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ENDOGENOUS NATRIURETIC FACTORS 3: ISOLATION AND CHARACTERIZATION OF HUMAN NATRIURETIC FACTORS LLU-α, LLU-β_1, AND LLU-γ

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Summary

A low molecular weight endogenous substance believed to be responsible for extracellular fluid homeostasis in mammals has been sought for many years. Our goal is to isolate and structurally characterize this putative "natriuretic hormone." We have developed an assay using the conscious rat to measure prolonged natriuresis (Benaksas et al (1993) *Life Sciences*, 52, 1045-1054), the activity originally described for this putative substance. Using this assay we have identified a number of natriuretic compounds isolated from human uremic urine. The collected urine is processed by ultrafiltration (≤ 3 kDa), gel filtration chromatography (G-25) and extraction with isopropanol and diethyl ether. The organic soluble material is then subjected to sequential high-performance liquid chromatography. We report here the initial characterization of two pure isolates (LLU-α and LLU-γ) obtained by this method, and the structural elucidation of a third pure compound, LLU-β_1, a natriuretic and previously unreported metabolite of the drug diltiazem.

Key Words: natriuresis, natriuretic hormone, urine

Over thirty years ago the existence of the "natriuretic hormone" was proposed by de Wardener as a result of his classic parabiotic experiments in the dog (1). In those experiments a prolonged natriuresis was produced in a normal animal by the infusion of plasma from a volume expanded animal (1). Subsequently, this putative humoral substance has been thought to be responsible for not only the sustained natriuresis but also for the inhibition of sodium transport and an increase in vascular reactivity. Many *in vitro* assays have been used to search for the hormone based on the tenet of sodium pump inhibition by the hormone. These assays include Na^+/K^+-ATPase inhibition, ouabain displacement from the pump, and cross-reaction with anti-cardiac glycoside antibodies (2). While ouabain (3-5) and digoxin (6) or their isomers have been isolated recently using these methodologies, these materials are not natriuretic. As yet, the putative natriuretic hormone has not been identified.

It has been our premise that, while it is possible that the putative hormone is a sodium pump inhibitor, inhibitory activity does not necessarily lead to natriuresis. In fact it is more likely to

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produce kaliuresis (7-10). We concluded that the use of search tools based on the binding to and inhibition of the Na^+/K^+-ATPase have been poor at best for seeking the putative hormone (2). Therefore, in order to isolate and purify a natriuretic substance the assay used must measure natriuresis. Consequently, we developed a sensitive *in vivo* assay to measure natriuresis, kaliuresis, diuresis, and blood pressure changes in the conscious female rat for this purpose (11).

Using this *in vivo* assay we have isolated numerous natriuretic materials from human uremic urine (11, 12). In our earlier reports these isolates had been only partially purified (11, 12). We report here further purification of these isolates to high purity. One natriuretically active compound has been purified to homogeneity from each of the crude isolates α, β, and γ in sufficient quantities to permit the determination of their physical, chemical, and biochemical characteristics. These are now designated LLU(Loma Linda University)-α, LLU-β1, and LLU-γ. LLU-β1 has been completely characterized and found to be (+)-(2*S*, 3*S*)-3-hydroxy-5-(2-hydroxymethyl)-2,3-dihydro-2-(4-methoxyphenyl)-1,5-benzo-thiazepin-4(5*H*)-one.

Materials and Methods

Prepurification--The collection of human uremic urine, initial processing by ultrafiltration (3 kDa) and lyophilization, and the isolation of the post-salt fraction from Sephadex G-25 gel filtration chromatography have been reported previously (first purification step, Table I) (11). This crude material was further purified by one of two procedures. One procedure involved four sequential high performance liquid chromatography (HPLC) steps, and the second procedure included organic solvent extraction followed by up to five sequential HPLC steps. Table I summarizes the two methods.

Chromatographic Isolation Method--The four-step sequential HPLC procedure was a modification of that reported previously (11). The first C_{18} RP-HPLC (second purification step, Table I) was performed on a Beckman Ultrasphere ODS column (10 μm; 21.2 x 150 mm) eluting at 6 mL/min. with a gradient of 0.2 M pyridinium acetate pH 5.5 (A) and methanol (B) (80% A:20% B for 22 min., a linear gradient to 40% A:60% B over 48 min., a linear gradient to 100% B over 10 min.). The column was washed with 70% toluene:30% methanol, then reequilibrated at initial conditions for at least 20 min. This column wash method was implemented in every chromatography employing a methanol eluant. The eluant was monitored with a Beckman 166 UV detector at 290 nm. Eighty, one-minute fractions were collected and dried under reduced pressure in a centrifugal vacuum concentrator.

Based on bioassay evaluation (11, and below) and chromatographic comparison of previous HPLC runs, fractions 50-80 were combined for the second RP-HPLC step (third purification step, Table I). A Beckman Ultrasphere ODS (C_{18}) column (5 μm; 10 X 250 mm) was eluted at 2 mL/min. with a gradient of 0.2 M acetic acid (A), methanol (B) and 70% toluene: 30% methanol (C), (60% A:40% B for 5 min., a linear gradient to 50% A:50% B over 5 min., a linear gradient to 30% A:70% B over 55 min., a linear gradient to 100% B over 2 min., 100% B for 3 min., 100% C for 8 min., 100% B for 7 min.). The eluant was monitored for fluorescence (exc. 310-410 nm; emm. 475-610 nm: Beckman 157 detector) and absorbance at 290 nm with a Beckman 168 diode array detector. Ultraviolet spectra (202-390 nm) were collected by diode array at 2 second intervals. Eighty fractions of 1 min. duration were collected.

The chromatographic region encompassing the three natriuretically active isolates (α, β, and γ), as determined by our bioassay was pooled and rechromatographed using a modified acetic acid/methanol gradient for the third RP-HPLC (fourth purification step, Table I). The solvents and column were the same as the second RP-HPLC above; however, the gradient was changed (60% A:40% B for 5 min., a linear gradient to 40% A:60% B over 5 min., a linear gradient to 30% A:70% B over 28 min., a linear gradient to 100% B over 2 min., 100% B for 3 min., 100% C for 8 min.) and only 50 one-minute fractions were collected.

Each of the 3 partially purified natriuretic isolates (α, β, and γ) was subjected individually to normal phase chromatography on silica gel (Beckman Ultrasphere, 5 μm, 10 X 250 mm) eluting at 2 mL/min. with an isopropanol (A) hexane (B) gradient (6% A:94% B for 25 min., a linear gradient to 100% A over 30 min., 100% A for 20 min., a linear gradient to 6% A:94% B over 5 min. and an equilibration period at 6% A:94% B for 35 min.). Seventy, one-minute fractions were collected from this fifth purification step (Table I). Fluorescence was monitored as described above. Based upon their UV spectra, chromatography of the first isolate (α) was monitored at 295 nm, that of the second isolate (β) at 270 nm, and that of the third (γ) at 267 nm. Fractions exhibiting UV absorbances characteristic of α and γ were bioassayed (see below).

Extraction Method--Freeze-dried material obtained from gel filtration chromatography was stirred with 9 volumes of isopropanol for 18 hours. The isopropanol solution was then removed and evaporated to dryness on a rotary evaporator under reduced pressure. The resulting thick, dark brown oil from the isopropanol soluble phase was weighed and then alternately stirred and sonicated for 6 hours and finally stirred for an additional 18 hours, with 10 volumes of diethyl ether. The ether solution was then decanted and 4 volumes of ether were added to the remaining insoluble material. After stirring for 72 hours, the ether solution was again decanted. Two volumes of deionized distilled water and 2 volumes of diethyl ether were added to the residue. After stirring for 2 hours, the ether phase was separated and the aqueous phase was washed three times with one volume of ether. The combined ether extracts were washed with saturated aqueous NaCl and water, and taken to dryness on a rotary evaporator under reduced pressure. The residue was redissolved in 95% ethanol and again taken to dryness.

The ether extraction residue was dissolved in 40% aqueous methanol and subjected to acetic acid-methanol RP-HPLC (third purification step as described above, Table I). The chromatographic region from α to γ, as identified by their characteristic UV spectra, was pooled, dried, resuspended and chromatographed on the second (modified) acetic acid-methanol RP-HPLC (fourth purification step, Table I and see above). Only α and γ were detected after this chromatography step. Isocratic acetic acid-methanol RP-HPLC (fifth purification step) was performed on α. Employing a Beckman Ultrasphere ODS (C_{18}) column (5 μm; 10 X 250 mm), α was eluted at 2 mL/min. with 45% 0.2 M acetic acid and 55% methanol for 35 min. collecting 70 half-minute fractions. The eluant was monitored for absorbance at 290 nm (diode array) and fluorescence. Alpha was identified by its UV spectrum and subjected to the silica gel HPLC (sixth purification) step (described above). The fractions containing LLU-α from the silica gel HPLC, as identified by UV and natriuresis in our bioassay, were pooled and subjected to another C_{18} RP-HPLC step. In this seventh purification step (Table I), a Beckman Ultrasphere ODS column (5 μm; 4.6 X 250 mm) was eluted at 1 mL/min. with a gradient of 50 mM acetic acid (A) and 45 mM acetic acid in acetonitrile (B) (85% A:15% B for 3 min., a linear gradient to 100% B over 42 min., 100% B for 5 min.). The column was washed with 1:1 methylene chloride: acetonitrile for 5 min. followed by reequilibration at initial conditions for 16 min. Chromatography was monitored at 265 and 295 nm with the diode array detector. Fifty, half-minute fractions were collected starting at 10 min.

Modified Extraction Method for Isolation of LLU-β1--The isopropanol solution obtained from the extraction of the freeze-dried material from gel filtration chromatography (as described above) was evaporated to dryness under reduced pressure in a rotary evaporator. The dried residue was dissolved in 20% methanol and subjected to chromatography on RP-HPLC eluting with pyridinium acetate/methanol (see above; second purification step Chromatographic Method, Table I). The isolation procedure for LLU-β1 involved the rest of the Chromatographic Method (steps 3-5, Table I). The peak that contained LLU-β1 (see Results) was identified after silica gel HPLC. The fractions containing LLU-β1 were chromatographed on RP-HPLC utilizing a procedure similar to the seventh purification step of the extraction method (Table I). The solvents and column used were the same but the gradient was modified (80% A:20% B for 5 min., a linear gradient to 60% A:40% B over 5 min., a linear gradient to 50% A: 50% B over 40 min., a linear gradient to 100% over 5 min., 100% B for 10 min.). Sixty-five, one-minute fractions were collected.

Preparation of nuclear magnetic resonance (NMR) sample of LLU-β1--The fractions containing pure LLU-β1 from the last purification step were combined, dried, and weighed. The dried sample was dissolved in 0.5 mL of CD_3OD (99.8%) and solvent removed under a stream of argon. This step was repeated once. The vial containing the sample was transferred to an argon-filled glove box. The sample was then dissolved in 150 µL of CD_3OD (^{12}C 99.95%, D 99.5%) and transferred to a 2 mm NMR tube which was capped and sealed with Parafilm®.

Treatment of LLU-α with CH_2N_2--Diazomethane was generated by treatment of 1-methyl-3-nitro-1-nitrosoguanidine (112 mg, 760 µmol) with 400 µL 50% KOH (aq). The diazomethane was distilled into 1 mL diethyl ether at -7°C (13). This solution was then added to 700 µg (2.6 µmol) LLU-α in 0.5 mL diethyl ether at 0°C. The reaction mixture was warmed to ambient temperature, then allowed to stand for 40 min. (14, 15). The solvent was removed under a stream of N_2. The residue was dissolved in 15% 45 mM acetic acid in acetonitrile/85% 50 mM acetic acid and subjected to the acetic acid - acetonitrile RP-HPLC (seventh) purification step as described above. The approximate yield of the methyl ester was 53%.

In vivo bioassay--The assay for natriuresis in conscious rats has been described previously (11), but will be briefly reiterated here. Female Sprague-Dawley (Harlan) rats (200-250 g) were cannulated in the femoral artery and vein for monitoring of mean arterial pressure (MAP) and infusion of saline and samples, respectively. The bladder was catheterized for collection of urine in ten-minute periods. Furosemide (100 µg, approximately 0.4 mg/kg bwt; 1 mg/mL in 0.17% saline) was infused as a positive control at the beginning of the sixth ten-minute period. The sample was infused at the beginning of the seventeenth ten-minute period. Urine was collected for another 150 minutes. The volume of the urine was determined gravimetrically and the Na^+ and K^+ concentrations determined with a Beckman E2A electrolyte analyzer. From these data the sodium excretion values (UNaV; [UNaV = urine sodium concentration X urine volume per time]) were calculated.

The natriuretic response of a sample was normalized to the dose of furosemide infused. The net sodium excretion for the infusion of furosemide or sample was calculated as follows. The median sodium excretion value (µmoles Na^+/10 minute period) for the five periods before infusion of furosemide or sample was used to establish a baseline value for the calculation of ΔUNaV (= µmoles Na^+ period - baseline µmoles Na^+) for the administration of either furosemide or sample respectively. The sum of ΔUNaV for the four periods following infusion of furosemide was the net sodium excreted for furosemide, defined as furosemide response or FR. The sum of ΔUNaV for the fifteen periods (150 minutes) following infusion of the sample was the net sodium excreted for the sample defined as sample response or SR. The natriuretic ratio (R) (or normalized natriuretic response) of a sample was calculated by dividing SR by FR (R = SR/FR). A sample is considered natriuretically active if the R value for that sample was greater than or equal to 0.55 (greater than 99% confidence limits).

Na^+/K^+-ATPase inhibition assay--The assay in Madin-Darby bovine kidney (MDBK) cells has been described previously (11), and a very brief synopsis is provided here. Madin-Darby bovine kidney (MDBK) cells (ATCC: CCL22) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 5% Fetal Bovine Serum and 5% Bovine Calf Serum in a 5% CO_2/95% humidified air atmosphere at 37°C and split (1:2) once per week. Inhibition of the Na^+/K^+-ATPase was assayed by measuring reduction of $^{86}Rb^+$ uptake into the MDBK cells plated out in a 96-well plate. Ouabain was assayed over the range of 10^{-5}-10^{-8} M as a control for inhibition. All samples were assayed in quadruplicate and corrected for uptake that was inhibitable by ouabain.

Analytical spectroscopy-- Spectroscopy other than UV for LLU-α was performed at the Chemistry Department at the University of California, Riverside. ^{13}C- and ^1H-NMR (nuclear magnetic resonance) spectra were recorded at 500.1357 MHz in deutero-chloroform (99.9%) in a GN-500 spectrometer (General Electric). High resolution Electron-Impact (EI) mass spectra with a resolution of 2000 were recorded at an ionization voltage of 70 eV, source temperature of 220°C and

introduction of sample by direct probe on a VG7070 EHF high resolution mass spectrometer. Fourier-transform infrared (FT-IR) spectroscopy was performed on a Nicolet 5DX with 4 wavenumber resolution. Spectroscopy for LLU-β_1 was performed at the Chemistry and Biochemistry Department at UCLA. ^{13}C- (125.758 MHz) and ^{1}H-(500.134 MHz) NMR and COSY spectra were recorded on a Bruker ARX 500 using the UX NMR software. Mass spectra were performed on a VG Autospec instrument at UCLA, and electron-impact mass spectroscopy (EI-MS) were done at University of California, Riverside as described above.

TABLE I

Summary of Steps Used in the Chromatographic and Extraction Isolation Procedures
A detailed description of each purification step can be found in "Materials and Methods"

Purification Step	Chromatographic Method	Extraction Method
First	3K ultrafiltration, lyophilization and G-25	3K ultrafiltration, lyophilization and G-25
Second	0.2 M pyridinium acetate pH 5.5/ methanol C_{18} RP-HPLC	Extraction of isopropanol/ diethyl ether soluble compounds
Third	1st 0.2 M acetic acid/methanol C_{18} RP-HPLC	1st 0.2 M acetic acid/methanol C_{18} RP-HPLC
Fourth	2nd (modified) 0.2 M acetic acid/ methanol C_{18} RP-HPLC	2nd (modified) 0.2 M acetic acid/ methanol C_{18} RP-HPLC[b]
Fifth	Isopropanol/hexane silica gel HPLC[a]	Isocratic 0.2 M acetic acid/methanol C_{18} RP-HPLC[c]
Sixth		Isopropanol/hexane silica gel HPLC
Seventh		50 mM acetic acid/acetonitrile C_{18} RP-HPLC[d]

[a] Amount of resulting material of LLU-α, β, or γ was so small that further purification was not pursued.
[b] LLU-γ is pooled at this step due to instability upon further purification.
[c] This HPLC step was only used for isolation of LLU-α.
[d] LLU-α methyl ester was also purified using these HPLC conditions.

Results

Natriuretic Assay. The assay for natriuresis, kaliuresis and blood pressure changes has been described previously (11). Because of the variation in response to a standard dose of furosemide (100 μg) noticed early in the assay development, the assay was set up to eliminate the detection of false positives. Now that a large number of assays have been run, the great range of the furosemide response has become more apparent. In over 1900 assays (n=1912) the mean for the furosemide response was 253 μmoles of Na^+ (standard deviation [S.D.] 81) with a range of 1093 μmoles of Na^+ to 16 μmoles of Na^+. The assay as currently developed was used as a means to detect natriuretic activity in chromatography fractions.

In addition to examining purified urine isolates in the *in vivo* assay, other natriuretic and putative natriuretic compounds have been investigated using this assay. These were examined not only to determine their natriuretic activity but also as a means to validate the assay, in addition to the controls reported previously (11). Among those examined were atrial natriuretic factor (ANF) (Figure1C) and the putative endogenous digitalis-like factors, ouabain (3-5) and digoxin (6) (Figure 1A and B). ANF infused as a bolus into the rat produces an immediate short term natriuresis (Fig. 1C). Both

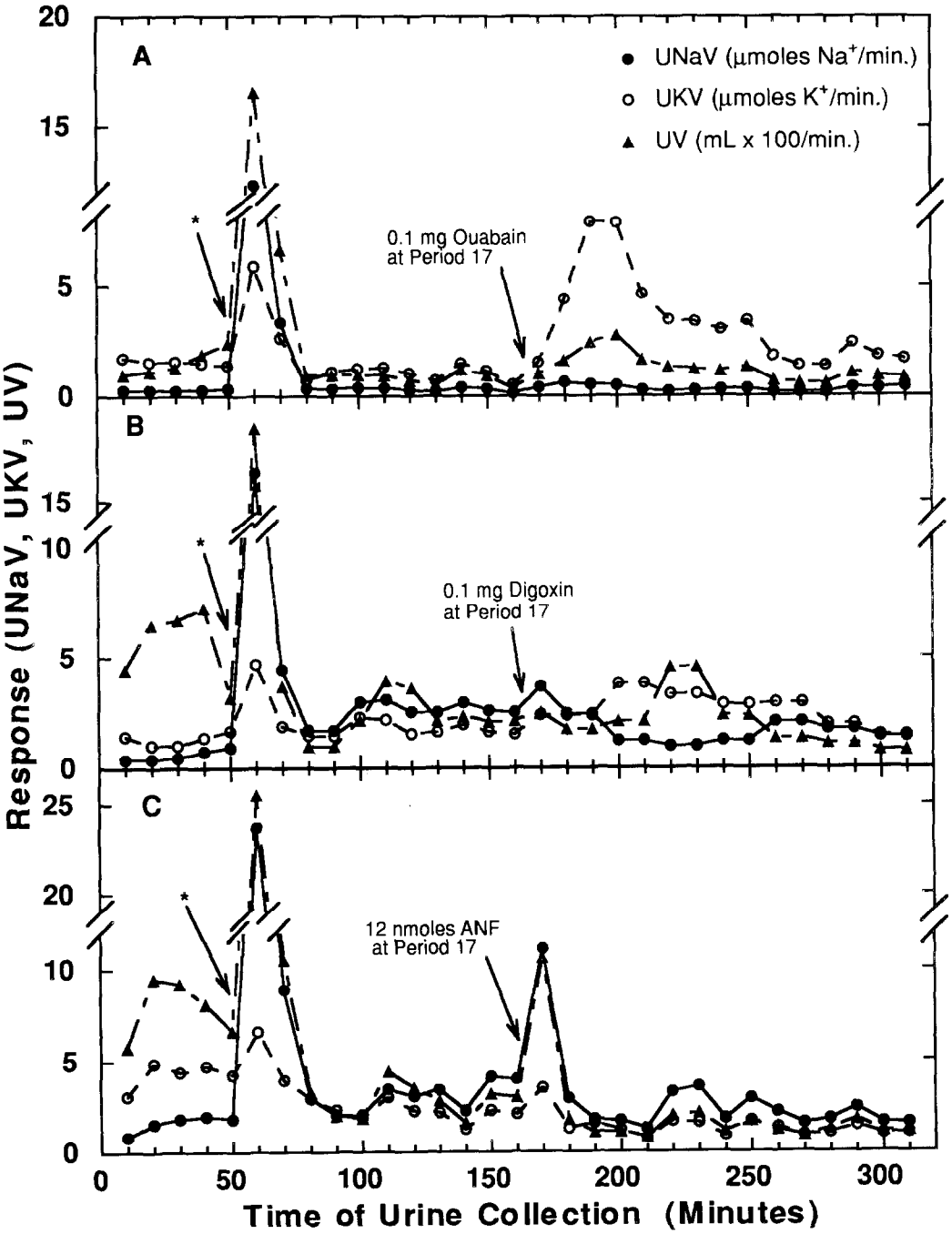


Fig. 1

In vivo bioassay of Ouabain (A), Digoxin (B) and Atrial Natriuretic Factor (ANF) (C). Assays performed as described in "Materials and Methods." Natriuresis (UNaV; closed circles), kaliuresis (UKV; open circles), and diuresis (UV; closed triangles) were measured. Furosemide infusion is indicated by the arrow with the asterisk. Samples were infused at the beginning of the periods indicated.

ouabain and digoxin, when infused, elicited an intense relatively long-lived kaliuresis with no natriuresis and some diuresis (Fig. 1A and B).

Isolation of Natriuretically Active Materials. We have previously reported the partial purification of isolates from human uremic urine that produced natriuresis in conscious rats (11, 12). It was evident from those initial studies that structural characterization of any one of these isolates would require a several fold increase in the amount of isolated material.

During the first aqueous acetic acid-methanol RP-HPLC (third purification step, Table I) UV spectra corresponding to the natriuretically active materials could be identified when processing different batches of urine. These UV spectra were used as marker chromophores in subsequent chromatographic steps. By rechromatographing fractions 38-58 and 63-66 using a modified acetic acid-methanol method (fourth purification step, Table I) employing a shorter gradient, the three natriuretically active marker chromophores, designated α, β and γ, reproducibly eluted at 27.8, 32.5, and 35.4 minutes, respectively (Figure 2). This fourth purification step has allowed consistent identification of the three natriuretically active crude isolates.

Alpha obtained from 935 L of uremic urine by this procedure was further purified on silica gel HPLC (fifth purification step, Table I). The yield of LLU-α was less than 1 mg; thus it was only possible to obtain a mass spectrum and ^{1}H-NMR. To increase the yield of LLU-α, extraction of the G-25 product with organic solvents was examined (see Extraction Method, Table I and Material and Methods). The G-25 material was sequentially extracted with isopropanol and diethyl ether. At each extraction step the organic solvent soluble material was taken on to the next step. Ultimately the ether soluble material was chromatographed in order to verify the presence of the natriuretic markers. The yield of LLU-α was increased by this method when compared to the purely chromatographic method of purification. In the chromatographic procedure, encompassing a total of five purification steps, less than 1 mg of LLU-α was obtained from about 105 g of lyophilized G-25 material (yield less than 9×10^{-4}%)[1]. Approximately 1.8 mg of LLU-α resulted from the extraction procedure (seven purification steps) applied to about 155 g of lyophilized G-25 product (yield approximately 1.2×10^{-3}%). The yield of LLU-γ appeared to increase comparably. Beta was detected only in the isopropanol extraction step (also see below). This extraction method was used to obtain LLU-α and LLU-γ. The minimum estimated concentration of LLU-α in urine based on the amounts isolated, described above, from 935 L and 1375 L, respectively, would be approximately 5 nM and the maximum concentration might well be several times that amount.

Characterization of LLU-α, LLU-β, and LLU-γ. LLU-α was purified from a total of 1375 L of uremic urine by the extraction methodology. Partially purified LLU-α from silica gel-HPLC (the sixth purification step, Fig. 3) was assayed for natriuretic activity utilizing the *in vivo* bioassay. It was active in the 4-8 μg/kg dose range and showed no activity at lower or higher doses (Table II). LLU-α was also active at the same doses when evaluated in the *in vivo* bioassay (Table II) after being further purified on acetic acid/acetonitrile RP-HPLC (elution time 24.8 min., seventh step of extraction method). When LLU-α was assayed in the Na^+/K^+-ATPase inhibition assay it exhibited no inhibition in the range of 0.2-200 ng/well(data not shown). LLU-α was methylated with CH_2N_2 and rechromatographed on acetic acid - acetonitrile RP-HPLC (see Materials and Methods). LLU-α methyl ester eluted as an apparently homogenous single peak (retention time: 31.5 min.) from acetic acid - acetonitrile RP-HPLC. Approximately 0.9 mg of LLU-α methyl ester was isolated and subjected to chemical characterization by ultraviolet, infrared, ^{13}C- and ^{1}H-NMR and mass spectroscopy. The physical chemical characteristics, molecular weight and inferred molecular formula of both LLU-α and its methyl ester are listed in Table III. LLU-α methyl ester did not produce natriuresis in our assay (data not shown).

[1]Due to the very complex mixtures (many thousands of compounds) obtained in the fractions from early chromatographic steps (steps 1-3) and due to the somewhat qualitative nature of the *in vivo* bioassay as employed for assay of chromatographic fractions, yield, as opposed to specific activity, is reported as a measure of the progress of purification.

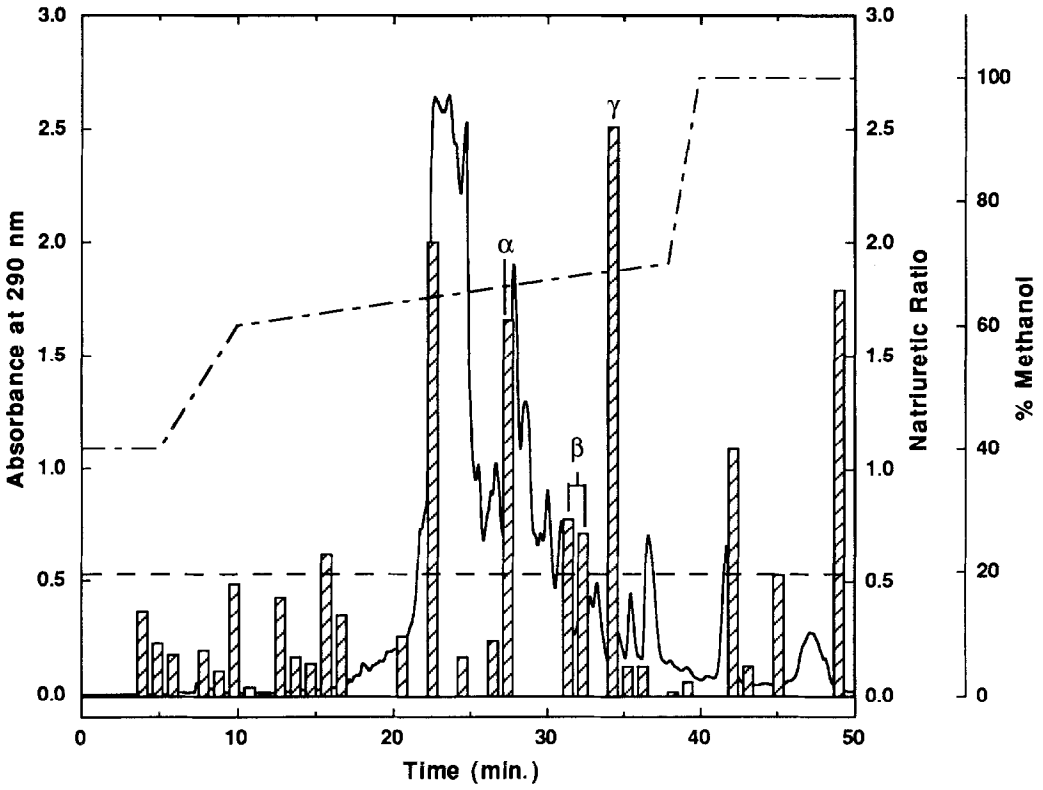


Fig. 2

HPLC chromatogram of acetic acid/methanol rechromatography step and natriuretic responses. Absorbance at 290 nm of eluant collected from the acetic acid/methanol rechromatography (fourth) step of the chromatographic purification procedure is shown (solid line). The programmed methanol gradient is plotted(—— - ——-). Ten percent of the fractions collected were assayed in the *in vivo* bioassay as described in "Materials and Methods." Non-negative Natriuretic Ratios (cross-hatched bars) are shown. The dashed line at 0.55 on the Response axis represents the cut-off above which samples are deemed natriuretically active (99% confidence limits). Natriuretic responses are from a single determination. Fractions corresponding to α, β and γ are indicated.

Because of its instability, LLU-γ can only be recovered without extensive decomposition from either purification method through the modified acetic acid-methanol RP-HPLC chromatography step (step 4). The UV absorbance maxima determined from diode array scans of the peak eluting from the acetic acid-methanol RP-HPLC rechromatography are shown in Table III. Loss of both the UV chromophore for LLU-γ and its natriuretic activity is observed upon further purification (data not shown). Addition of 100 μg dithiothreoitol to the fractions collected from hexane/isopropanol silica gel HPLC preserved the chromophore sufficiently so that it could at least be detected by UV in the appropriate fractions approximately 20 minutes after collection. The chromophore was no longer evident after the fractions were dried under reduced pressure. Assay of crude LLU-γ obtained from the acetic acid-methanol RP-HPLC rechromatography step in the sodium pump inhibition assay showed no inhibition (data not shown).

Beta was obtained from isopropanol extraction of the lyophilized G-25 product of 55 L of uremic urine and chromatographed through the modified acetic acid-methanol RP-HPLC rechromatography

(fourth purification step). It was then separated into at least six natriuretically active components (LLU-β_{A-F}) by silica gel HPLC (Fig. 4). The material remaining after *in vivo* assay was insufficient

TABLE II

Dose Response of LLU-α from the Silica Gel and RP HPLC Purification Steps (6 & 7) of the Extraction Procedure Assayed *In vivo* for Natriuretic Activity as Described in "Materials and Methods."

Purification Step	Dose	Natriuretic Response (R)[a]
	μg/kg	
Silica	0.8	0.24, -0.14
	4	0.93, 0.27
	8	1.14, 0.75
	40	0.26, 0.09
RP-HPLC	4	0.90, 0.04
	8	0.77, 0.23

[a] Natriuretic ratio greater than 0.55 indicates that a sample is natriuretically active (99% confidence limits).

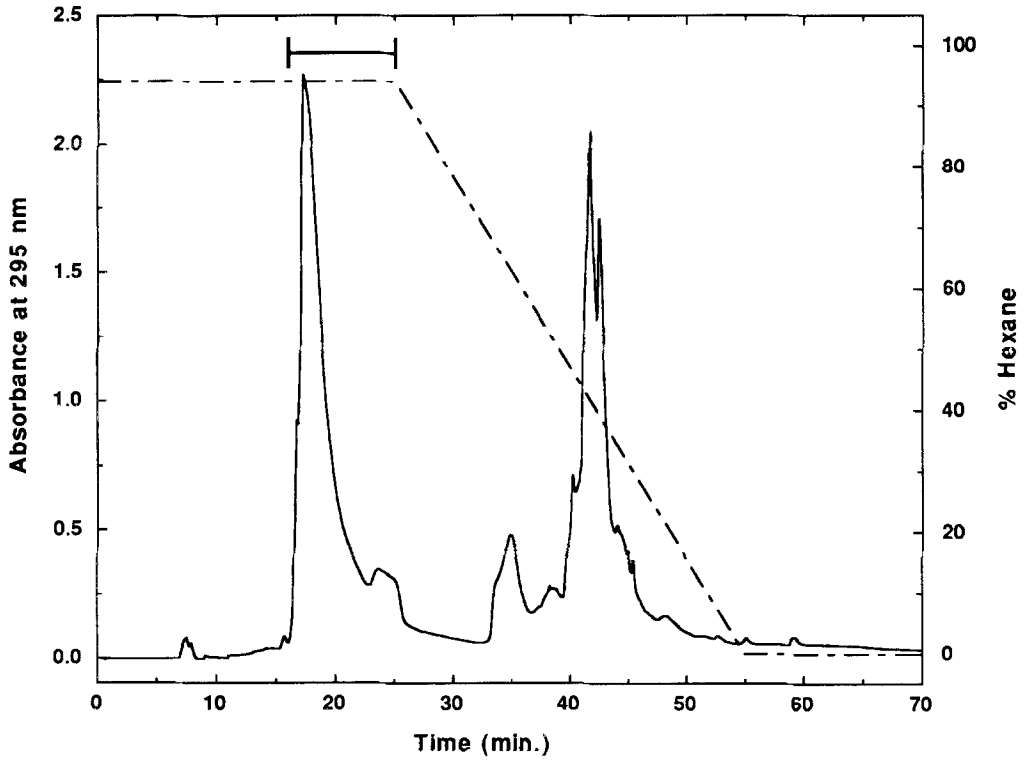


Fig. 3

Chromatogram of the isopropanol/hexane silica gel HPLC (sixth) step of the isolation of LLU-α by the extraction procedure. Absorbance at 295 nm of eluant collected is shown (solid line). The programmed hexane gradient is plotted (——- - ——-). UV spectra corresponding to α are detected between approximately 17 and 25 minutes and are marked with a bar (|——|) at the top of the figure.

TABLE III
Chemical Characteristics of LLU-α and LLU-γ

	LLU-α	LLU-γ
Exact Mass	264.1373	ND[a]
Empirical Formula	$C_{15}H_{20}O_4$	ND
UV Characteristics	λ_{max} 205nm λ_{max} 294nm	λ_{max} 220nm λ_{max} 268nm
Functional Groups Determined by IR	carboxyl hydroxyl vinyl ether	ND
Physical Properties	Unstable in Dilute Base Unstable in $CDCl_3$	Very Unstable when Purified Very Unstable in Dilute Base
Reaction with CH_2N_2	LLU-α methyl ester $C_{14}H_{19}O_2CO_2CH_3$ MW 278.1515 + Other Products	ND

[a] ND: Not Determined

for further characterization. The ultraviolet spectra of these natriuretic actives were obtained from the diode array and have provided markers for identification of these materials in subsequent larger scale isolations.

In one such isolation on 800 L of processed uremic urine in which the ether extraction step was not used, the LLU-β_E material was detected and subjected to modified acetic acid/acetonitrile RP-HPLC (see Materials and Methods). LLU-β_E separated into six peaks eluting over the time range of about 20.5-28 minutes one of which was natriuretic (R = 0.95 and 2.88; assayed in duplicate) when approximately 0.3% of the total material was evaluated in the *in vivo* bioassay. This material eluting at 21.0 minutes was designated LLU-β_1. This purified LLU-β_1 (0.92 mg) was then subjected to chemical characterization by ^{13}C- and ^{1}H- NMR, COSY and mass spectroscopy. The proton NMR is shown in Figure 5. The MS data are listed in Table IV. The ^{13}C-NMR was taken in CD_3OD and the following data were obtained (δ in ppm): 173.27 (C-4); 161.15 (C-17); 147.18 (C11); 136.21 (C-9); 132.62 (C-15, C-19); 132.04 (C-7); 129.96 (C-14); 128.78 (C-8); 128.18 (C-10); 126.28 (C-6); 114.18 (C-16, C-18); 70.47 (C-3); 59.79 (C-13); 57.93 (C-2); 55.53 (C-20); 53.05 (C-12). From these data the structure for this compound was determined to be a metabolite of the drug diltiazem (Figure 6). The overall yield for LLU-β_1 was approximately 1 x 10^{-3}%, and its minimum estimated concentration in the 800 L pool of urine, from which it was isolated and the donors had received diltiazem as therapy, would be 3 nM.

Activity of the LLUs in the in vivo bioassay. LLU-α, -β_1 and -γ when infused into the rat produced a long-lasting natriuresis (Figure 7A, B and C). There was no detectable kaliuresis when LLU-α or LLU-β_1 were infused (Fig. 7A and 7B). However, mild kaliuresis was occasionally observed after the infusion of LLU-γ. Neither LLU-α nor -γ caused a significant change in mean arterial pressure of the assay animal (data not shown). A decrease in MAP of up to 15 mm Hg was observed for LLU-β_1 in one of the two natriuretically positive samples tested (data not shown).

Discussion

Isolation of natriuretic substances. Both LLU-α and LLU-β_1 have been purified to apparent homogeneity as verified by the complete structure determination of LLU-β_1 (Fig. 6) and initial structural characterization of LLU-α (Table III). Neither LLU-α nor -β_1 are not similar to any of the

20 previously described isolates (reviewed in 2; Table 2 therein), nor are they similar to any of the more recently reported isolates such as ouabain (or an isomer, [3-5]), digoxin (or an isomer, [6]), resibufogenin (16), 20-hydroxyeicosatetraenoic acid or 20-carboxyarachidonic acid (17), urodilatin (18), or pregnenolone sulfate (19).

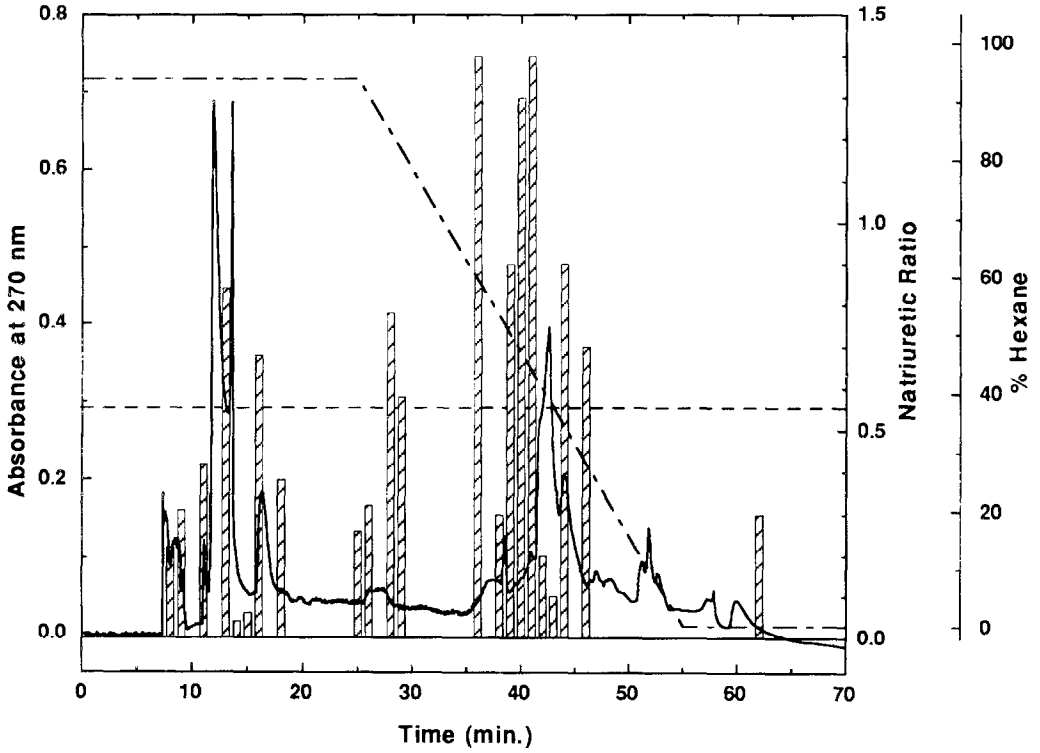


Fig. 4

Chromatogram of the isopropanol/hexane silica gel HPLC (Sixth) step and natriuretic response for β from the extraction procedure. The extraction was done only with isopropanol on the equivalent of 55L of urine and then subjected to the chromatography steps as described in "Materials and Methods." Absorbance at 270 nm was monitored (solid line). The programmed hexane gradient is included (—— - —— -). Two-thirds of each of the fractions collected were assayed for natriuresis in the rat as described in "Materials and Methods." Positive Natriuretic Ratios are shown (cross-hatched bars). The dashed line at a Natriuretic Ratio of 0.55 indicates the cut-off above which samples are deemed natriuretically positive. Natriuretic Ratios are from a single determination. LLU-β_A is fraction 13, LLU-β_B is fraction 16, LLU-β_C is fractions 28 and 29. LLU-β_D is fraction 36, LLU-β_E is fractions 39-41, LLU-β_F is fractions 44 and 46.

As anticipated when first identified during the modified acetic acid/methanol RP-HPLC (fourth purification step of the Chromatographic Method, Table I), β had turned out to be a very complex mixture. When further chromatographed on silica gel HPLC it gave rise to six regions of natriuretic activity (Fig. 4). One of those regions (LLU-β_E) afforded six peaks during modified acetic acid/acetonitrile RP-HPLC, one of which is LLU-β_I. With this purification procedure, LLU-β_I was purified to the point to permit complete structural characterization. The ^{1}H-NMR indicates that LLU-β_I was essentially homogeneous and at worst contains much less than 10% of any contaminant (Fig. 5). LLU-β_I is a previously unidentified metabolite of the drug diltiazem (20-22). It most likely

originates by an oxidative deamination of the —CH_2—$CH_2N(CH_3)_2$ moiety of diltiazem to —CH_2COOH followed by reduction to —CH_2CH_2OH with concomitant deacetylation of the 3-O-acetate (23). That a metabolite of diltiazem was found is not surprising since some of the urine donors were being medicated with this anti-hypertensive agent. Even though LLU-β_1 is not a candidate for the "natriuretic hormone," this metabolite may be associated with the natriuresis observed with diltiazem administration (24-26). However, other studies suggest that diltiazem itself is natriuretic (27, 28).

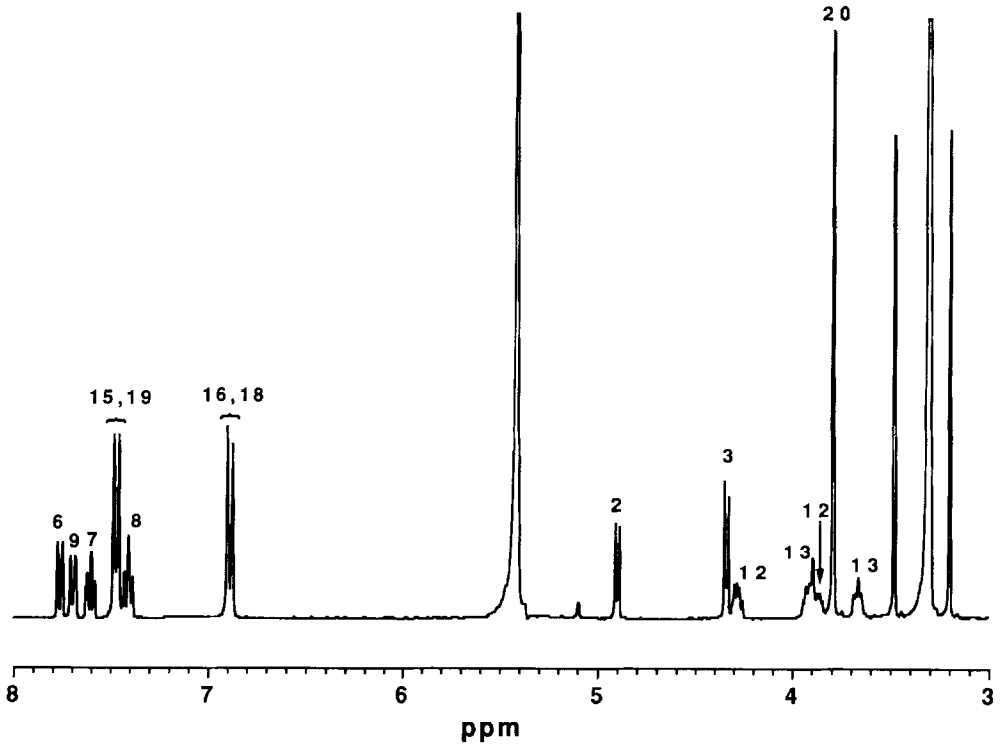


Fig. 5

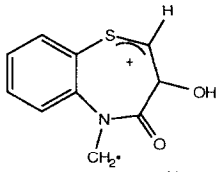
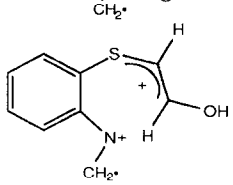
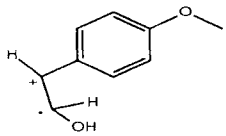
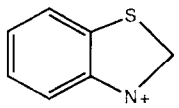
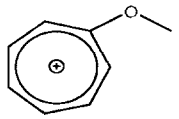
Proton NMR of LLU-β_1. Spectrum was taken in CD_3OD as described in "Materials and Methods." The two peaks at approximately 3.2 and 3.5 ppm are due to C^{12}-O^{18} coupling (^{13}C depleted CD_3OD). Peak at about 5.45 ppm is due to water. Peak at about 3.3 ppm is due to methanol. Numbers above peaks correspond to the carbon atoms to which the proton is attached (see Fig. 6).

By the chromatographic procedures employed, LLU-α is apparently homogeneous. Its detailed structure was investigated. Its exact mass is 264.1368 daltons corresponding to the empirical formula $C_{15}H_{20}O_4$. The IR and ^{13}C-NMR spectra of LLU-α provided evidence of homogeneity and the presence of a carboxylic acid group. This explained the tailing of LLU-α observed upon elution from isopropanol/hexane silica gel HPLC (sixth step of extraction procedure; Figure 3). The presence of a carboxyl group was verified when the reaction of LLU-α with diazomethane resulted in a product that was less polar on RP-HPLC and had an exact mass 14 units greater than LLU-α as determined by MS (Table III). This is consistent with the formation of a methyl ester.

The extraction purification procedure has increased the yield of isolated LLU-α by about 50% (see Results) and the two additional RP-HPLC steps of this procedure lead to essentially pure LLU-α. Methylesterification of LLU-α followed by RP-HPLC also yielded essentially pure LLU-α

methyl ester. Also from this extraction procedure there should also be sufficient LLU-γ to attempt to solve its structure by direct HPLC/MS which is presently underway.

TABLE IV
Summary of EIMS data (70 eV) of LLU-β_1

Mass	Fragment Formula and/or Name	Probable Structure
m/z (%)[a]		
346 (2)	$C_{18}H_{20}NO_4S$ (MH^+)	
345 (9)	$C_{18}H_{19}NO_4S$ (M^+)	LLU-β_1
317 (10)	—[b]	
316 (46)	$C_{17}H_{18}NO_3S$ (M^+ – CHO)	
209 (4)	—[b]	
208 (22)	$C_{10}H_{10}NO_2S$	
180 (8)	$C_9H_{10}NOS$	
151 (8)	—[b]	
150 (30)	$C_9H_{10}O_2$	
137 (8)	—[b]	
136 (23)	C_7H_6NS	
135 (9)	—[b]	
122 (11)	—[b]	
121 (100)	C_8H_9O Tropilium-OCH_3	

[a]Percent is relative to the ion of the highest intensity. Only major ions are listed.
[b]There are too many probable structures or molecular formulas for these ions to list them.

The extraction procedure was also performed on 55 L of normal human urine. LLU-α was isolated after the acetic acid/acetonitrile RP-HPLC (seventh) step and LLU-γ was detected during the silica gel HPLC (sixth) step. The detection of these substances in normal urine is encouraging, suggesting that they are endogenous and not the product of uremia. Further, they may be involved in volume homeostasis in normal individuals and uremics.

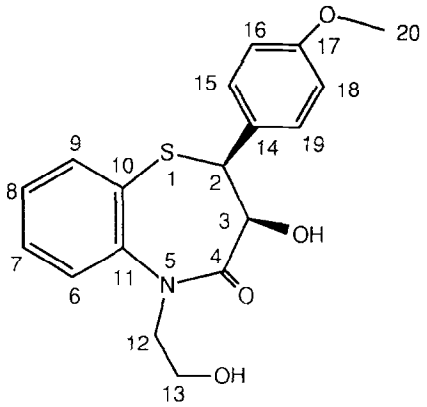


Fig. 6
Structure of LLU-β_1.
Exact Mass: 345.1034. Inferred Molecular Formula: $C_{18}H_{19}O_4NS$
(+)-(2*S*,3*S*)-3-Hydroxy-5-(2-hydroxyethyl)-2,3-dihydro-
2-(4-methoxyphenyl)-1,5-benzothiazepin-4(5*H*)-one

From uremic urine purified through the modified acetic acid/methanol (fourth) step, there are five regions of natriuretic activity of which two regions (α, γ) are being actively pursued (Figure 2). The activities at retention times of about 42 minutes (δ) and 49 minutes (ε) have little ultraviolet absorbance making them very difficult to follow in subsequent chromatography steps using ultraviolet detection. However, these peaks are being saved for further purification using refractive index detection. The natriuretic activity at about 22 minutes is associated with a complex mixture with no discrete UV spectrum. This isolate is being subjected to further chromatography to determine the number of natriuretically active isolates that it may contain. These isolates will eventually be subjected to chemical characterization.

From previous work in this laboratory (11, 12), there were regions of natriuretic activity that have not been pursued. Due to the modification of the isolation methodology to allow for more efficient purification of LLU-α, -β_1, and -γ numerous very small quantities of natriuretically active materials previously reported (12) have not been defined. In addition, the later eluting region of the second purification step of the chromatographic method (elution time approximately 55-60 min.) has not been pursued due to the low ultraviolet absorbance associated with those fractions. These are presently under investigation employing a refractive index detector.

In vivo and in vitro activities of the LLU's and other compounds. In the mid-1970's a hypothesis linking the putative natriuretic hormone to inhibition of the Na^+/K^+-ATPase and the role it might play in hypertension (29) has led many investigators in the field to use assays such as inhibition of the Na^+/K^+-ATPase, displacement of [^{3}H]-ouabain from the pump, or cross-reactivity with anti-digoxin or anti-ouabain antibodies as a search tools for the putative hormone (2). The primary search tool employed by this laboratory is an *in vivo* assay for natriuresis in the mildly hydropenic conscious rat. It was, therefore, important to test the various identified available endogenous digitalis-like factors and other natriuretic materials in our *in vivo* assay.

Ouabain, digoxin and ANF were examined in the *in vivo* bioassay for their effects on sodium, potassium, and water excretion and MAP. ANF gave rise to a strong short-lived natriuresis and decrease in MAP in this assay system (Figure 1C) as was observed in the anesthetized rat (30, 31). This short-lived natriuresis is not consistent with the original observation for "natriuretic hormone" as described by de Wardner (1). As ouabain and digoxin (or isomers thereof) have been reported recently to be endogenous digitalis-like factors (EDLFs, [3-6]), we were interested in the effects of these compounds in our *in vivo* assay system. Ouabain has been previously reported to produce

kaliuresis in the anesthetized rat (7). Here, ouabain also produced kaliuresis accompanied by diuresis (Figure 1A) and an increase in MAP at the time of injection (data not shown). Digoxin also produced kaliuresis and diuresis (Figure 1B) with a slight increase in MAP at injection. Neither ouabain nor digoxin proved natriuretic and are therefore not candidates for the putative hormone,

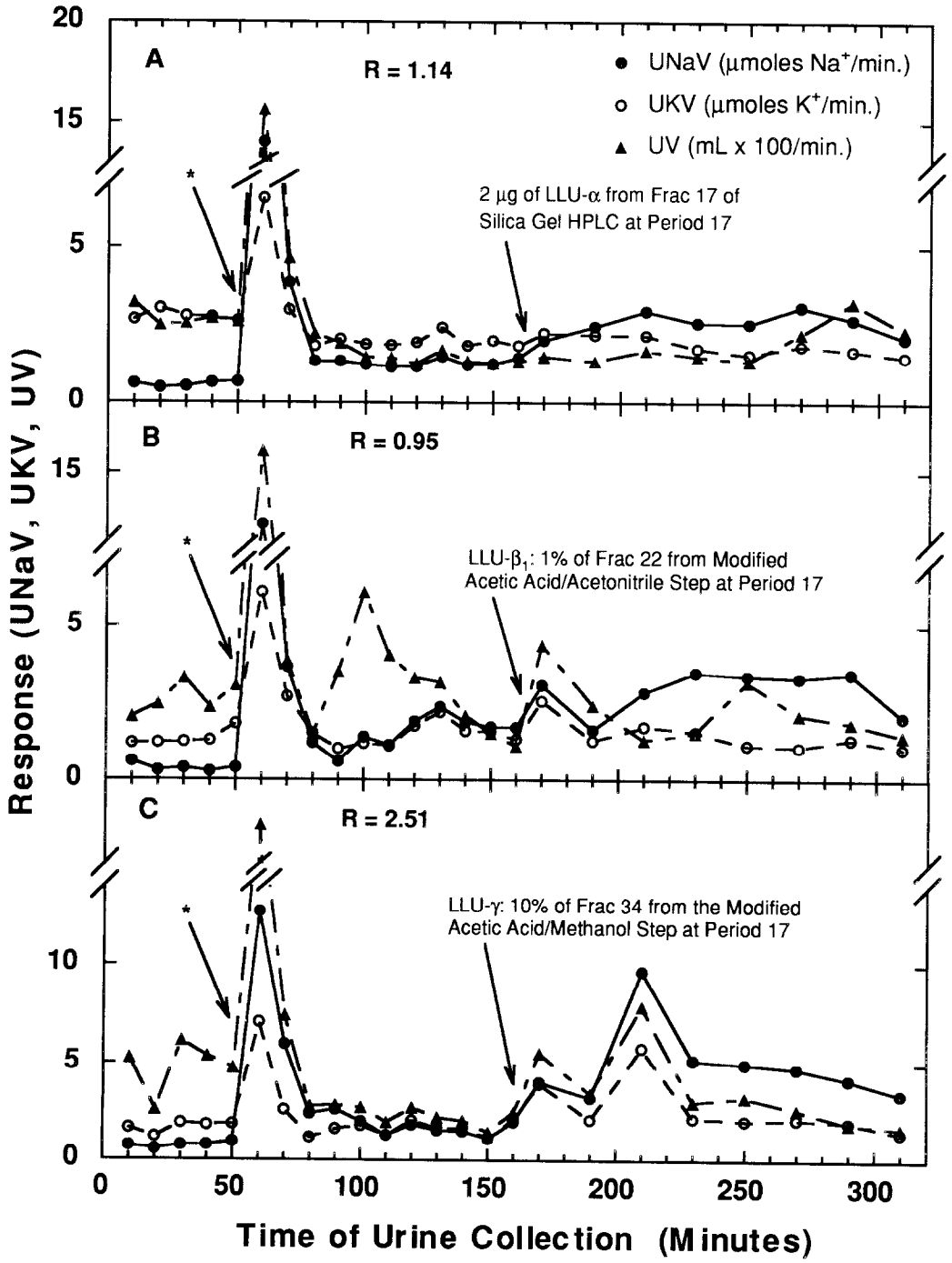


Fig. 7
In vivo bioassay of LLU-α (A), LLU-β_1 (B) and LLU-γ (C). See legend to Figure 1.

even though they have some of the properties which are the basis of many search strategies for the hormone (2). Exactly what functions these EDLFs may have in the body or whether they are truly endogenous have been questioned by various investigators (2, 32).

Both LLU-α and -γ produce a sustained natriuresis (Figure 7) with no effect on blood pressure, while LLU-β_1 also produces a sustained natriuresis but with an occasional effect on blood pressure (data not shown). The effect on blood pressure exhibited by LLU-β_1 seems to be much less than that exhibited by the drug diltiazem in the *in vivo* bioassay (data not shown). Sufficiently pure and stable LLU-γ has not been isolated to obtain a dose-response curve for natriuresis. LLU-α displays a narrow and parabolic natriuretic dose-response curve (Table II). The reason for this complex response is not understood at present. Part of the explanation for the inconsistency in the dose-response experiments for LLU-α is due to animal to animal variation as evidenced by the furosemide response (see "Results"). We are in the process of determining the false negative rate of our assay in order to increase the precision of the assay for subsequent dose-response studies. A true dose-response curve will be generated for LLU-β_1 once it has been synthesized and the structure of the synthetic material has been verified to be identical to that of the isolated LLU-β_1. Even though the bioassay as used is not quantitative, the two isolated compounds studied in depth, LLU-α and -β_1, can be compared with other natriuretic substances run in the bioassay. LLU-α (4-8 μg/kg) and LLU-β_1 lead to more sodium excretion than ANF (3 nmole/kg) over an extended time, yet they are not as potent as furosemide (400 μg/kg).

A question that must be addressed by all investigators in this field, including ourselves, is whether any isolated material is truly endogenous. It is readily apparent that LLU-β_1 is not endogenous since it is the product of an exogenously administered drug. A recent paper reports the presence, in various commercial teas and herbs, of digoxin immunocross-reactivity and inhibition of ouabain binding to Na^+/K^+-ATPase (33). Since these are some of the assays used to look for endogenous digitalis-like factors it brings to the forefront the question whether EDLFs detected and isolated from human sources are possibly of dietary origin.

With the isolation of a multiplicity of "endogenous" compounds which affect sodium transport it may well be that no "natriuretic hormone" as such exists, but rather extracellular fluid homeostasis is the product of multifactorial control. Having determined the structure of one (LLU-β_1) of our isolates and in the process of synthesizing it, we are now in a position to fully characterize the biological effects of this substance. Presuming that the structures of the other natriuretic isolates allow for the possibility of their being of endogenous origin, elucidation of their structures, followed by their synthesis and the characterization of their biological effects may provide insight into the possibility of multifactorial control of ECF volume.

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