Urinary and plasma urodilatin measured by a direct RIA using a highly specific antiserum

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Urodilatin (95–126) (URO) appears to play a major physiologic role in fluid homeostasis and produces major changes when administered intravenously. Here we describe a monospecific, high-affinity antiserum against URO with no cross-reactivity (<0.01%) against the structural highly homologous atrial natriuretic peptide 99-126 (ANP-99-126), ANP analogs, and related peptides such as brain natriuretic peptide. A competitive RIA was developed, based on this antiserum. Urine samples with or without ethanol extraction and plasma samples without pretreatment were analyzed by the RIA, which had a detection limit of 10.5 ng/L, a linear measuring range between 10.5 and 1000 ng/L, and recoveries of 93–102% in urine and 90–104% in plasma. The intraassay CVs were 8.2% and 8.1% for urine samples with 269 and 669 ng/L URO; the interassay CV was 9.7% at 839 ng/L. Using this assay, we present URO data for urine from healthy volunteers receiving low and routine sodium diets and from clinical urine specimens; we also present pharmacokinetic data for URO in plasma from patients suffering from bronchial asthma and treated by URO infusion.

The past decade has led to the identification and characterization of several hormones of the natriuretic peptide family (1) involved in the regulation of salt and water homeostasis. The prototype of the natriuretic hormone is the circulating atrial natriuretic peptide 99-126 (ANP-99–126),6 which is processed from the atrial prohormone ANP-1–126 (2, 3). Additional members of the natriuretic peptide family include brain natriuretic peptide (BNP) (4) and C-type natriuretic peptide (5). The main physiological effects of ANP-99-126 and BNP are natriuresis, diuresis, and vasodilation (1, 6).

Urodilatin (URO, ANP-95-126), another member of the natriuretic peptide family, is isolated from human urine (7) and has an N-terminal extension of four additional amino acids compared with the circulating form of ANP-99-126. This hormone, which is synthesized in the kidney (8), exerts potent paracrine renal effects (9). Several studies have suggested that URO plays an important role in the physiological regulation of renal function, especially in the control of renal sodium and water excretion. A concomitant increase in sodium and URO excretion was observed after acute volume load (10) and after dilation of the left atrium (9). Furthermore, close correlations between sodium and URO excretion were found in the circadian rhythm (11) and in healthy volunteers receiving different sodium diets (12).

Infusions and bolus injections of URO in rats (13) and healthy volunteers (14) revealed the pharmacological potency of this natriuretic peptide. Profound diuresis and natriuresis as well as a slight reduction in blood pressure are the most prominent effects. The strength and duration of these effects differ considerably from ANP-99-126. When healthy volunteers received bolus injections of URO, the resulting natriuresis and diuresis were twice those of ANP-99-126, with a hypotensive effect occurring only at the highest doses used (14). In addition, in cardiomyopathic dogs the natriuretic and diuretic effects of ANP-99-126 showed no marked effects on renal excre-

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6 Nonstandard abbreviations: ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; URO, urodilatin; BSA, bovine serum albumin; PPNE, phosphate buffer containing sodium azide and EDTA; PBS, phosphate-buffered saline; and KLH, keyhole limpet hemocyanin.
tory function in heart failure (15). In contrast, Riegger et al. (16) showed that URO causes a strong diuresis and natriuresis in equimolar doses.

These physiological and pharmacological results that indicated stronger renal effects of URO compared with ANP-99-126 encouraged us to use URO in different clinical settings such as prophylaxis and treatment of acute renal failure (17,18) and treatment of bronchial asthma (19,20).

To investigate the physiological role and pharmacokinetics of URO, a quantitative measurement of URO concentrations in body fluids is essential. However, until now only polyclonal antisera purified by immunoaffinity chromatography with a specificity for URO of <5% have been available (21,22). Furthermore, because of the homology of URO and ANP-99-126, the development of a specific RIA for URO without ANP-99-126 cross-reactivity has been difficult.

Here we report the development of a direct RIA for plasma without sample pretreatment, using an antisemum monospecific for URO with high affinity. With this RIA, we measured URO concentrations in the urine of healthy volunteers receiving low and routine sodium diets and in patients undergoing cardiac catheterization; we also established pharmacokinetic data for URO in plasma from patients suffering from bronchial asthma and treated by URO infusion.

**Materials and Methods**

**REAGENTS, PEPTIDES, AND PROTEINS**

Bovine serum albumin (BSA) was supplied by Sigma Chemical Co.; all other chemicals of analytical grade were supplied by Merck.

Synthetic human URO and human ANP-99-126 (hANP-99-126) was kindly provided by HaemoPep Pharma GmbH, Hannover, Germany. hBNP was a kind gift from Biotop, Oulu, Finland. hBNP was purchased from Peninsula Laboratories.

**BUFFERS**

The assay buffer was a phosphate buffer (pH 7.4) containing 800 mL of 67 mmol/L Na2HPO4, 200 mL of 67 mmol/L KH2PO4, 1 g/L sodium azide, and 400 mg/L EDTA (PPNE) to which 20 g/L BSA was added (PPNE-BSA).

Phosphate-buffered saline (PBS; pH 7.4) containing 13.5 mmol/L KH2PO4, 2.7 mmol/L KCl, 13.5 mmol/L Na2HPO4, 2H2O, and 13.7 mmol/L NaCl was used as the buffer for the radioactive labeling of URO.

For calibration, stock solutions of URO were prepared by dissolving the lyophilized peptide in 0.1 mol/L acetic acid and subsequently stored at 4°C. For calibrators, stock solutions were freshly diluted in PPNE-BSA and used for the RIA. In addition, ANP-99-126 was diluted in PPNE-BSA and stored at −20°C.

**PREPARATION OF IMMUNOGEN AND IMMUNIZATION PROCEDURE**

Two rabbits and 10 sheep were immunized with a synthetic URO fragment (ANP-95-102-Cys) coupled to key-hole limpet hemocyanin (KLH) via the −SH group, according to the method described by Sofroniew et al. (23) to raise specific antibodies. The immunogen was mixed with nonulcerative Freund’s adjuvant (Guildhay). After the priming immunization of each rabbit with 150 µg of KLH-conjugated URO (each sheep received 500 µg) and two booster injections with 50 µg of antigen (each sheep received 500 µg), additional boosts were supplemented with free URO to induce the appropriate affinity and high titers. A polyclonal antisheep IgG serum was developed by immunizing donkeys with sheep IgG.

**IODINATION PROCEDURE**

The tracer (125I-URO) was produced according to the chloramine-T method described by Hunter and Greenwood (24) in a modified version by use of 1 µg of URO in 10 µL of PBS, pH 7.4, 500 µCi of Na125I-solution (Amersham Buchler), and 10 µg of chloramine-T in 10 µL of PBS. After incubation for 60 s, the reaction was stopped by the addition of 25 µg of Na2S2O5 in 10 µL of PBS. The tracer was purified on a SepPack C18 cartridge according to the method of Schöneshöfer et al. (25). The specific activity of the tracer was in the range of 370–546 Ci/g (n = 20).

**SAMPLE PREPARATION**

Ice-cold ethanol (1 mL) was added to 0.5 mL of urine sample and mixed thoroughly. After 5 min, the sample was centrifuged for 10 min at 1500–1800 g. The supernatant was decanted into a new tube, and 1 mL of 700 mL/L ice-cold ethanol–300 mL/L H2O was added to the pellet. The sample was centrifuged again under the same conditions. Both supernatants were pooled and evaporated to dryness. Before use in the RIA, urine samples were redissolved in 0.5 mL of PPNE-BSA.

Alternatively, samples were used without pretreatment in the RIA. To avoid matrix effects, samples were diluted 1:2 or 1:4 (by volume) in assay buffer.

EDTA plasma samples were used in the RIA without pretreatment.

There were no repeated freeze-thaw cycles in any of the samples analyzed, except those samples used for the comparison of two different antibodies.

**RIA**

One hundred microliters of calibrator or sample was preincubated with 100 µL of specific antibody (1:80 000 dilution in PPNE with 1 g/L BSA) at 4°C for 16–20 h. Thereafter, 100 µL of 125I-URO (~30 000 cpm) in PPNE-BSA was added and incubated for 3 h at 4°C. The bound and free ligands were separated by incubation with 100 µL of antigoat precipitating reagent (1L of PPNE containing 1 g/L BSA, 47.6 mL/L donkey antisheep serum, 60 g/L polyethylene glycol (PEG 6000), and 3 mL/L normal human serum) for 1 h at 4°C. The samples were then centrifuged for 10 min at 1500–1800 g.

For calibration, stock solutions of URO were prepared by dissolving the lyophilized peptide in 0.1 mol/L acetic acid and subsequently stored at 4°C. For calibrators, stock solutions were freshly diluted in PPNE-BSA and used for the RIA. In addition, ANP-99-126 was diluted in PPNE-BSA and stored at −20°C.
goat serum in PPNE-BSA] for 1 h at room temperature, followed by centrifugation at 1500–1800 g for 10 min. The supernatants were removed, and the pellets were treated with 250 µL of washing solution (9 g/L NaCl and 60 g/L PEG 6000 in distilled water) and subsequently centrifuged at 1800 g for 10 min. The radioactivity in the precipitate was determined in a γ-counter (MultiCrystal/Gamma-Counter LB2103; Berthold) for 1 min.

STATISTICS
Values are given as mean ± SD. The statistical methods used include least-squares regression analysis and determination of the linear correlation coefficient.

PATIENT CHARACTERIZATION
Healthy male volunteers (n = 8) participated in a study in which they received a constant sodium diet of 52 (according to the recommendations of the German Society of Nutrition considered as a low-sodium diet) and 173 mmol of Na+ per day (routine sodium diet) for 1 week. Urine samples were taken at the end of the sodium diet phase because at that time stable Na+ and H2O homeostasis could be assumed.

Spontaneously voided clinical urine specimens (n = 64; 51 spot urines and 13 24-h urines) were analyzed for URO and sodium excretion.

Five clinically stable asthmatics attending a clinical phase II trial received URO infusion 30 ng·kg body weight−1·min−1 over 1 h (0–60 min) to test the bronchodilatory capacity of the peptide. Blood samples were taken before infusion [0 min, (sample 1)]; during URO infusion [at 20 min (sample 2), 40 min (sample 3), and 60 min (sample 4)]; and after URO infusion [at 80 min (sample 5), 100 min (sample 6), 120 min (sample 7), and 150 min (sample 8)].

All procedures concerning human subjects were in accordance with the Helsinki Declaration of 1975, as revised in 1983.

Results
CHARACTERIZATION OF ANTISERUM S70/94
Rabbits and sheep were immunized with human URO-95-102-Cys-KLH. After the second booster immunization, all animals responded, and URO was added to the immunogen for the next boosts. All rabbit antisera contained antibody titers of 1:>20 000; all sheep antisera showed titers of 1:>50 000. After all antisera were screened, antiserum S70/94 from sheep was selected for RIA development because of its high antibody titer of 1:240 000.

Synthetic human URO exhibited 100% reactivity with antiserum S70/94, whereas ANP-99-126 (tested in a concentration range of 10−3 000 000 ng/L), different ANP fragments (ANP-95-100, ANP-95-104, ANP-1-98, ANP-104-123, and ANP-75-98), and BNP (all tested in a concentration range of 10−1000 ng/L) had no cross-reactivity (<0.01%) as immunogens.

DEVELOPMENT AND CHARACTERISTICS OF THE URODILATIN RIA
Antiserum S70/94 was used in a final dilution of 1:240 000 in the test tube, adding ~30 000 cpm 125I-URO for detection and giving a binding of 30% of the total radioactivity.

The antibody was characterized by an affinity constant $K_a$ of 5 × 10^9 L/mol, derived from the Scatchard plot analysis (26). Kinetic studies demonstrated that the equilibrium of the reaction was reached after 3 h of incubation.

The sequential saturation technique described above decreased the detection limit by a factor of 3. A typical calibration curve is depicted in Fig. 1, with a $B_{80}$ (80% binding relative to the maximal binding) of 30 ng/L, a $B_{50}$ (50% binding relative to the maximal binding) of 145 ng/L, and a $B_{20}$ (20% binding relative to the maximal binding) of 605 ng/L.

The linearity of the RIA was confirmed by measurement of URO concentrations in serial dilutions of three urine samples with different URO concentrations (659, 872, and 994 ng/L). The linear range was from 10.5 to 1000 ng/L. Regression analyses of these three serial dilutions yielded the following correlation coefficients: $r = 0.9963$ for 659 ng/L; $r = 0.9933$ for 872 ng/L; and $r = 0.9990$ for 994 ng/L.

Recovery of 100 ng/L synthetic URO added to three
urine samples, initially containing 62 (urine I), 307 (urine II), and 436 (urine III) ng/L was 102% for urine I, 94% for urine II, and 102% for urine III, and was independent of sodium concentration (urine I, 100.5 mmol/L; urine II, 125.4 mmol/L; and urine III, 54.9 mmol/L) or osmolality (urine I, 289 osmol/kg; urine II, 595 osmol/kg; and urine III, 396 osmol/kg). All analyses were performed in duplicate within a single assay. The recovery of URO added to plasma samples was 90–104%.

Studies of serial dilutions of two plasma samples (1067 and 320 ng/L, respectively) yielded the following least-squares regression equations: 

\[ y = 11.3 x + 5.0 \text{ ng/L}, \quad r = 0.985; \]

\[ y = 3.52 x - 35.6 \text{ ng/L}, \quad r = 0.996. \]

The intraassay CVs for two urine specimens were 8.2% and 8.1% [means, 269 and 669 ng/L, respectively (n = 24)]. The interassay CV (n = 12) was 9.7% (mean, 839 ng/L). For plasma samples the intra- and interassay CVs were comparable to those measured for urine.

The lower limit of detection (determined by 12 replicate analyses of the zero calibrator and calculation of the concentration that corresponds to the \( B_{\text{max}} - 3 \text{ SD of the counts obtained} \)) was 10.5 ng/L. We used calibrators with the following assigned values: 0, 4.1, 12.3, 37, 111, 333, and 1000 ng/L.

The URO concentration was measured in 27 urine samples with and without pretreatment (ethanol precipitation). There was a close correlation between the two treatment procedures (\( y = 1.2x - 141; \ r = 0.89)\), suggesting that the RIA can be simplified by using the urine samples without pretreatment.

We assayed 32 urine samples under the present RIA (with extraction) and compared the results with those obtained in the samples 3 years ago with the RIA conditions (rabbit antiserum) described previously by Drummer et al. (21). Although the samples had been stored at \(-20^\circ C\) for 3 years, there was a close agreement between the results (Fig. 2) despite the use of different antisera and the long-term storage of the urine samples.

**PLAUSIBILITY STUDY BY MEANS OF BIOLOGICAL MATERIAL.**

In healthy volunteers, the URO concentration in urine increased from 53 ± 39 to 110 ± 81 ng/L, dependent on the sodium intake (low to routine), which corresponded to the increase in natriuresis (from 19 to 77 mmol/L).

In the clinical urine specimens the values ranged from 91 to 1145 ng/L. The URO:creatinine ratio correlated significantly with the urinary sodium concentration (\( r = 0.41; \ P < 0.01; \) Fig. 3).
Pharmacokinetic data in patients suffering from bronchial asthma and receiving URO infusion for 1 h demonstrated a sharp increase of URO concentration in plasma during URO infusion, to ~2000 ng/L during the infusion period, followed by a strong decrease in the period thereafter, to ~250 ng/L (Fig. 4).

**Discussion**

Immunosassays are routine methods sensitive enough to determine peptides in physiological concentrations in organs and body fluids. Because URO exhibits an important role in sodium and water homeostasis (1, 6) and also has beneficial effects as a drug in different clinical indications (17, 19), quantitative analysis of the peptide concentration as a tool for physiological characterization as well as for pharmacokinetic analysis is mandatory. However, the high structural homology of the natriuretic peptides URO and ANP-99-126 has impeded the generation of specific antiseras.

Soon after the discovery of URO, Solc et al. (27) developed a method to discriminate physiological amounts of URO and ANP-99-126 in the urine of healthy volunteers. In their study, Solc et al. used an ANP-RIA that could not differentiate between the two peptides. However, the combination of HPLC followed by ANP-RIA of the separated fractions led to the identification of URO and ANP on the basis of their different chromographic properties.

Because HPLC separation requires a considerable amount of time and large quantities of peptides, antibodies were generated to specifically detect physiological concentrations of URO in urine. Drummer et al. (21) and our group developed an RIA that uses an affinity-purified polyclonal antibody that does not cross-react with ANP-99-126, ANP analogs, or human proANP, which is the peptide most likely to be detected by this antibody. The polyclonal antibody was raised in rabbits by immunization against the four N-terminal amino acids of URO and three additional amino acids that are also contained in ANP-99-126. In this RIA, urine samples were pretreated with ethanol to remove interfering substances.

Carstens et al. (22) have also produced a URO-specific antibody and used it in an RIA for the detection of URO in human urine. In contrast to Drummer et al. (21), Carstens et al. immunized with the whole 32-amino acid peptide, which contains the immunogenic loop formed by the disulfide bridge. The rationale was to avoid the poor immune response often seen with low-molecular mass immunogens. However, as expected, they generated an antiserum that cross-reacted with ANP-99-126. Therefore, their method required affinity chromatography using three different columns, on which BSA, ANP-99-126, and URO were immobilized to absorb unwanted cross-reactivity. Similar to the method of Drummer et al., the samples in the assay developed by Carstens et al. were also extracted with ethanol prior performing the RIA.

Our method represents a major improvement over previous assays. We successfully generated for the first time a URO-specific antibody without cross-reactivity against ANP-99-126 as a high-titer antiserum. Similar to the method of Drummer et al. (21) and in contrast to Carstens et al. (22), we immunized with the four N-terminal amino acids of the URO molecule plus three additional amino acids corresponding to ANP-99–102, extended at the C-terminal end by a cysteine residue to allow selective coupling to KLH, leading to a monospecific antibody. Therefore, no affinity chromatography such as was required by Carstens et al. had to be carried out. In contrast to the earlier reported methods, we succeeded in generating a very high antibody titer, allowing a final dilution of 1:240,000 in the assay used.

For the RIAs described by Drummer et al. (21) and Carstens et al. (22), urine samples had to be pretreated with ethanol before use in the RIA. Sample pretreatment with ethanol to remove interfering substances was not necessary for the RIA presented here. We clearly show a close correlation of URO concentrations in samples with and without ethanol precipitation, suggesting the direct application of the urine samples in the RIA. This correlation may be caused by the monospecificity as well as the high affinity of the antiserum, which is also reflected by the tolerance to pH changes and different salt concentrations.

To validate the RIA, we remeasured the URO concentrations in urine samples that had been already determined in the RIA described by Drummer et al. (21); however, this time the concentrations were measured using the RIA conditions presented here. Except for the antibody used in each RIA (rabbit vs sheep), the procedures were comparable, including sample pretreatment. When both series of URO data were compared, the close correlation proved the equivalency of both the RIA of Drummer et al. and the RIA presented here, particularly regarding the specificity of the antibody used.

Previous studies have suggested that URO plays a key role in the physiological regulation of extracellular volume, especially in the control of renal sodium and water excretion (1, 9). Increased excretion of URO and sodium was observed after acute volume load by saline infusion (10) and after balloon dilation of the left atrium (9). Drummer et al. (11) investigated the role of URO in sodium homeostasis and demonstrated that the circadian rhythm of urinary sodium excretion parallels URO excretion. Furthermore, our group (12) and Heer et al. (28) demonstrated a sodium intake-dependent natriuresis and URO excretion. In the present study, URO concentrations in urine obtained from volunteers receiving low and routine sodium diets revealed a close correlation between URO excretion and natriuresis. In the clinical specimens, we found a close correlation between the URO:creatinine ratio and natriuresis, which also supports the important physiological role of URO in sodium and water homeostasis and gives additional evidence for the plausibility of the assay results.
In addition, for the first time pharmacokinetic data for URO in plasma were measured in patients suffering from bronchial asthma and receiving URO as an intravenous infusion for 1 h. URO concentrations increased to 2000 ng/L during URO infusion and decreased after termination of infusion to 250 ng/L.

In conclusion, we demonstrated the development of an RIA utilizing an antibody with monospecificity, high affinity, and high titer. We showed that for the detection of URO in urine and plasma, samples can be applied directly to the assay, thereby simplifying the performance of the RIA. These improvements represent a major step toward a URO-specific antiserum for the investigation of the physiological role and pharmacokinetic characteristics of URO. In the near future, we plan to develop other immunoassays, such as a sandwich-type ELISA (29) or a competitive enzyme immunoassay (using selectively biotinylated URO), based on this antiserum.

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References