Identification of xanthurenic acid 8-O-β-D-glucoside and xanthurenic acid 8-O-sulfate as human natriuretic hormones

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Hormonal regulation of salt excretion and water balance by the kidneys is well documented. Before 1961, it was widely believed that the glomerular filtration rate and the steroid hormone aldosterone controlled sodium balance in the body. In 1961, deWardener et al. [de Wardener HE, Mills IH, Clapham WF, Hayter CJ (1961) Clin Sci 21:249–258] showed that when these two variables were controlled, the kidney was still able to increase sodium excretion in response to a salt load. Several lines of evidence argued for a small-molecule signal as a definitive modulator of sodium excretion by the kidney. However, the chemical nature of the suspected natriuretic agent remained unknown. Here we report the identification and natriuretic activity of a closely related small molecule isolated from human urine, xanthurenic acid 8-O-β-D-glucoside and xanthurenic acid 8-O-sulfate. The two compounds were partially purified by activity-guided fractionation and subsequently identified by using NMR spectroscopic analyses of enriched active fractions. Both compounds caused substantial and sustained (1- to 2-h) natriuresis in rats and no or minimal concomitant potassium excretion. We believe these compounds constitute a class of kidney hormones that also could influence sodium transport in nonkidney tissues given that these tryptophan metabolites presumably represent evolutionarily old structures.

In 1985, de Wardener and Clarkson (1) reviewed the status of the search for the putative natriuretic hormone, a signaling molecule that could regulate sodium excretion by the kidney (2, 3). In numerous experimental models, several groups had consistently observed natriuretic activity from a small molecule (<1,000 Da) contained in mammalian urine that elicited as a postsalt peak in Sephadex G-25 gel chromatography. A natriuretic substance was shown to be present in urine and/or serum extracts of patients with chronic renal disease (CRD) (4, 5), normal man (6), salt-loaded man (7, 8), volume-loaded man (9), and volume-loaded rat (10). Although inferred in the experiments mentioned earlier, no molecule was ever isolated or identified. Numerous isolation and assay techniques have been applied to natriuretic urine fractions derived from Sephadex G-25 chromatography to further characterize the small-molecule components with varied results (1). Other laboratories, initially in pursuit of the low-molecular-weight natriuretic hormone, focused on a digitalis-like substance that specifically and with high affinity inhibited sodium/potassium ATPase (11). In contrast to the compounds described herein, the digitalis-like substance is kaliuretic and inconsistently natriuretic (11, 12).

Additional evidence supporting the existence of a natriuretic hormone includes experiments in which small-molecule-containing fractions from serum (13) or urine extracts of CRD patients were shown to inhibit short-circuit current (SCC) across the frog skin (13, 14) and toad bladder (15). These urine extracts also inhibited sodium transport in isolated perfused rabbit renal tubular collecting ducts (16). Furthermore, the urine of salt-loaded dogs was reported to contain a small molecule that inhibited sodium current in the toad bladder (17, 18).

Results

In the present study, we used the frog skin assay (Rana pipiens) (19) to screen for an inhibitor of sodium transport obtained from the urine of CRD patients (15). It was assumed that the CRD patients’ urine would be a rich source of natriuretic hormone because it would be the signal to the remaining nephrons, in advancing CRD, to increase sodium excretion per nephron, per unit of sodium intake (20). Its low molecular weight also should make it ultrafiltrable and thus more likely to appear in the urine.

Urine from CRD patients was lyophilized and fractionated by Sephadex G-25 chromatography (5, 13) (see Materials and Methods). Fractions eluting immediately after the salt peak were collected, lyophilized, reconstituted in water, and then assayed for inhibition of SCC in the frog skin when applied to the serosal surface. The active fractions (equivalent to 6 h of original urine) decreased the SCC from 40–50 to 20–30 μamp/cm². SCC values returned to or toward initial levels upon washout and replacement with fresh anuran Ringers. Urine fractions obtained by using a similar protocol were previously shown to induce natriuresis in the uremic rat (5). In the present study, these active fractions were purified further by two iterations of reversed-phase HPLC again by using the frog skin SCC inhibition assay for monitoring activity. In addition, HPLC fractions were monitored for fluorescence and UV absorbance as previously reported (5).

Active fractions from the second stage of HPLC were lyophilized, reconstituted in isotonic saline, and infused into a uremic rat that responded with strong and sustained natriuresis (see Fig. 14). Natriuresis began within 10 min and continued for 100 min, during which time the mean Δ sodium excretion rate (ΔUNaV: experimental UNaV – control UNaV) was 4.21 ± 1.5 SEM μEq/min. In contrast, the mean Δ potassium excretion rate was


Conflict of interest statement: Five of the six authors hold stock in Naturon, which is incorporated in the State of Delaware. The sixth author (F.C.S.) participated as a paid consultant. All costs for the present studies were paid for by Naturon. No dividends have been issued. A patent application on the two molecules (NH and NH-1) has been submitted (patent application no. US 2006-0217322-A1).

This article is a PNAS Direct Submission.

Abbreviations: CRD, chronic renal disease; NH, xanthurenic acid 8-O-β-D-glucoside; NH-1, xanthurenic acid 8-O-sulfate; SCC, short-circuit current.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0705553104DC1.

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www.pnas.org/cgi/doi/10.1073/pnas.0705553104
remaining virtually unchanged (ΔUkV = −1.05 (± 0.15 SEM) μEq/min. Concomitantly, the mean urinary sodium concentration increased from a mean control value of 55.0 (± 19.0 SEM) mEq/liter to 150 (± 49.8 SEM) mEq/liter, reaching a peak of 249 mEq/liter at 80 min. The mean urinary volume increased from a control rate of 57 (± 1.5 SEM) nl/min to 77 (± 4.5 SEM) nl/min during the first 40 min and gradually decreased to 26 nl/min 100 min after administration of the fraction. The natriuretic and nonnatriuretic responses of the isolated urine fraction, both in terms of magnitude and duration, are consistent with previous reports that followed a similar HPLC fractionation scheme (5).

The natriuretic HPLC fractions consistently showed two peaks [xanthurenic acid 8-O-α-d-glucoside (NH) and xanthurenic acid 8-O-sulfate (NH-1)] with strong fluorescence (excitation, 332 nm; emission, 430 nm) and characteristic UV absorption (UV-max at 338 nm) (Fig. 2). These spectroscopic signatures were subsequently used as the main pooling criteria for further HPLC-based purification of NH (Fig. 3B) and NH-1 (Fig. 3C).

The fluorescence and characteristic UV spectra suggested an aromatic or heteroaromatic moiety as part of NH and NH-1 (Fig. 3 B and C). Furthermore, their chromatographic properties suggested that these compounds may have acidic and/or basic properties because chromatographic separation was generally poor unless carefully buffered solvents were used. In addition, prolonged exposure to acidic conditions (pH < 5) resulted in precipitation and loss of fluorescence of NH, indicating decomposition of the active principles. The later-eluting NH-1 was noted to have similar fluorescent properties, but a slightly prolonged exposure to acidic conditions (pH 2) revealed that both fractions represent mixtures each containing two major aromatic compounds, as well as smaller amounts of other aromatic and aliphatic components. However, even the major components in these mixtures represented <50 μg of material. Therefore, to prevent additional losses of material, further fractionation aiming at the isolation of individual components was not pursued. Instead, building on recent examples for successful NMR-based characterization of individual compounds in complex small-molecule mixtures (21, 22), we continued with a comprehensive series of MS and NMR-spectroscopic experiments designed to characterize the individual components in the active fractions without further purification. Additional (1H,13C)-HMOC and (1H,13C)-HMBC NMR spectra of the fraction containing NH suggested the presence of a trisubstituted benzene derivative (compound 1) and a heteroaromatic bicyclic compound featuring a glucosylated phenol (compound 2) [supporting information (SI) Tables 1 and 2]. In combination with high-resolution positive-ion electrospray mass spectra, these data indicated that compound 1 corresponded to 2-(6-amino-3-hydroxyphenyl)-2-hydroxypropanoic acid, whereas compound 2 corresponded to NH (Fig. 3). Because the natriuretic activity of the active urine fractions did not appear to correlate with the amounts of compound 1 present, stereochemical characterization of compound 1 was not pursued. To confirm the identity of compound 2, an authentic sample of NH was chemically synthesized (see Materials and Methods). Because the NMR-spectroscopic data of compound 2 were highly pH- and concentration-dependent, we conducted an NMR-spectroscopic mixing experiment (NMR-spectroscopic coanalysis) (23), which provided final proof that compound 2 corresponded to NH (Fig. 4). Subsequent assays using synthetic NH clearly showed that this compound represents the suspected natriuretic factor NH (see Fig. 1B).

Similarly, NMR-spectroscopic analysis of the other natriuretic HPLC fraction revealed a major component whose structure appeared to be closely related to that of NH. In conjunction with
Isolation. First, 2- to 4-liter samples of human urine were lyophilized and then reconstituted with 100 ml of water (5). Aliquots of reconstituted urine were applied to a Sephadex G-25 medium column (Spectra/Chrom LC Column; 500 ml; i.d., 2.5 cm; 4.91 ml/cm). The mobile phase was 0.01 M ammonium acetate (pH 6.8) with a flow rate of 1.0 ml/min at 4°C. The UV absorbance was monitored at 285 nm, and conductivity was monitored online. After the elution of the salt peak (5), the osmolality dropped to <100 mOsm/kg. Following this step, ten 10-ml
fractions were collected, lyophilized, resuspended (in 1.0 ml of water), and assayed for inhibition of SCC in the frog skin (10).

Active fractions, as already defined, from the Sephadex column were then injected into a semiprep C18 column (Syringe 4 μ Hydro-RP 80A 250 × 10 mm, 4 μm; Phenomenex, Torrance, CA). The HPLC system included a Waters 600E Multisolvent Delivery System (Milford, MA). The detectors included a Waters 474 Fluorescent Detector and a 996 Photodiode Array Detector. Chromatograms were extracted at 290 and 338 nm. The mobile phase was 0.1 M pyridium acetate (pH 5.5) (solvent A) and methanol (solvent B). In the HPLC I method, sample volumes were 0.8–1.0 ml. The flow rate was 4 ml/min with 100% solvent A for 3 min and then in a linear gradient from 0–40% solvent B for the next 11 min. Subsequently, the column was washed with solvent B and then equilibrated with 100% solvent A. The retention time for NH was 13 min, as determined by the combination of a fluorescent peak (excitation, 332 nm; emission, 430 nm) and the UV spectrum (maximum at 338 nm) (Fig. 2). Then 2-ml fractions from multiple runs were pooled, and the volume was reduced by centrifugation under vacuum with medium heat. In the pooling and volume reduction of HPLC I fractions, the recovery of the fluorescent peak was typically 80–90%. Samples were periodically spiked with pyridine to keep the pH >5. Typically, 10 HPLC I runs were pooled together for each HPLC II injection of 1.0 ml. The HPLC II method differed from the HPLC I method only in the use of an isocratic gradient of 8% methanol, which was run for 13 min. Retention time for NH ranged from 11.2–11.6 min. Typically, 10 HPLC II runs were pooled for each HPLC III injection. The HPLC III method was the same as the HPLC II method, except that an analytical column was used with a flow rate of 1.0 ml/min by using a 0.15-ml injection volume (Syringe 4 μ Hydro-RP 80A 250 × 4.6 mm, 4 μm; Phenomenex). The retention time for NH was 10.6 min. After the HPLC III chromatography, NH was relatively pure based on the lack of any additional UV absorbance at 290 nm and its characteristic spectrum at 338 nm (Fig. 3B). The largely purified NH was then applied to the same analytical column by using only methanol (20%) and water (80%) as solvents to eliminate pyridium acetate.

During the HPLC I chromatography, a second fluorescent peak, subsequently determined to be NH-1 (retention time 13.7 min), was eluted after NH. This peak had a UV spectrum similar to NH as noted earlier (Fig. 3B and C). From this point on, NH-1 was purified through all of the same steps as NH. The final HPLC III retention time was 11.6 min.

The amounts of NH in the urine of CRD patients ranged from 2–130 nmoles per 24-hr urine collection, with an average of 10 nmoles. This calculation is based on a standard curve of synthetic NH and the fluorescence peaks from HPLC I method chromatograms (Fig. 2B). Similarly, amounts of NH-1 ranged from 0–105 nmoles per 24-hr urine with an average of 8 nmoles.

**Rat Assays.** The protocol to measure sodium and potassium excretion in rats has been used previously at multiple institutions and has been described in detail (5). In summary, following an equilibration period of 60 min (to allow for stabilization from the anesthesia and surgical procedures), urine was collected from rats for three 10-min control periods. At time 0, the test material was infused in 1.0 ml of saline by constant infusion over 10 min. Thereafter, urine was collected at 10-min intervals. U_{\text{Na}} V is sodium excretion rate in μEq/min, and U_{\text{K}} V is potassium excretion rate in μEq/min. Sodium and potassium concentrations were measured with ion specific probes.

**NMR Spectroscopy.** All NMR-spectroscopic analyses were carried out by using a Varian Inova 600 MHz NMR spectrometer (Palo Alto, CA), which was equipped with a 5-mm inverse-detection HCN probe. Shigemi tubes were used as needed. NMR spectra were acquired at 25°C by using the standard pulse sequences provided by Varian. For each sample, a standard phase-cycled, phase-sensitive dqf-COSY spectrum was obtained by using the following parameters: 600-ms acquisition time, 512 increments in F1, and 16 or 64 scans per increment. For further characterization, nongradient HMQC (phase-sensitive), gradient HSQC (phase-sensitive), nongradient HMBC, and phase-cycled, phase-sensitive NOESY spectra (using a 600-ms mixing time) were acquired. HMQC spectra were acquired without decoupling.

**MS.** Positive- and negative-ion electrospray ionization mass spectra were acquired using a Micromass (Manchester, U.K.) Quattro I mass spectrometer. High-resolution mass spectra were recorded on a Micromass Autospec instrument operated in a positive- or negative-ion electrospray mode. High-resolution positive-ion electrospray mass spectra of synthesized NH revealed a strong ion at 390.0811 (C_{19}H_{19}NO_5Na); the calculated molecular weight of this ion was 390.0801. After removal of the sodium, the measured molecular weight was 368.0995 (C_{19}H_{19}NOS), with a calculated molecular weight of 368.0982. Negative-ion electrospray MS of NH-1 showed a strong peak at 390.0801.
and dried to yield 215 mg of xanthurenic acid 2,3,4,6-tetra-turitated with 28 ml of 4:1 dimethyl sulfoxide:water and filtered. 283.9865 and a calculated molecular weight of 285.9803 8-2,3,4,6-tetra-


First, 4.53 mmol of xanthurenic acid was dissolved in 10 ml of aqueous 1 M NaOH and cooled to 10°C. Then 4.94 mmol of O

Step 2. Synthesis of xanthurenic acid 2,3,4,6-tetra-O-acetyl 8-O-β-d-glucoside.

First, 0.35 mmol of xanthurenic acid 2,3,4,6-tetra-O-acetyl 8-O-β-d-glucoside from step 1 was added to a 0.7-mmol solution of sodium methoxide in 5 ml of methanol and stirred for 1 h. The mixture was adjusted to pH 3.5 with aqueous 1 M HCl. The slurry was diluted with 20 ml of diethyl ether and filtered. The filter cake was washed with 1:1 methanol:diethyl ether and dried in vacuo to give 118 mg of pure NH.

Synthesis of NH-1. First, 2.92 mmol of sulfurtioxide complex and 5 ml of acetone were added to a 1.46-mmol solution of xanthurenic acid 2.9 ml of 1 M NaOH and 2.1 ml of water at room temperature. Then the reactor tube was sealed under nitrogen and stirred at 70°C for 16 h. It was cooled to room temperature and concentrated to dryness under reduced pressure. The residue was washed with acetone, acetonitrile, dichloromethane, ethyl acetate, and diethyl ether consecutively and then dried under vacuum overnight. The brown solid was dissolved in 3 ml of water and loaded on Sephadex SP-C25 column (swollen in water) to remove the sodium salt. The yield was 92.8%.

We thank Boaz Cotton, Carrie Cottrell, Sam Gbadebo, Tony Mai, and Deborah Thomas for providing technical assistance with the isolation and bioassays.