Development of a urodilatin-specific antibody and radioimmunoassay for urodilatin in human urine

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Urodilatin (URO) (95–126) is a renal-derived natriuretic peptide that is isolated only from human urine. This study describes the development of a URO-specific antibody and a RIA for URO in urine. At present, there is no commonly available URO-specific antibody. We produced a URO-specific antibody without cross-reactivity with atrial natriuretic peptide (ANP) analogs by immunization of rabbits with the URO (95–126) peptide and subsequent purification of the resulting URO antiserum with affinity chromatography with CNBr-activated Sepharose 4B. The urine samples were ethanol-extracted before assay. The CVs were 6.7% (intraassay) and 14.1% (interassay). This study reports the circadian urinary excretion of URO in 24 healthy subjects with seven sampling periods per 24 h.

INDEXING TERMS: atrial natriuretic peptide • sodium • diuresis • circadian rhythm

Urodilatin (URO) (95–126) is a 32-amino acid peptide isolated from human urine that was characterized by Forssmann et al. [1] in 1988.3 URO belongs to the natriuretic peptide family and is identical in structure to the circulating 28-amino acid human atrial natriuretic peptide (ANP) (99–126), with addition of four amino acids (Thr-Ala-Pro-Arg) at the NH2 terminus. URO is measurable in human urine, but not in human plasma, suggesting that URO is a peptide synthesized and secreted in the kidney [2]. Studies have shown that apparently URO is cleaved in the kidney from a prohormone identical to the ANP prohormone found in the atria [3]. Probably URO is synthesized in the distal cortical nephron and secreted luminally to have its paracrine action downstream in the nephron at the inner medullary collecting duct, where the peptide inhibits Na+ entry through the amiloride-sensitive sodium channels via the guanylate cyclase-coupled receptors previously described for ANP [4, 5].

Studies have shown that urinary excretion of URO correlates closely with variations in salt excretion in humans [6, 7], and that urinary excretion of URO may have a closer correlation with urinary sodium loss than plasma ANP [8]. URO seems to be an important natriuretic peptide, but numerous questions about the role of URO in human physiology and pathophysiology are still open. Therefore, a reliable specific assay for measuring URO in urine is needed to investigate further the biological role of URO. Drummer et al. [9] developed a URO-specific RIA involving a polyclonal URO antibody specific for the four N-terminal residues of URO. This URO antibody is not commonly available and the immune response that is correlated to immunogens with low molecular mass often is poor; therefore we report here the development and purification of a URO antibody raised in rabbits immunized with the whole 32-amino acid URO peptide containing the immunogenic ring structure.

The aims of the present study were (a) to raise a URO antibody in rabbits by immunization with the synthetic URO peptide of 32 amino acids and to purify the resulting URO antiserum with CNBr-activated Sepharose 4B affinity chromatography to a degree without cross-reactivity with ANP analogs, (b) to develop a specific and sensitive RIA for measuring URO-like immunoreactivity (irURO) in human urine, and (c) to investigate if the excretion rate...
of URO in urine has a circadian variation in healthy volunteers.

**Materials and Methods**

**REAGENTS**

URO (95–126) was from Pharma Bissendorf Peptide, Hanover, Germany; α-ANP (human, 99–126), pro ANP (human, 1–30), pro ANP (human, 31–67), and C-type natriuretic peptide-22 (human CNP-22) from Peninsula Labs, Europe, St. Helens, Merseyside, UK; brain natriuretic peptide (BNP) from Clinalfa®, Lävijättingen, Switzerland; arginine vasopressin (AVP) from Ferring, Malmö, Sweden; aldosterone (Aldo), bovine thyroglobulin (BTG), bovine serum albumin (BSA), 1,3,4,6-tetrachloro-3-(dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (CD1) from Fluka, Buchs, Switzerland; Freund’s incomplete adjuvant from Statens Seruminstitut, Copenhagen, Denmark; CNBr-activated Sepharose-4B and DEAE-Sephadex A-25 from Pharmacia Biotech, Uppsala, Sweden; human serum albumin (HSA) from Hoechst-Behring, Marburg, Germany; Triton X-100, EDTA, Tween 20, methanol, ethanol, trifluoroacetic acid (TFA), and acetonitrile from Merck, Darmstadt, Germany; and polyethylene glycol 6000 (PEG) from Merck, Hohenbrunn, Germany.

**PREPARATION OF IMMUNOGEN**

URO (95–126) (MW 3506) was covalently conjugated to BTG. URO (2.4 mg) dissolved in 3.2 mL of demineralized water was coupled to 7.4 mg of BTG with 0.25 mg of CD1 as a coupling agent. The molar ratio of URO:BTG:CD1 was 60:1:112. The solution was kept for 24 h at 20 °C with constant stirring. The mixture was then dialyzed against demineralized water for 48 h at 4 °C.

**IMMUNIZATION PROCEDURE**

Aliquots of 160 μg each (100 μL) of the URO-thyroglobulin complex were emulsified with 100 μL of incomplete Freund’s adjuvant and injected subcutaneously into the backs of white rabbits. Immunizations were performed every 2 weeks, and after day 42, a booster injection was given at 4-week intervals. Blood was drawn 12 days after each booster injection [10].

**AFFINITY CHROMATOGRAPHY**

As the resulting polyclonal URO antibodies cross-reacted with ANP, CNBr-activated Sepharose 4B affinity chromatography was used for purification of the rabbit antiserum [11]. In short, three different columns of CNBr-activated Sepharose-4B were coupled to BSA, ANP, and URO, respectively. Freeze-dried CNBr-activated Sepharose-4B was suspended in ice-cold HCl (1 mM/L). The gel was washed and reswelled three times. It was then immediately transferred to a solution of the ligand, 30 mg of BSA, 1 mg of ANP, and 2 mg of URO dissolved in coupling buffer (0.1 mol/L NaHCO₃ containing 0.5 mol/L NaCl, pH 7–8). The three protein-gel suspensions were rotated end-over-end for 2 h at room temperature. After coupling, the solutions were transferred to a 0.2 mol/L glycine buffer, pH 8.0, standing overnight at 4 °C, to block remaining active groups on the gel. The three ligand-Sepharose conjugates were packed in Rheodyne columns. To remove excess uncoupled ligand, the absorbent was washed alternately with high- and low-pH buffer solutions (coupling buffer followed by 0.1 mol/L acetate buffer, pH 4, containing 0.5 mol/L NaCl). Finally, 10 mmol/L Tris buffer, pH 7.5, was used to wash away the blocking agent. The rabbit serum was applied to the BSA column with free flow; the unbound fraction of the serum was transferred to the ANP column, and subsequently the nonadsorbed amount was applied to the URO column. A glycine buffer (0.1 mol/L, pH 2.5) was used as the eluting agent on the URO column. The eluted fractions were collected in tubes containing a small amount of 1 mol/L Tris buffer, pH 8.0. The fraction with the highest protein content measured at wavelength 260 nm was selected, stored at −20 °C, and investigated for URO-specific antibodies.

**IODINATION PROCEDURE**

URO was iodinated by the Iodo-Gen method according to Salacin et al. [12] with minor modifications [13]. In short, Iodo-Gen was dissolved in dichloromethane/trichloromethane (40 mg/L). Aliquots (150-μL) of the Iodo-Gen solution were evaporated by dry nitrogen atmosphere in cryotubes. Ten microliters of a solution containing 0.2 mg of synthetic URO per mL of 0.1 mol/L acetic acid was transferred to the Iodo-Gen tube and mixed with 100 μL of 0.5 mol/L sodium phosphate buffer, pH 7.4, and 7 μL of Na₂¹²⁵I (25.9 MBq). The iodination reaction was allowed to run for 10 min. The solution was then applied to a DEAE-Sephadex A-25 column equilibrated with 0.1 mol/L sodium phosphate buffer, pH 7.4, which also was the eluting buffer. The fraction with peak radioactivity was selected and diluted with assay buffer. Aliquots of the URO tracer were stored at −20 °C for up to 6 weeks.

**SAMPLE EXTRACTION**

Human urine samples were extracted by addition of absolute ethanol (1:1.5 dilution of samples) with subsequent centrifugation and lyophilization of the supernatant. Dried extracts were resuspended in assay buffer.

**RADIOIMMUNOASSAY**

A 0.04 mol/L sodium phosphate solution (pH 7.4) containing 0.5 g/L sodium azide, 12 g/L EDTA, 1 mL/L Triton X-100, and 2 g/L HSA was used as assay buffer. The URO calibrators (range 0–128 fmol/tube) were prepared from a stock solution (10 nmol/L) and performed in triplicate. One hundred microliters of calibrator or urine extract (duplicates) were incubated with 100 μL of
URO antibody for 24 h at 4 °C. One hundred microliters of [125I]URO (~3000 cpm) was added and the mixture was incubated for a further 24 h. PEG (2 mL, 200 g/L) and 100 μL of pig γ-globulins (15 g/L per tube) were used for separation.

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

The identity of the immunoreactive material in human urine was studied by HPLC. A HPLC system from Pharmacia LKB was used. Reversed-phase HPLC was performed on a C18 column (250 × 4.6 mm, 5 μm) (The Separations Group, Hesperia, CA). Eluent A consisted of water with 1 g/L TFA and eluent B was a mixture of water:acetonitrile (20:80 by vol) with 1 g/L TFA. Eluent A (1000 mL/L) was applied for 2 min followed by 30 min of a linear gradient to 1000 mL/L eluent B with a resulting content of acetonitrile in the eluent ranging from 0 to 800 mL/L. The flow was 1 mL/min. Samples with synthetic URO and extracted human urine were dissolved in eluent A before injection.

HEALTHY VOLUNTEERS

Twenty four healthy volunteers, all men, with a mean age of 44 years, range 23–66 years, were studied during a 24-h period in the hospital. The inclusion criteria were: (a) healthy male volunteer, (b) age between 20 and 70 years, and (c) written informed consent to undergo the study. The exclusion criteria were: (a) clinical and/or laboratory evidence of renal, hepatic, cardiovascular, endocrinological, allergic, infectious, or neoplastic disease; (b) history of bladder dysfunction; and (c) alcohol abuse. They received a standard hospital diet and a fluid intake of 1500 mL/m2 per 24 h. The meals were given at 0730, 1200, and 1800 and there was no fluid intake during the night. The subjects were only in the supine position during the night, and only minimal activity was allowed during the daytime. Urine was collected in the following seven periods: 1500–1800, 1800–2100, 2100–2400, 0000–0700, 0700–1000, 1000–1300, and 1300–1500. The subjects gave written informed consent, and the study was approved by the local Ethics Committee.

Results

ANTISERA

Two of four rabbits produced detectable URO antibodies within 12 weeks after initial immunization. The antisera from one rabbit was usable at a dilution of 1:2000, but this polyclonal URO antibody cross-reacted 25% with synthetic ANP (99–126).

The polyclonal URO antibody was purified by CNBr-Sepharose affinity chromatography. The fraction eluting from the URO column with the highest protein concentration contained a highly specific URO antibody with a cross-reactivity of <0.1% with human ANP (99–126) and human pro-ANP (31–67) (Fig. 1). No cross-reactivity was observed with pro-ANP (1–30), BNP, CNP, Aldo, AVP, and cGMP. The purified antiserum was used at a final dilution of 1:4000. Scatchard plot analysis [14] of the purified antiserum exposed a homogeneous antibody population. The antibody is characterized by a K of 1.05 × 1011 L/mol.

IODINATION OF URO

Twenty three percent of the radioactive iodine was incorporated in the final tracer preparation. The specific activity of the iodized URO substance was 15.84 MBq/μg, calculated by the self-displacement ability as described by Morris [15]. Approximately 0.47 mol of iodide/mol of URO was available during the iodination process. Assuming a specific activity of 647.5 GBq/mg Na125I and monoiodination of URO, it could be calculated that ~64% of the URO molecules in the tracer solution were labeled.

CALIBRATION CURVE

The RIA for URO was optimized. Reproducible calibration curves were obtained and a typical zero calibrator binding, 50% inhibitory dose (ID50), and nonspecific binding was ~50%, 19 fmol/tube, and 7%, respectively. Nonspecific binding of [125I]URO in human urine was ~4%, indicating that interference by substances in urine was not significant.

LOWER LIMIT OF DETECTION

The lower limit of detection was 0.5 fmol/tube (P <0.05, paired test, n = 12) with a 95% level of confidence, corresponding to a urine concentration of 7.5 pmol/L URO if 400 μL of urine was extracted and resuspended in 600 μL of assay buffer, using 100 μL for assay.
The imprecision for analysis of irURO in human urine was assessed from measurements of internal quality-control pools in 12 consecutive assays over a period of 3 months. The intraassay CV was 6.7% and the interassay CV 14.1% at a concentration of 200 pmol/L.

The recovery of [125I]URO added to human urine samples before extraction was 89.9% ± 2.8% in 10 consecutive assays. The recovery of 30 fmol and 60 fmol of unlabeled synthetic URO added to morning urine samples from 12 healthy adults was 112.1% ± 20.7% and 104.9% ± 16.7% (mean ± SD), respectively.

The amount of URO measured is a linear function of the volume of urine extract assayed in RIA. The y-axis intercept is not significantly different from zero as seen in Fig. 2. The measurable irURO in urine extract diluted parallel to the calibration curve.

Varying the ethanol volume between 0 and 500 µL in the extraction of 500 µL of urine (n = 4) showed that irURO gradually decreased from 114.5 pmol/L to 93.5 pmol/L.

Urine samples (n = 8) kept at room temperature for 24 h to 72 h before extraction did not show a decay of endogenous URO immunoreactive material. Storage of urine samples at −20 °C for up to 3 months or repeated cycles (n = 4) of freezing and thawing did not change the measured immunoreactivity of URO.

HPLC tracing of 1 pmol of URO injected directly to the column eluted with a major peak after 17 min. HPLC tracing of 0.5 mL of ethanol-extracted human urine supplemented with 1 pmol of URO showed a major peak after 17 min and a small peak after 4 min. If human urine supplemented with 1 pmol of URO was stored at room temperature for 24 h, the elution profile showed that the majority of irURO eluted after 4 min and a minor part after 17 min. This early peak of irURO could represent a decomposition product of URO. The eluting pattern of ethanol-extracted human urine consisted of a major peak after 4 min and in addition two minor peaks after 12 min and 17 min. Thus, the identity of irURO in human urine extracts seems to have an elution profile identical to URO and a degradation product of URO.

The urinary URO excretion in 24 healthy adults during a 24-h study period with seven urine collections is illustrated in Fig. 3. The excretion of URO did not change significantly during the 24 h, but there was a tendency towards a lower URO excretion rate in the urine collected from 0000 to 0700.

Discussion

A method allowing measurement of URO in human urine is of importance in assessing the physiological and pathophysiological roles of this natriuretic peptide. At present, there is no commonly available URO-specific antibody. We produced a URO-specific antibody by immunization of rabbits with the URO (95–126) peptide and subsequent purification of the resulting URO antiserum with affinity chromatography using CNBr-activated Sepharose 4B. The purified polyclonal URO antibody cross-reacted ≤0.1% with the ANP analogs. Thereby, we solved the difficulties inherent in the generation of a URO-specific antibody. Compared with the immunization procedure used by Drummer et al. [9], we succeeded in developing a URO-specific antibody without using an immunogen with low molecular mass.

The immunoextracted rabbit antiserum contains one homogeneous population of antibodies against URO, indicated by linearity of the Scatchard plot. The affinity of the purified antibody population is approximately the same as the affinity of the URO-specific antibody developed by Drummer et al. (K_d = 9.5 pmol/L compared with K_d = 15 pmol/L). The sensitivities of the assays were
almost identical, with detection limits in the range 7–7.5 pmol/L.

Problems associated with reduced sensitivity and nonspecific absorption have been described for ANP assays involving the charcoal procedure for separation of bound and free ligand [16]. In the present assay, the lowest nonspecific bound values (~7%) were obtained when using a mixture of PEG and porcine γ-globulin as carrier instead of PEG alone or dextran-coated charcoal. This is a simple, fast, cheap, and reproducible precipitation method [17].

The Iodo-Gen method was chosen as Rasmussen et al. [13] reported about this simple and successful iodination method for preparation of an ANP tracer without loss of immunoreactivity during iodination as described by Gutkowska et al. with the chloramine T method [18]. The method was reproducible in the present study.

The irURO in urine proved to be stable at room temperature for 72 h. Likewise, the present data showed no loss of irURO after prolonged storage at −20 °C or by repeated cycles of freezing and thawing. In contrast, Drummer et al. [9] observed a significant decay of immunoreactive material if human urine samples were stored at room temperature. As well, conflicting data exist about the stability of ANP [19–22]. An explanation of the unchanged concentration of irURO could be that the purified URO antibody cross-reacted also with a degradation product of URO generated during storage. This is supported by HPLC analyses that showed that the irURO in stored human urine had an elution profile identical to URO and a degradation product of URO.

The circadian study showed that the excretion rates of URO in urine were almost unchanged during day and night. Despite a tendency towards a lower URO excretion rate during the night, we did not find a significant circadian 24-h rhythm with minimal excretion rates during the night as indicated by Drummer et al. in their study with a small number of subjects [6]. The URO excretions, determined with the use of our specific RIA, were higher than indicated by Drummer et al. [6] and Kentsch et al. [23], but in all three studies the excretion rates were in the range of 20–200 fmol/min. The proportion between the amount of urine and ethanol used in the extraction procedure might have an important influence on the measured concentration of irURO.

In conclusion, the present combination of immunization of rabbits with the URO (95–126) peptide and subsequent purification of the resulting URO antiserum with CNBr-activated Sepharose affinity chromatography was a simple way of producing a URO-specific antibody without cross-reactivity with ANP analogs. The RIA for URO demonstrated specificity and sensitivity for URO in human urine. The usefulness of the assay has been presented in the circadian study, and it seems to be convenient when a large number of clinical samples are to be assayed. Further studies are needed to elucidate the physiological significance of URO in urine under various physiological and pathological conditions.

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