Ouabain Is Not Detectable in Human Plasma

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Abstract An enzyme-linked immunosorbent assay is described for the measurement of ouabain in human plasma. This assay is specific for ouabain, strophanthidin, and ouabagenin, with other steroids, including digitoxin and vasopressor hormones, exhibiting negligible cross-reactivity. Assay sensitivity was 0.06 nmol/L if 1 mL plasma was extracted and less than 0.005 nmol/L when 20 mL plasma was analyzed. Extracted plasma samples showed ouabain-like immunoreactivity that diluted in parallel with the ouabain standard curve. Repeated extraction and assay of single plasma samples, however, did not produce consistent results in the assay. Increased specificity was obtained by high-performance liquid chromatography of sample extracts before assay. When high-performance liquid chromatographic profiles of plasma spiked with ouabain standard or following bolus intravenous injections of ouabain into normal human volunteers were compared with profiles of unspiked plasma, there was no support for the immunoreactive material in the latter samples being ouabain. We propose that if ouabain is present in the human circulation, its concentration is less than 0.005 nmol/L. (Hypertension. 1994;24:S49-555.)

Key Words • ouabain • hypertension, essential • sodium

Ouabain is a cardiac glycoside found in certain plant species and may also be found in animals.1,2 Recently, Ludens et al1 and Hamlyn and colleagues2-6 suggested that ouabain is secreted by the adrenal glands and circulates in plasma in humans. This raises the question of whether ouabain might be the, or one of the, digoxin-like compounds thought to play a role in the regulation of sodium balance, arterial pressure, and vascular smooth muscle tone under normal circumstances and in disorders such as essential hypertension, pregnancy-induced hypertension, cardiac failure, and chronic renal failure. Uncertainty surrounding the involvement of ouabain in the regulation of blood pressure and electrolyte balance should be resolved in part by the availability of a reliable method for measuring ouabain concentrations in plasma. Published enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay methods can measure picomolar concentrations of ouabain.3-6 We describe an alternative ELISA method based on the methodology of Lewis et al3 that is sensitive and precise and compare the results obtained by assay of plasma extracts with those obtained after extraction and high-performance liquid chromatographic (HPLC) fractionation.

Methods

Buffers

Phosphate-buffered saline (PBS) solution (60 mmol/L Na2HPO4, 40 mmol/L NaH2PO4, 150 mmol/L NaCl, pH 7.0) containing 1% bovine serum albumin and 1% thiomersal was used to reconstitute extracted samples and to dilute standards. PBS (pH 7.4) containing 1% gelatin and 0.05% Tween 20 was used for diluting antibody, and PBS (pH 7.4) containing 0.6% Tween 20 was used for washing microtitre plates. Substrate for horseradish peroxidase was prepared immediately before use. We added 40 mg o-phenylene-diamine and 60 µL hydrogen peroxide (30% vol/vol) to 100 mL substrate buffer (50 mmol/L Na2HPO4, 25 mmol/L citric acid buffer, pH 5.0).

Antibody Preparation

Antibody raising procedures were approved by the Animal Ethics Committee, Christchurch School of Medicine. Ouabain dialdehyde was coupled to C6-ovalbumin following the method of Harris et al. The resulting solution was freeze-dried. Ouabain-ovalbumin (10 mg) was suspended in 5 mL 0.9% saline and emulsified with 5 mL Freund's complete adjuvant. Each of two New Zealand White rabbits received a subcutaneous injection of the ouabain-ovalbumin solution with boosters at monthly intervals for 2 months. Blood samples were collected into EDTA tubes through a small incision on a marginal ear vein 2 weeks after the booster injections. Immunoreactivity to ouabain was detected in EDTA plasma from both rabbits. Plasma from the second bleed of one rabbit (Gil-2) was diluted 1:100 with assay buffer and stored in 100-µL aliquots at -20°C.

Conjugate Preparation

Preparation of thyroglobulin-ouabain conjugate followed the procedure outlined by Harris et al except mannitol was used instead of meso-erythritol in the preparation of ouabain dialdehyde and the lyophilized conjugate was not purified on a G-25 Sephadex column. A 50 µg/mL stock solution of ouabain-thyroglobulin conjugate was prepared by rehydrating lyophilized conjugate in distilled water. This solution had sodium azide added as a preservative and was stable at 4°C for at least 3 months. A working conjugate solution was prepared by adding 10 µL of stock conjugate solution to 10 mL of 6 mol/L guanidine hydrochloride.

Standards

A stock standard of 1 µg/mL ouabain (Sigma Chemical Co) was prepared in ethanol and diluted in phosphate buffer to provide a series of standards with concentrations of 0.023, 0.69, 2.1, 6.2, 18.7, and 56 nmol/L. Sodium azide was added as a preservative. Standards were stable at 4°C for at least 3 months.
Extraction of Ouabain From Plasma Samples

Venous blood samples were drawn from healthy volunteers and patients into chilled tubes containing EDTA (1.5 mg/mL blood) and were centrifuged at 1500g and 4°C for 5 minutes within 10 minutes of blood collection; the plasma was stored at −20°C. After thawing, plasma samples were centrifuged (1500g for 5 minutes at 4°C), and the supernatant was used for extraction. One of two extraction methods was used. Extraction method 1 was used unless otherwise stated.

Extraction Method 1 (Acetonitrile Cartridge)

Bond Elut C18 cartridges (Varian) were washed under vacuum with 3 mL methanol, followed by 3 mL distilled water with a Super Separator Unit (Amersham). The plasma sample (1 mL) was added to the cartridge and allowed to pass through without vacuum, followed by three 3-mL washes with distilled water. Then 3 mL of 25% acetonitrile/0.1% trifluoroacetic acid (TFA) solution was used to elute ouabain. The eluate was dried under air at 45°C and reconstituted with 400 μL phosphate buffer (pH 7.0) for use in the ELISA or with 200 μL 5% isopropanol for HPLC analysis. For extraction of 20-mL plasma samples, the procedure was the same as outlined above except 5 mL plasma was gravity fed through each of four cartridges and the eluates were dried down, reconstituted, and combined before HPLC analysis. In some specified instances, the plasma was collected after passing through the cartridge and reanalyzed with extraction method 2.

Extraction Method 2 (Meohanol Precipitation)

Plasma (20 mL) was mixed with 100 mL methanol for 5 minutes and centrifuged (1500g for 20 minutes at 4°C). The supernatant was aspirated, recentrifuged, and dried under air. The extract was reconstituted with 5% isopropanol, transferred into Eppendorf tubes, and reconstituted. This step was repeated until no visible precipitate remained. The extract was then injected onto the HPLC column.

HPLC

A 10-cm Brownlee Spheri5 RP18 column was used for HPLC analysis (series 410 HPLC system, Perkin-Elmer) running a gradient of 4% to 16% isopropanol over 20 minutes, followed by 80% isopropanol for 2 minutes. Fractions (1 mL) were collected from the HPLC column for 35 minutes, dried under air at 50°C, and stored at −20°C before assay.

ELISA

All steps except plate activation and sample addition were performed on an automatic ELISA processing machine (Behring Elisa processor II, BPIII, Behring). For activation of microtitre plates (Falcon 3912, Microtest III, Becton Dickinson & Co), working conjugate solution (100 μL) was added to each well of a 96-well microtitre plate and the plate was incubated overnight at 4°C. A 2.5-hour incubation at room temperature on the assay day provided similar adsorption of the conjugate to the microtitre plate and could be used as an alternative to overnight incubation. All subsequent steps in the assay were performed at room temperature.

The plate was inverted and shaken dry onto a paper towel to remove unbound conjugate. The plate then was washed four times with 200 μL wash buffer per well and dried by inversion. Assay buffer (150 μL) was added to each well, and the plate was incubated for at least 1 hour to allow protein to adsorb to nonspecific binding sites. Plates were emptied by inversion and shaken dry, and standard or sample extract (50 μL per well) was added in duplicate to the appropriate wells, except for the zero standard, which was added in quadruplicate. Anti-ouabain plasma was then added (50 μL per well) at a dilution of 1:2000 000 to give a final in-well dilution of 1:400 000. Plates were incubated for at least 2 hours and then washed as above. Goat anti-rabbit serum conjugated with horseradish peroxidase (Amersham) was added (100 μL per well) at a dilution of 1:2000. After incubation for at least 1.5 hours, the plate was washed. Substrate solution (100 μL per well) was added, and development of color was left to proceed in the dark for approximately 10 minutes. Then the reaction was stopped by the addition of 100 μL per well of 1.25 mol/L H2SO4. Absorbance was read at 492 nm, with a reference wavelength of 650 nm using the ELISA processor.

Results were interpolated from the standard curve using a four-parameter logistic function.

Specificity

Serial dilutions (ranging from 10 μg/mL to 10 pg/mL, ie, approximately 10 μmol/L to 10 pmol/L) of various steroid and vasoactive hormones were prepared in PBS and assayed to assess cross-reactivity with the ouabain antibody.

The HPLC retention times of strophanthidin, ouabagenin, and ouabain standards were determined by ultraviolet absorbance at 220 nm.

Extraction Recovery

Charcoal-stripped human plasma was spiked with [3H]ouabain ([21,22-3H]ouabain, Amersham) to 0.2 or 2 nmol/L, and 2, 1, or 0.5 mL was extracted using method 1 to assess recovery through the extraction system. Duplicate tubes containing [3H]ouabain only were dried down and reconstituted to compare the amount of tracer added to that recovered from the cartridge. Ouabain in all tubes was reconstituted with 400 μL phosphate buffer. A subsequent elution (3 mL 25% acetonitrile/0.1% TFA) was performed to determine carryover from the cartridge. The amount of [3H]ouabain in each tube was determined by liquid scintillation counting in a beta counter (Rackbeta, LKB).

Precision and Recovery

Buffer and plasma were spiked with either 0.39 or 1.9 nmol/L ouabain, and each sample was assayed to determine recovery through the extraction and ELISA. Repeated measurement (n=29) of a plasma sample and the same sample spiked with 1.4 nmol/L ouabain was carried out to determine within-assay variation.

HPLC Analysis

Sensitivity

Assay sensitivity was calculated from the means and SDs of the baseline values for 12 assays. The sensitivity was calculated as the concentration of ouabain that gave a response 2 SDs greater than the mean baseline. To determine whether ouabain could be detected in plasma, 11 HPLC runs with CIS-extracted plasma were compared. HPLC fractions 12 through 14 were combined, as ouabain in spiked plasma consistently eluted in these fractions. A similar comparison was performed on the 20-mL plasma extracts, comparing them with buffer extracts. The combined fractions were analyzed pairwise by Student's t test.

Parallelism

Plasma obtained from a healthy volunteer 10 minutes after an intravenous infusion of 0.25 mg ouabain given over 30 seconds was extracted, and 100-, 50-, 25-, and 12.5-μL aliquots of the extract were injected into the HPLC system and subsequently assayed to check linearity through the system. The same sample was also assayed without HPLC separation.

Ouabain standard (0.32 nmol/L) was added to plasma, and this solution was diluted with plasma 1/2, 1/4, and 1/8 before extraction to assess parallelism through the assay system.

To determine the effect of heating plasma, 1 mL of spiked (0.22 nmol/L) or unspiked plasma was extracted in duplicate and subjected to heating at 37°C (or held at room temperature) for 3 hours.
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Bulk plasma samples (20 mL) collected from healthy volunteers were extracted by both acetonitrile cartridge and methanol precipitation followed by HPLC and ELISA. Plasma was also collected after elution off the acetonitrile cartridge and reextracted by methanol precipitation before HPLC and ELISA.

**Ouabain Bolus Study**

Healthy volunteers (n=7) were given 0.5 mg ouabain by intravenous injection over 2 minutes, and venous samples were obtained from the opposite arm at -1, 0, 0.5, 1, 2, 3, 4, 5, and 6 hours after injection for measurement of plasma ouabain. Plasma ouabain was measured by both HPLC (0.5 mL extracted) and non-HPLC (1 mL extracted) methods.

**Clinical Studies**

Plasma obtained from individuals with different clinical disorders (congestive heart failure, primary aldosteronism, chronic renal failure, and pregnancy-induced hypertension), during normal pregnancy, and from umbilical cord blood was screened for ouabain with both the non-HPLC and HPLC methods.

**Comparison With Assay Kit**

Plasma ouabain concentrations were also measured with an ELISA kit (Du Pont—NEN) in conjunction with a 3,3',5,5'-methylbenzidine (TMB) substrate kit (Bio-Rad, Life Sciences Group). Plasma samples were extracted according to the kit methodology and assayed directly or after HPLC fractionation. Briefly, the extraction was as follows. Plasma (1 mL for non-HPLC assay or 5 mL for HPLC assay) was combined with 2 vol 0.1% TFA and incubated at room temperature for 1 hour and then overnight at 4°C. Then the sample was centrifuged and the supernatant extracted by using C18 cartridges. The same sample extracts were analyzed in parallel by using our assay as described above.

**Results**

Adsorption of ouabain-thyroglobulin conjugate to the microtitre plate, as tested in the absence of sample or standard, was 1.669±0.034 absorbance units with a coefficient of variation of less than 2.1%.

Fig 1 shows a typical standard curve for ouabain, along with doubling dilutions of a human plasma sample analyzed without HPLC. All compounds tested, except ouabagenin and strophanthin, exhibited negligible cross-reactivity with the anti-ouabain antibody (Table 1). Recovery of [3H]ouabain from the extraction technique was greater than 92% over the range of 0.2 to 2 nmol/L.

**Fig 1.** Serially diluted plasma sample (•) analyzed without high-performance liquid chromatography shows parallelism with a typical ouabain standard curve (©).

**Table 1.** Cross-reactivity, Defined as 50% Displacement From the Zero Standard, of Anti-Ouabain Serum

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cross-reactivity, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ouabain</td>
<td>100</td>
</tr>
<tr>
<td>Aldosterone</td>
<td>0.04</td>
</tr>
<tr>
<td>Estradiol</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Cortisol</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>Testosterone</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Deoxycorticosterone</td>
<td>0.07</td>
</tr>
<tr>
<td>Progesterone</td>
<td>0.07</td>
</tr>
<tr>
<td>Digoxin</td>
<td>1.25</td>
</tr>
<tr>
<td>Ouabagenin</td>
<td>77.24</td>
</tr>
<tr>
<td>Strophanthin</td>
<td>70.38</td>
</tr>
<tr>
<td>Angiotensin II</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Dehydroepiandrosterone</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Prednenediol glucuronide</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Estriol glucuronide</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Estradiol glucuronide</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Recovery of "cold" ouabain from spiked plasma was 90% to 108% (after subtraction of blank values) when samples were assayed without HPLC.

Repeated extraction of aliquots of plasma samples spiked to two different concentrations with ouabain produced coefficients of variation of 42% and 11.6% (0.43±0.18 and 2.1±0.24 nmol/L), respectively. Recoveries were 150%. This high recovery may indicate interference by another substance in the assay. For this reason, samples were subsequently separated by HPLC, and the fractions were assayed by ELISA to determine whether immunoreactivity in plasma samples occurred in the same fractions as ouabain standard.

Standard solutions of ouabain and ouabagenin were not resolved by HPLC analysis with ultraviolet detection, but both were distinct from strophanthin (Fig 2).

**HPLC Analysis**

Isopropanol (5%) run as a blank in the HPLC system produced immunoreactive ouabain values less than 0.06 nmol/L (Fig 3). A plasma sample spiked with ouabain showed a peak of immunoreactivity in fractions 12 through 14 (Fig 3) with no peak detected in unspiked plasma.

**Sensitivity**

The median sensitivity for 12 assays was 0.06 nmol/L (range, 0.02 to 0.13 nmol/L). When buffer samples were compared with plasma samples, there was no significant difference between plasma and buffer. The minimal detectable difference between buffer and plasma was 0.06 nmol/L. Using 20 mL plasma, the detection limit was 0.005 nmol/L.

**Parallelism**

A plasma extract, obtained from a volunteer given ouabain intravenously, diluted in a dose-response man-
when decreasing amounts of the extract were injected into the HPLC system (Fig 4). All dilutions gave a plasma ouabain value of approximately 3 nmol/L, whereas non-HPLC assay of the same plasma sample produced a ouabain concentration of 4.5 nmol/L.

Serially diluted plasma samples spiked with 0.32 nmol/L ouabain produced concentrations of approximately 1 nmol/L when assayed without HPLC. The ouabain concentration calculated from the HPLC peak (fractions 12 through 14) was 0.38 (undiluted), 0.15 (diluted 1/2), 0.084 (diluted 1/4), and 0.032 nmol/L (diluted 1/8) with up to another 0.26 nmol/L from a peak of immunoreactivity at fractions 28 through 30.

Plasma spiked with “cold” ouabain showed immunoreactivity where standard ouabain eluted, whereas unspiked plasma did not. Heating did not abolish ouabain immunoreactivity in spiked samples.

Ouabain peaks were not detected when plasma (20 mL) from 10 healthy volunteers was extracted by either acetonitrile cartridge or MeOH precipitation before HPLC separation (Fig 5A through 5C). An immunoreactive peak was observed in fractions 28 through 32 with all extraction methods and in fractions 2 and 3 with MeOH precipitation. The identity of these immunoreactive moieties was not pursued. Table 2 shows ouabain concentrations in 1-mL plasma extracts from various
TABLE 2. Ouabain Concentrations Determined by Local Assay or NEN Assay Kit in Plasma Samples From Different Patient Groups Measured With or Without HPLC Coupled to ELISA

<table>
<thead>
<tr>
<th>Patient Group</th>
<th>Local assay Mean</th>
<th>SEM</th>
<th>Range</th>
<th>HPLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy volunteers (n=9)</td>
<td>0.32</td>
<td>0.13</td>
<td>0.13-0.56</td>
<td>ND</td>
</tr>
<tr>
<td>Congestive heart failure (n=6)</td>
<td>0.65</td>
<td>0.36</td>
<td>0.25-1.6</td>
<td>ND</td>
</tr>
<tr>
<td>Chronic renal failure* (n=6)</td>
<td>0.44</td>
<td>0.15</td>
<td>0.24-0.62</td>
<td>ND</td>
</tr>
<tr>
<td>Pregnancy</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Healthy volunteers (n=5)</td>
<td>0.40</td>
<td>0.15</td>
<td>0.21-0.58</td>
<td>ND</td>
</tr>
<tr>
<td>Preeclampsia† (n=5)</td>
<td>0.27</td>
<td>0.09</td>
<td>0.16-0.38</td>
<td>ND</td>
</tr>
<tr>
<td>Cord plasma (n=8)</td>
<td>1.81</td>
<td>0.06</td>
<td>1.61-2.16</td>
<td>ND</td>
</tr>
<tr>
<td>Conn’s syndrome (n=1)</td>
<td>0.33</td>
<td></td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>NEN assay kit</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Healthy volunteers (n=5)</td>
<td>0.06</td>
<td>0.004</td>
<td>0.05-0.07</td>
<td>ND</td>
</tr>
<tr>
<td>Cord plasma (n=8)</td>
<td>0.07</td>
<td>0.005</td>
<td>0.06-0.10</td>
<td>ND</td>
</tr>
<tr>
<td>Conn’s syndrome (n=1)</td>
<td>&lt;0.05</td>
<td></td>
<td></td>
<td>ND</td>
</tr>
</tbody>
</table>

HPLC indicates high-performance liquid chromatography; ELISA, enzyme-linked immunosorbent assay; and ND, not detectable.

*Before maintenance dialysis.
†Weeks 30 to 40 of pregnancy.

patient groups without HPLC. Whereas ouabainlike immunoreactivity was detectable by non-HPLC assay, there was no evidence of ouabain in plasma from patients with congestive heart failure or chronic renal failure or in normotensive or hypertensive pregnant women when samples were analyzed by HPLC and ELISA.

Non-HPLC immunoreactive ouabain levels in plasma 30 minutes after the 0.5-mg bolus intravenous injection of the cardenolide in seven healthy volunteers reached approximately 8 nmol/L and then declined steadily to a plateau of approximately 1.7 nmol/L at 4 hours. Ouabain concentrations obtained from the assay of the same plasma samples after HPLC fractionation paralleled those from the non-HPLC assay but were lower (Fig 6).

Assay Kit

The NEN assay kit could detect ouabain to less than 0.05 nmol/L from 1-mL plasma extracts (Fig 7), indicating that it was more sensitive than our assay. Endogenous ouabain concentrations ranged from 0.05 to 0.1 nmol/L in plasma analyzed by the ouabain ELISA kit, but this immunoreactivity was abolished with HPLC analysis of the plasma extract (Table 2). Measured ouabain concentrations in spiked or infused plasma extracts tended to be higher in our assay than in the kit assay.
**Discussion**

A circulating sodium-transport inhibitor has been implicated in the pathogenesis of human disorders such as essential hypertension, pregnancy-induced hypertension, primary aldosteronism, cardiac failure, chronic renal failure, and acromegaly.\(^6\) Over the last 20 years, numerous and largely unsuccessful attempts have been made to identify such digoxin-like inhibitors of Na,K-ATPase.

Ludens et al\(^{14}\) reported the isolation of ouabain, or an isomer of ouabain, from 3000 L of human plasma. Its identification was facilitated by chromatography, mass spectroscopy, and its immunoreactivity and binding characteristics. An ELISA was developed and showed in the rat and dog\(^2\) that the adrenal glands were the likely source of circulating ouabain. Immunoreactive ouabain levels in plasma were reported to be elevated in patients with cardiac failure\(^15\) and hypothyroidism,\(^16\) elevated in acenatal cases of primary aldosteronism and pheochromocytoma,\(^7,^{17}\) and reduced in hyperthyroidism.\(^16\) These data suggested that ouabain might be the, or one of the, circulating inhibitors of Na,K-ATPase and could be central to the pathogenesis of certain disorders in humans. This possibility was reinforced by the observation that sustained intraperitoneal infusion of ouabain at high doses raised arterial pressure after 5 weeks in normal rats, an effect that was exaggerated in animals with reduced renal mass.\(^19\) Additional information obtained under experimental circumstances over many years provided mechanisms by which ouabain might alter circulatory homeostasis, particularly to raise arterial pressure in humans. These actions include augmentation of myocardial contractility, direct arterial constriction, inhibition of endothelium-dependent vasodilatation, augmentation of endothelin secretion, a mitogenic effect on vascular smooth muscle, and enhancement of the pressor effects of angiotensin II, norepinephrine, and mineralocorticoids.\(^2,^{16},^{20,26}\)

Despite widespread interest after publication of the reports of Hamlyn and colleagues,\(^2,^{3,4,14}\) editorial reviews were cautious in assigning a physiological or pathophysiological role to ouabain.\(^27,^{29}\) Furthermore, data contrary to the findings of Hamlyn's group have appeared. For example, some workers\(^30,^{32}\) identified digoxin-like factors and Na,K-ATPase inhibitors in human plasma that were distinct from ouabain. Naruse and colleagues\(^6\) reported plasma immunoreactive ouabain levels in human plasma that were much lower than those recorded by Hamlyn's group and Smith\(^33\) was unable to detect ouabain in either plasma or urine samples from 30 patients. Furthermore, Naruse et al\(^6\) concluded that because no step-up in immunoreactive ouabain levels was seen in adrenal venous versus peripheral venous blood, it was unlikely that the adrenal glands were the source of circulating immunoreactive ouabain.

In view of uncertainties regarding the circulating levels of ouabain, its site of secretion, and its biological functions in humans, we developed an ELISA to measure the glycoside in human plasma. By coupling the ELISA to HPLC methodology, we have developed a specific, reliable, and sensitive procedure for the measurement of plasma ouabain. Assay sensitivity was 0.06 nmol/L; recovery of exogenous ouabain through both the non-HPLC and HPLC assay exceeded 90%. Measurable cross-reactivity with the ouabain antibody was observed with strophanthidin and ouabagenin, compounds structurally similar to ouabain, and to a lesser extent with digoxin, but not with other steroids or vasoactive compounds (Table 1).

Other conjugate preparations involving the use of succinyl dihydrazide and hexane diamine as bridges between ouabain dialdehyde and thyroglobulin were compared with the conjugate used in the present assay, but they had less affinity for the antibody and were not pursued.

Our non-HPLC assay yielded results similar to those reported by Hamlyn's group\(^15\) (ranging from 0.2 to 1.6 nmol/L [Table 2]). Displacement of the antibody in serially diluted plasma samples assayed without HPLC was parallel with the ouabain standard curve. Repeated extraction and assay of single plasma samples, however, did not produce consistent results in the ELISA, suggesting the presence of interfering factors. When our ELISA was then coupled to HPLC, no immunoreactive peak was detected where ouabain was expected to elute, suggesting that ouabain itself was not contributing to this plasma immunoreactivity. An immunoreactive peak was observed around fractions 28 through 32 from the HPLC profile, but its identity has not been pursued.

Naruse et al\(^6\) suggested that ouabain circulates at lower levels (0.04 to 0.08 nmol/L) than those proposed by others.\(^2,^{15}\) Extraction of 20 mL plasma increased our assay sensitivity to 0.005 nmol/L, yet we still did not detect endogenous ouabain by HPLC. This indicates that the sensitivity of our ELISA assay was not a limiting factor in ouabain detection.

Recovery of ouabain from the HPLC peak around fractions 12 through 14 concurred with the amount of ouabain spiked into plasma. Heating or adding TFA to plasma (both of which should denature protein) before extraction did not increase ouabain concentrations. Administration of a bolus intravenous dose of ouabain to healthy volunteers resulted in high plasma ouabain concentrations, which then decreased rapidly over the first 3 hours but did not reach baseline levels within the 6-hour study period (Fig 6). This biphasic decline in ouabain concentration agrees with pharmacokinetic studies reported previously.\(^34\)

To the best of our knowledge, the NEN ouabain ELISA kit uses the same antibody as Hamlyn's group did, yet we did not obtain endogenous ouabain-like immunoreactivity above 0.1 nmol/L in non-HPLC samples using this method. Immunoreactivity was abolished by HPLC. Plasma spiked with varying amounts of ouabain and separated by HPLC before the NEN ELISA could be measured down to 0.05 nmol/L (Fig 7). Results obtained from the NEN ELISA were generally lower than those obtained by our assay, even though the NEN ELISA standard curve was more sensitive and had smaller coefficients of variation, particularly at lower ouabain concentrations.

An isomer of ouabain has been characterized from bovine hypothalamus.\(^35\) One possible explanation for the discrepant observations from various ouabain assays may be that human endogenous ouabain is a stereoisomer of plant ouabain and is detectable by some antibodies and assay systems\(^6,\) but not by others, including our own. However, the NEN ELISA apparently used Ham-
lyn's antibody, and in our hands, ouabain was not detected.

In conclusion, we have developed and validated a sensitive and specific system for the assay of ouabain in human plasma. Without preliminary HPLC fractionation, ELISA (using our own antiserum or that from NEN) initially yields fallacious values for apparent plasma ouabain concentrations. When this source of error was eliminated, we could find no evidence for the presence of endogenous ouabain in the plasma of healthy volunteers or from patients with a variety of circulatory disorders.

Acknowledgments

The Health Research Council of New Zealand provided financial support for this project. We thank Dr John Livesey for statistical advice.

References

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Hypertension. 1994;24:549-555
doi: 10.1161/01.HYP.24.5.549

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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