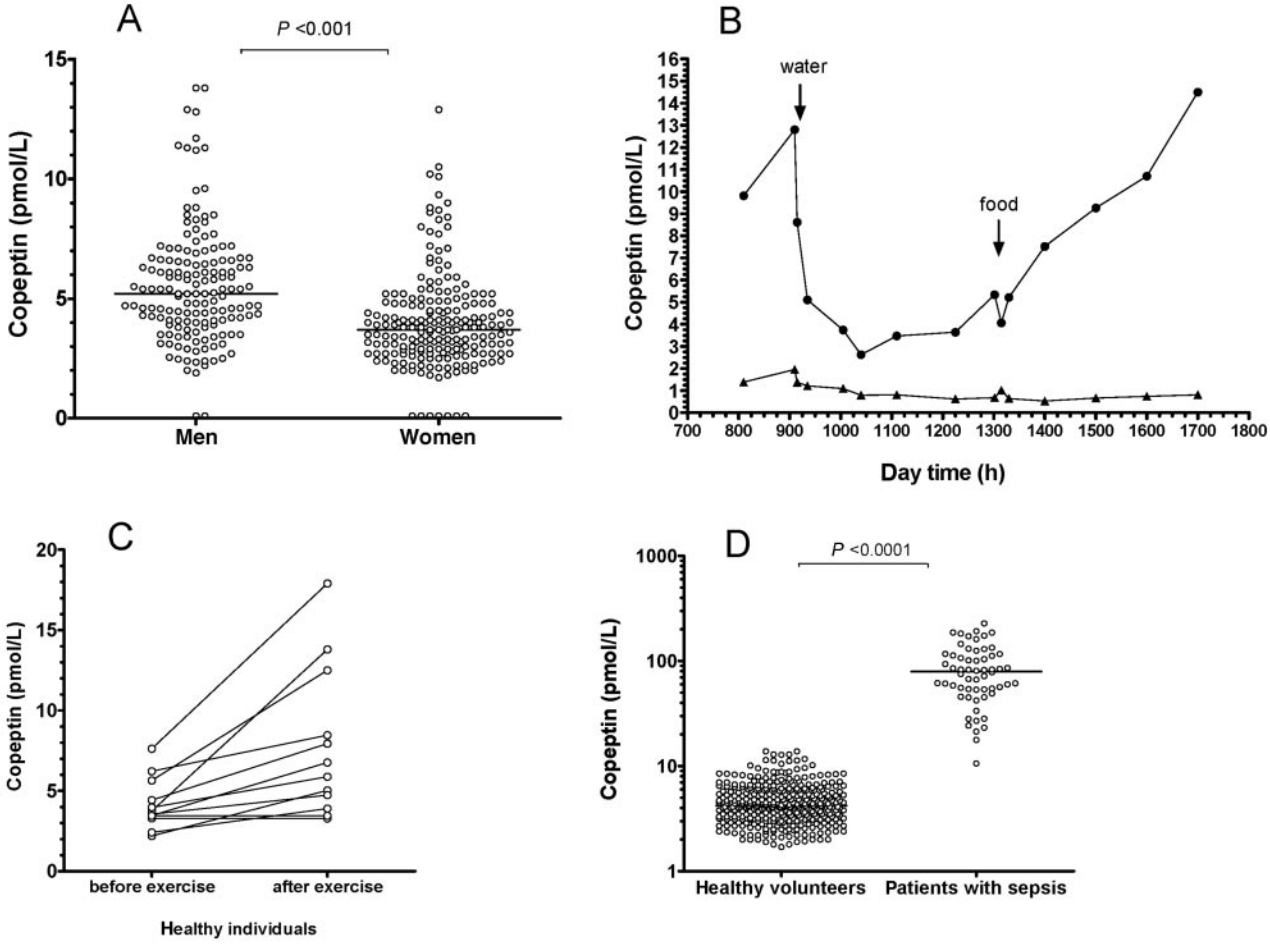

Fig. 4. Copeptin in healthy individuals and patients.

(*A*), distribution of copeptin values in 153 healthy men and 206 healthy women. There was a significant difference in median values (*horizontal lines*) between the 2 groups. (*B*), copeptin values for 2 representatives of 6 healthy individuals over a 9-h period. The man (●) was 45 years of age with a body mass index of 23 kg/m^2; the woman (▲) was 23 years of age with a body mass index of 19 kg/m^2. *Arrows* indicate a water load (1 L within 5 min) and a standardized meal (~1200 kcal over 45 min). (*C*), copeptin concentration immediately before and after a typical exercise regimen in 6 healthy women and 6 healthy men. (*D*), distribution of copeptin values in 60 patients with sepsis, severe sepsis, or septic shock compared with the 359 healthy individuals. The median values are indicated by *horizontal lines*. Differences between the 2 groups are significant.

Table 1. Copeptin in 359 healthy blood donors stratified by age.[a]

	Blood donor age, years					
	18–24	**25–34**	**35–44**	**45–54**	**55–64**	**65–80**
n	46	65	76	48	84	40
Copeptin, pmol/L						
Mean (SD)	5.1 (2.4)	4.1 (1.8)	4.6 (2.0)	4.8 (2.4)	4.5 (2.6)	5.0 (2.0)
Median	4.9	3.9	4.1	4.1	4.1	4.7
Range	1.8–13.8	1.0–8.8	1.0–11.4	1.0–12.9	1.0–13.8	2.4–11.3

[a] All age groups showed gaussian distribution (by Kolmogorov–Smirnov test). Differences in mean or median copeptin values between groups were not significant (by Kruskal–Wallis ANOVA).

low, and initially detectable values decreased to below the detection limit of the assay.

To estimate the influence of exercise on copeptin values, 6 male and 6 female adult volunteers performed a bicycle exercise test. EDTA-plasma samples were collected immediately before and after exercise. After exercise, the median copeptin concentration increased significantly ($P = 0.002$), from 3.6 pmol/L to 6.3 pmol/L. Three participants showed a strong increase of copeptin values after exercise (>2.5-fold), 7 showed a moderate increase, and 2 showed no increase (Fig. 4C). In 2 individuals, copeptin concentrations were monitored after exercise. In both individuals, the copeptin concentration had returned to the original preexercise value 1 h after exercise completion.

COPEPTIN IN CRITICALLY ILL PATIENTS

In 60 intensive care unit patients with sepsis, severe sepsis, or septic shock, copeptin concentrations were significantly increased ($P < 0.001$) compared with those of healthy individuals. Median (range) values in the sepsis patients were 79.5 (10.6–228.0) pmol/L, ~20-fold higher than those in healthy individuals. There was no significant difference in copeptin concentrations in patients with sepsis (no organ failure), severe sepsis (organ failure), or septic shock. The distribution of the data is shown in Fig. 4D.

AVP data were available for 39 of the study patients. The correlation between copeptin and AVP was highly significant (Spearman $r = 0.74$; 95% CI, 0.55–0.86; $P < 0.0001$; Fig. 5). Also shown is the correlation for both analytes for 71 samples from healthy controls (Spearman $r = 0.31$; 95% CI, 0.078–0.51; $P = 0.008$). The overall correlation for both groups together was $r = 0.78$ (95% CI, 0.69–0.84; $P < 0.0001$).

Discussion

We determined that our sandwich immunoassay for the measurement of copeptin has a functional assay sensitivity (defined as the lowest value with an interassay CV <20%) of 2.25 pmol/L and allows the precise measurement of copeptin in a range of 2.25–1215 pmol/L. In dilution studies, the assay was linear at analyte dilutions up to 1:32. In contrast to mature AVP, copeptin was stable in all matrices at room temperature for at least 7 days and at 4 °C for at least 14 days. This assay therefore offers considerable advantages for the exact measurement of AVP production.

Released amounts of copeptin may directly reflect those of AVP, and measurement of the latter is technically challenging. AVP is known to be unstable, largely attached to platelets, and rapidly cleared *(15, 16)*. Furthermore, because of its small size, AVP cannot be detected by sandwich immunoassays, and the reported competitive assays *(15, 18)* require complex preanalytic procedures including peptide extraction. Thus, despite the important physiologic role of AVP in fluid homoeostasis, routine measurements of AVP have never truly been implemented in intensive care settings and have been reserved for specific endocrinologic questions such as the differential diagnosis of central and renal diabetes insipidus or the syndrome of inappropriate antidiuretic hormone. Because of its stoichiometric generation, copeptin offers a simple and readily available method for AVP determination. The

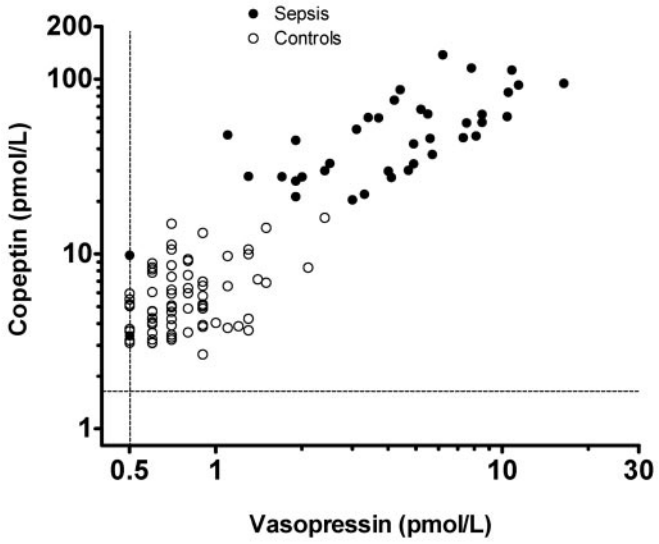

Fig. 5. Correlation between copeptin and AVP.

Data are shown for 39 patients with sepsis (●; Spearman $r = 0.74$; 95% CI, 0.55–0.86; $P < 0.0001$) and 71 healthy controls (○; Spearman $r = 0.31$; 95% CI, 0.08–0.51; $P = 0.008$). The overall correlation for both groups together was $r = 0.78$ (95% CI, 0.69–0.84; $P < 0.0001$). The *dashed lines* indicate the analytical detection limits of the assays.

good correlation found between copeptin and AVP in this study supports this reasoning.

In healthy individuals, copeptin showed a relatively broad distribution with a median (range) of 4.2 (1.0–13.8) pmol/L. This distribution is similar to that reported by Robertson et al. *(15)* for AVP. Stratification according to sex and age revealed lower values in females but comparable median values and distribution according to age for individuals 18–80 years of age. Most healthy individuals (97.5%) had copeptin concentrations detectable by this assay. There was no significant difference in median concentration or distribution in all tested matrices. Copeptin values increased markedly after exercise, although the response varied among individuals. After a water load, the copeptin concentration decreased rapidly in 1 patient and returned to original values during the remainder of the day. A similar pattern after a water load has also been described for AVP *(9, 19)*. This rapid reduction of copeptin in vivo is puzzling in light of the long-term stability of the peptide ex vivo, and the underlying mechanism has to be addressed in subsequent studies.

The concept of replacing the problematic measurement of a bioactive, rapidly cleared peptide with measurement of a nonfunctional, stable peptide derived from the cognate precursor is well known and has been applied with great success for the A- and B-type natriuretic peptides *(20–24)*. Our finding of such a peptide derived from the AVP precursor opens the door to a better method than has previously been available to assess the actual release of AVP gene products under pathologic conditions. One practical example in this study is the measurement of copeptin in patients with sepsis, severe sepsis, and septic shock.

In light of the use of AVP therapy during septic shock *(12–14, 25–27)*, copeptin measurement may be of benefit in monitoring such therapy. Not only is copeptin much easier to measure than mature AVP, it is also undisturbed by therapeutically administered AVP and would allow the assessment of endogenous AVP production during AVP administration (similar to the detection of C-peptide in diabetes mellitus to assess endogenous insulin release under insulin therapy).

In conclusion, the proposed assay can measure copeptin in the serum and plasma of healthy individuals and of patients. This assay may be useful to evaluate the clinical importance of copeptin concentrations in a variety of pathologies in which AVP secretion is reportedly disturbed.

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