Effect of salt intake on excretion of endogenous ouabain-like substance, measured by RIA

YEMANE K. SEMRA, ASIF N. BUTT, AND R. SWAMINATHAN*

Recent studies suggest that ouabain or a ouabain-like substance (OLS) may be present endogenously in humans. We developed a RIA for ouabain with antisera raised in goat against ouabain conjugated to keyhole limpet hemocyanin and ovalbumin. The antiserum was of high antibody titer (200 000) and was specific for ouabain, with little cross-reactivity with common steroids and structurally related compounds such as ouabagenin (4%), strophanthidin (4%), and dihydroouabain (2%). The RIA had a working range of 0.06–2.0 nmol/L, and the intra- and interassay CV was 6.5% at a concentration of 1.7 nmol/L. With this assay the effect of salt loading on urinary excretion of OLS was examined in 10 healthy volunteers (ages 18–22 years) who increased their salt intake (sodium) for 5 days and reduced it for the next 5 days. Urine was collected and OLS concentration was measured by RIA after solid-phase extraction with a Bond Elut C_{18} column. Excretion of OLS and sodium were maximal on day 5 and lowest on days 9 and 10. Urine excretion of OLS on day 5 (2.66 ± 1.22 mmol/24 h) was significantly higher (P < 0.0001) than on day 10 (1.47 ± 0.69 mmol/24 h). We conclude that (a) the assay developed has sufficient sensitivity and specificity to detect endogenous OLS present in biological fluids, and (b) salt intake increases the excretion of OLS.

INDEXING TERMS: endogenous inhibitor • hypertension • sodium • potassium • ATPase

The presence of endogenous sodium transport inhibitor(s) in several species including humans is well documented [1–4]. This sodium transport inhibitor(s) has been suggested to play a role in the regulation of sodium excretion and in the pathogenesis of hypertension. Increased concentrations of sodium transport inhibitor(s) have generally been found during plasma volume expansion, during high salt intake, and in hypertension [1–5]. Various techniques have been used to demonstrate the presence of this inhibitor, including displacement of labeled ouabain from intact cells or isolated membranes, measurement of Na^+•K^+• ATPase activity of purified enzyme, and immunoassay involving cross-reactivity with digoxin antibodies [4]. The chemical nature and source of this inhibitor(s) remains controversial [3, 4, 6, 7]. Previously, an endogenous sodium transport inhibitor with immunological, biochemical, mass spectrometric, and pharmacological characteristics similar to ouabain was identified in human plasma [8]. An immunoassay for ouabain was developed and the presence of ouabain in human plasma was demonstrated [9]. However, Doris et al. [10] and Lewis et al. [11] were not able to demonstrate the presence of endogenous ouabain or ouabain-like substance (OLS) in human plasma.1 The low specificity of the antibody used in these studies may account for these results.

The aim of this study was to develop a specific and sensitive immunoassay for ouabain and to examine the effect of salt loading on the excretion of OLS in healthy subjects.

MATERIALS

Unless otherwise stated, chemicals and reagents were Analar grade and purchased from Sigma Chemical Co (St. Louis, MO). Dextran grade C, M, 60 000–90 000, and methanol were from BDH (Poole, UK), and [21,22-3H]ouabain, specific activity 1.18 TBq/mmol, was from Amersham Life Science (Amersham, UK).

Assay buffer. Phosphate-buffered saline (PBS), 145 mmol/L, pH 7.4 (7.82 mmol/L Na_2HPO_4, 1.47 mmol/L K_2HPO_4, 137 mmol/L NaCl, and 2.68 mmol/L KCl) containing 1 g/L bovine serum albumin (BSA) and 0.5 g/L sodium azide was used for the assay and to reconstitute extracted samples.

Charcoal. Dextran-coated charcoal (25 g/L) was prepared by adding 2.5 g of washed charcoal to 100 mL of PBS containing 0.25 g of dextran grade C.

1 Nonstandard abbreviations: OLS, ouabain-like substance; PBS, phosphate-buffered saline; BSA, bovine serum albumin; KLH, keyhole limpet hemocyanin; SDH, succinyl dihydride; and OVA, ovalbumin.
Ouabain calibrators. Stock ouabain calibrator (1 μmol/L) was prepared in 800 mL/L methanol in water and stored at −20 °C. This was diluted in PBS to provide concentrations of 0.16–10.00 nmol/L.

Radioactive ouabain. [21,22,3H]Ouabain, specific activity −1.18 TBq/mmol, was dissolved in methanol and stored at −20 °C.

Production of anti-ouabain antibody
Preparation of conjugates.
1) Ouabain–keyhole limpet hemocyanin (KLH) conjugates: Ouabain was conjugated to KLH by the method of Butler and Tse-Eng [12]. In this method, ouabain (0.56 mmol) was dissolved in 20 mL of distilled water and 92.5 kBq of [3H]ouabain was added. Freshly prepared 100 mmol/L sodium metaperiodate (20 mL) was added dropwise to the suspension over a 3-min period with continuous magnetic stirring. After 30 min, 0.6 mL of 1 mol/L ethylene glycol was added to inactivate excess periodate. KLH (34.5 mg) was dissolved in 20 mL of deionized water and the pH was adjusted to 9.5 with 0.36 mol/L K2CO3. The periodate-oxidized ouabain mixture was added dropwise to the protein solution with continuous magnetic stirring. The pH was maintained in the 9.3–9.5 range by simultaneous dropwise addition of 0.36 mol/L K2CO3. After the pH had stabilized in the 9.3–9.5 range, 7.9 mmol of sodium borohydride (freshly prepared in 20 mL of distilled water) was added to the mixture. The reaction mixture was transferred to a conical flask and left overnight at room temperature. The following morning the pH was adjusted to 6.5 with 1 mol/L ammonium hydroxide and dialyzed overnight against cold running tap water. The contents of the dialysis tube were lyophilized. A 1 g/L solution of the lyophilisate was prepared in distilled water and the protein content determined. Aliquots (30 μL) were also taken for counting to determine the radioactivity (dpm) associated with the lyophilisate. The mass of ouabain represented by the radioactivity (dpm) was calculated from the specific activity of the original material. From this we estimated that the KLH conjugate had 491 mol of ouabain per mol of KLH.

2) Ouabain–succinyl dihydrazide (SDH)–ovalbumin (OVA) conjugate: Conjugation of ouabain to OVA was carried out as described by Harris et al. [9]. One gram of SDH and 215 mg of OVA were added to water, pH 5, containing 3.5 mmol of 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide (EDAC). The mixture was stirred for 6 h at room temperature, after which it was dialyzed four times against 5 L of 49 mmol/L sodium acetate buffer, pH 4.5 at 4 °C over 72 h. Ouabain (0.919 mmol) was dissolved in 6.7 mL of water and 4.5 mL of acetone, and 92.5 kBq of [3H]ouabain was added. The mixture was warmed gently to dissolve ouabain. On cooling to room temperature 2.3 mmol of sodium periodate was added. After 30 min, 3 mmol K2HPO4 was added over 5 h to raise the pH. The reaction was quenched overnight by the addition of 0.4 mmol of meso-erythritol. The acetone/water containing the diadhyde was dried and OVA-SDH dialysate was added to it. The total volume was brought up to 25 mL with 30 mmol/L K2HPO4 and to a pH of 7. After 30 min, 1.1 mmol of sodium borocyanohydride (NaBH4CN) was added and the mixture was stirred continuously. A further 2.7 mmol of NaBH3CN was added 90 min later. After 20 h the mixture was dialyzed for 72 h against 25 mmol/L Tris buffer, pH 7.9, before being lyophilized. Determination of protein and associated radioactivity, as described earlier, indicated 27 mol of ouabain per mol of OVA.

Immunization. Ouabain conjugated to OVA was dissolved in 15 mmol/L NaCl at a concentration of 2 g/L and mixed with equal volumes of Freund's incomplete adjuvant, and four goats were immunized. Four weeks later, the goats were boosted with KLH-ouabain in Freund's incomplete adjuvant. Two weeks after the last injection, the goats were bled; serum was separated and stored at −70 °C. Antiserum with the best titer was selected for RIA.

Measurement of OLS in human urine and serum. Urine (3 mL) was applied directly to a C18 disposable Bond Elut column (Varian Associates, Harbor City, CA) that had been activated with methanol and equilibrated with distilled water. After washing with 10 mL of distilled water, OLS was eluted with 2 × 1.5 mL of methanol. The eluent was evaporated under nitrogen in an evaporator (Turbo Vap®; Zymark, Warrington, UK), reconstituted in assay buffer, and assayed for OLS.

Extraction recovery. To assess the recovery of the extraction step, urine samples were treated with charcoal to remove endogenous ouabain. These urine samples and assay buffer with added [3H]ouabain were extracted with C18 Bond Elut columns. To compare the amount of tracer added with that recovered from the column, duplicate tubes containing [3H]ouabain only were dried and reconstituted in 3 mL of assay buffer. The amount of [3H]ouabain in each tube was determined by liquid scintillation counting in a beta counter.

Precision and analytical recovery. Ouabain at different concentrations (0.2, 2, 4, 5, 8, or 10 nmol/L) was added to buffer or urine samples and each sample was extracted and assayed to determine recovery of the whole assay procedure. Intraassay precision was determined by analyzing a urine sample 10 times and interassay precision was determined by analyzing urine samples with and without added ouabain in 10 different assays.

Parallelism. Urine (30 mL) from a healthy volunteer was extracted and reconstituted in 3 mL of assay buffer. The concentrated extract was subsequently serially diluted and assayed. The above experiment was also repeated with a urine sample with added ouabain to increase the concentration by 10 nmol/L.

Specificity. Various cardiac glycosides, cardenolides, and related compounds were dissolved in methanol (or ethanol in some cases) to give concentrations at least 10 000 times greater than the highest concentration of ouabain calibrator. Serial dilutions of these compounds were prepared in PBS and assayed to assess cross-reactivity with the ouabain antibody. Percentage cross-reactivity was calculated as (a/b) × 100, where a and b are concentrations of ouabain and the cross-reactant that show 50% displacement, respectively.
RIA. Tritiated ouabain was diluted to 30 000 cpm/mL with assay buffer. Tracer solution, antiserum (final dilution 1:90 000), and urine extract or ouabain calibrator (100 μL each) were added to polystyrene tubes, mixed, and incubated for 1 h at 37 °C or overnight at 4 °C. The bound and free ouabain were then separated by adding 250 μL of cold 25 g/L dextran-coated charcoal. The samples were mixed, allowed to stand for 10 min in an ice/water bath, and then centrifuged at 2500g for 25 min at 4 °C. The supernatant was transferred to a scintillation vial, 4 mL of scintillant was added to each vial, and the radioactivity counted in a beta counter for 10 min. All samples and calibrators were assayed in duplicate. The ouabain concentration in test samples was estimated from a calibration curve.

SALT LOADING STUDIES

Subjects. To determine the effects of increased salt intake on excretion of OLS, 10 healthy male volunteers (ages 18–22 years) took part in a 10-day salt loading study. The volunteers had no history of hypertension, and were not taking any medication. On days 1 to 5 inclusive, subjects were given 200 mmol of additional sodium as slow-release sodium tablets (Slow Sodium, Ciba, UK). On days 6–10, subjects were placed on a low-sodium diet (<40 mmol Na/day). This study was approved by the Ethical Committee of the hospital. During the entire study period subjects were allowed to drink fluids ad lib. All urine passed over the entire study period was collected for the measurement of sodium, potassium, creatinine, and OLS. Urinary sodium and potassium concentrations were measured by flame photometry, and urinary creatinine concentrations were measured by a standard Jaffe reaction in an automated analyzer. Urinary OLS was measured by RIA as described above.

Statistics. The data are expressed as means ± SE and were analyzed by using analysis of variance for repeated measurements. Scheffé’s comparisons were performed on the means, and P ≤0.05 was considered significant.

Results

Titer. The antisera obtained were of high titer and the antibody dilution required to bind 50% of the tracer ([21,22,3H]ouabain) was 1:200 000.

Calibration curve. Fig. 1A shows a typical calibration curve for ouabain. Serial dilutions of a urine extract produced a curve parallel to the ouabain calibration curve (Fig. 1A), as did urine with added ouabain (10 nmol/L) (Fig. 1B).

Sensitivity. The detection limit of the assay, defined as 2 SD from the mean for the zero calibrator, was calculated as 0.06 nmol/L from 10 experiments.

Specificity. Table 1 shows the cross-reactivity of the antibody against some cardiac glycosides and related compounds. Their cross-reactivity was determined by comparison with a ouabain calibration curve (Fig. 2). Strophanthinid (4.1%), ouabagenin (4.0%), lanatoside C (2.1%), dihydroouabain (2.0%), digitoxigenin (1.5%), and bufalin (1.3%) showed little cross-reactivity, and all other compounds including natural steroids such as cortisol, 17α-hydroxyprogesterone, and androstenedione showed negligible cross-reactivity, indicating that the antiserum is highly specific for ouabain.

Recovery studies and precision. Extraction recoveries of added [21,22,3H]ouabain to urine or buffer were 90–107% and 90–103%, respectively. Recovery of cold ouabain was determined by adding ouabain to urine treated with charcoal to remove endogenous ouabain. Extraction and subsequent assay of these samples demonstrated that recovery ranged from 75% to 97.8% over the range of 0.2–10 nmol/L (Table 2). Precision data are also shown in Table 2. The intraassay CV was between 4.5% and 5.9% and interassay CV was between 6.5% and 7.7%.

Effect of salt loading on the excretion of sodium and ouabain. Table 3 shows sodium excretion; OLS excretion; urine volume; and sodium/creatinine, OLS/creatinine, and sodium/potassium ratios during the salt-loading study. Sodium excretion increased from 280 ± 55 mmol/day on day 1 to 419 ± 91 on day 3 and remained high on days 4 and 5. On low salt, sodium excretion rapidly decreased and on day 10 was 25 ± 20 mmol/day. Fractional excretion of sodium decreased from 2.42 ± 0.34% on
Table 1. Cross-reactivity of structurally related substances with anti-ouabain antisera.

<table>
<thead>
<tr>
<th>Substance</th>
<th>% Cross-reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ouabain</td>
<td>100</td>
</tr>
<tr>
<td>Ouabagenin</td>
<td>4.0</td>
</tr>
<tr>
<td>Strophanthidin</td>
<td>4.0</td>
</tr>
<tr>
<td>Lanatoside C</td>
<td>2.2</td>
</tr>
<tr>
<td>Dihydrouabain</td>
<td>2.0</td>
</tr>
<tr>
<td>Digitoxigenin</td>
<td>1.5</td>
</tr>
<tr>
<td>Bufalin</td>
<td>1.3</td>
</tr>
<tr>
<td>Digitoxin</td>
<td>0.67</td>
</tr>
<tr>
<td>Digoxin</td>
<td>0.4</td>
</tr>
<tr>
<td>Digoxigenin</td>
<td>0.1</td>
</tr>
<tr>
<td>Progesterone</td>
<td>0.016</td>
</tr>
<tr>
<td>Digiton</td>
<td>0.01</td>
</tr>
<tr>
<td>Aldosterone</td>
<td>0.001</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>&lt;0.000001</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>&lt;0.000001</td>
</tr>
<tr>
<td>Cortisol</td>
<td>&lt;0.000001</td>
</tr>
<tr>
<td>Deoxycholic acid</td>
<td>&lt;0.000001</td>
</tr>
<tr>
<td>17 α-hydroxyprogesterone</td>
<td>&lt;0.000001</td>
</tr>
<tr>
<td>β-Estradiol</td>
<td>&lt;0.000001</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>&lt;0.000001</td>
</tr>
<tr>
<td>Lyso phosphatidylcholine</td>
<td>&lt;0.000001</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>&lt;0.000001</td>
</tr>
<tr>
<td>Cortisone</td>
<td>nd</td>
</tr>
<tr>
<td>Androsterone</td>
<td>nd</td>
</tr>
<tr>
<td>Epiandrosterone</td>
<td>nd</td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>nd</td>
</tr>
<tr>
<td>Dihydroepiandrosterone</td>
<td>nd</td>
</tr>
</tbody>
</table>

Cross-reactivity was calculated as the ratio of the concentrations of the compound to ouabain that caused 50% displacement (see Materials and Methods); nd = not detected.

Table 2. Results of precision and recovery studies.

<table>
<thead>
<tr>
<th>Ouabain added, nmol/L</th>
<th>Ouabain measured, nmol/L</th>
<th>Mean recovery, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2 (5)</td>
<td>0.15 ± 0.02</td>
<td>75.0 ± 10.0</td>
</tr>
<tr>
<td>2.0 (6)</td>
<td>1.73 ± 0.06</td>
<td>86.5 ± 3.2</td>
</tr>
<tr>
<td>4.0 (10)</td>
<td>3.83 ± 0.17</td>
<td>95.8 ± 4.3</td>
</tr>
<tr>
<td>5.0 (10)</td>
<td>4.84 ± 0.26</td>
<td>96.7 ± 5.2</td>
</tr>
<tr>
<td>8.0 (10)</td>
<td>7.80 ± 0.56</td>
<td>97.5 ± 6.9</td>
</tr>
<tr>
<td>10.0 (10)</td>
<td>9.78 ± 0.71</td>
<td>97.8 ± 7.0</td>
</tr>
</tbody>
</table>

Precision at different concentrations of ouabain added to urine

<table>
<thead>
<tr>
<th>Mean concentration, nmol/L</th>
<th>SD</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Within-assay (n = 10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.34</td>
<td>0.02</td>
<td>5.9</td>
</tr>
<tr>
<td>4.74</td>
<td>0.25</td>
<td>5.3</td>
</tr>
<tr>
<td>9.61</td>
<td>0.43</td>
<td>4.5</td>
</tr>
<tr>
<td>Between-assay (n = 10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.68</td>
<td>0.13</td>
<td>7.7</td>
</tr>
<tr>
<td>3.85</td>
<td>0.25</td>
<td>6.5</td>
</tr>
<tr>
<td>7.6</td>
<td>0.51</td>
<td>6.7</td>
</tr>
</tbody>
</table>

* Results are mean ± SD; numbers in parentheses indicate the number of experiments.

day 5 to 0.17 ± 0.05% on day 10 (P < 0.001). Excretion of OLS increased from 2.11 ± 0.83 nmol/day on day 1 to 2.66 ± 1.22 nmol/day on day 5 (Fig. 3, Table 3). This trend was significant (by ANOVA, F = 3.23, P < 0.023), and OLS excretion on days 5 and 4 was significantly higher than on days 1 and 2 (P < 0.05 by Scheffe’s test). The maximal excretion of both sodium and OLS occurred on day 5. From day 6, OLS excretion decreased rapidly and by day 10 was 48.8% lower than on day 5. This trend was highly significant (by ANOVA, F = 6.53, P < 0.0001). Excretion of OLS on days 7 to 10 was significantly lower than on day 5 (P < 0.05). To exclude any effect due to changes in water excretion and to exclude possible incomplete urine collections, OLS excretion was also expressed as OLS/creatinine ratio (Table 3). OLS/creatinine ratio also showed a similar trend.

Discussion

Much evidence has accumulated about the role of a sodium transport inhibitor in the regulation of sodium balance in humans and in other mammals [1–4]. The nature of this substance has been elusive. Hamlyn et al. [8] identified this endogenous sodium transport inhibitor as ouabain or OLS. However, the reported circulating concentrations of OLS in humans vary widely, from 0.076 to 1.1 nmol/L [13]. Some of this variation in concentration of OLS appears to be due to the specificity of the antibody. The antisera selected in this study was of a high titer (200 000) and showed a high degree of specificity for ouabain, with little or no appreciable cross-reactivity with other structurally related compounds (Table 1); antisera used by other investigators showed variable degrees of cross-reactivity with structurally related compounds. Table 4 compares the immunoassays described so far. The antisera used by Lewis et al. [11] showed high cross-reactivity with ouabagenin (77.2%) and strophanthidin (70.4%). The antisera developed by Harris et al. [9] showed cross-reactivity for strophanthidin (66%), ouabagenin (40%), digitoxin (28%), and digoxin (5.2%). Antisera used by Doris [14] also showed significant cross-reactivity with ouabagenin (56.2%) and digoxin (4.5%). More recently, Komiyama et al. [16] reported cross-

Fig. 2. Calibration curve of RIA for ouabain and cross-reactivities with related compounds: ouabain (■), ouabagenin (+), bufalin (★), digoxin (□), digitonin (▲), digoxigenin (○).
reactivity for strophanthidin, digitoxin, and ouabagenin, being almost the same as ouabain in their ELISA system. Antisera produced by Masugi et al. [18] was not sufficiently characterized, and ouabain-like immunoreactivity was not specific because of an unstable peroxide. Our antisera showed high specificity to ouabain, and cross-reactivity with ouabagenin (4%) was the lowest reported so far, with only Gomez-Sanchez et al. [17] reporting a lower value (2.2%) by ELISA.

The types of assays used by investigators for ouabain measurement also vary. Initially, we developed an ELISA method using microtiter plates coated with ouabain conjugated to BSA [19]. The ELISA had a working range of 2–500 fmol, with a 50% inhibitory concentration (IC50) of 30 fmol/well. Cross-reactivity studies involving this method demonstrated very little cross-reactivity with digitoxin (1.5%), bufalin (0.4%), digoxin (0.2%), digoxigenin (0.1%), progesterone (0.016%), digitoxin (<0.0001%), and other related compounds [19]. However, the reproducibility was poor; intra- and interassay CVs were 26% and 35%, respectively. When extracted urine samples were assayed, nonspecific binding effects were noted, as indicated by false-positive readings in “blank” controls, indicating the presence of interference factors in urine (unpublished data). A recent report on the evaluation of a commercial ELISA for OLS shows similar results [20]. For these reasons we developed the RIA for measurement of ouabain.

In most of the immunoassay methods described so far, a solid-phase extraction step is used (C18 columns). We have used methanol to elute OLS, whereas others have used acetonitrile or ethanol (Table 4). The lowest detection limit of the assay was 0.06 nmol/L. Recovery of nonradioactive ouabain added to urine was 75% to 97.8% over the range of 0.2 to 10 nmol/L (Table 2), whereas recovery of [21,22-3H]ouabain in urine or buffer was consistently >90%. Dilution of a urine extract yielded a curve with slopes similar to the ouabain calibration curve, and urine samples with added ouabain gave a curve

<table>
<thead>
<tr>
<th>Day</th>
<th>Urine volume</th>
<th>Sodium, mmol/24 h</th>
<th>Sodium/creatinine ratio</th>
<th>Na/K ratio</th>
<th>OLS, nmol/24 h</th>
<th>OLS/creatinine, nmol/nmol</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.693 ± 0.213</td>
<td>280 ± 17.3</td>
<td>21.6 ± 1.6</td>
<td>3.54 ± 0.09</td>
<td>2.11 ± 0.26</td>
<td>0.16 ± 0.019</td>
</tr>
<tr>
<td>2</td>
<td>1.415 ± 0.121</td>
<td>324 ± 18.8</td>
<td>25.5 ± 1.7</td>
<td>5.02 ± 0.16</td>
<td>2.09 ± 0.22</td>
<td>0.19 ± 0.013</td>
</tr>
<tr>
<td>3</td>
<td>1.751 ± 0.151</td>
<td>419 ± 28.8</td>
<td>32.4 ± 2.2</td>
<td>5.49 ± 0.09</td>
<td>2.36 ± 0.28</td>
<td>0.18 ± 0.025</td>
</tr>
<tr>
<td>4</td>
<td>1.777 ± 0.117</td>
<td>431 ± 30.8</td>
<td>36.7 ± 4.4</td>
<td>5.67 ± 0.10</td>
<td>2.52 ± 0.19a</td>
<td>0.21 ± 0.022</td>
</tr>
<tr>
<td>5</td>
<td>2.091 ± 0.278</td>
<td>421 ± 25.2</td>
<td>35.6 ± 4.5</td>
<td>5.70 ± 0.11</td>
<td>2.66 ± 1.39a</td>
<td>0.24 ± 0.035</td>
</tr>
<tr>
<td>6</td>
<td>1.769 ± 0.155</td>
<td>209 ± 12.8</td>
<td>17.6 ± 2.2</td>
<td>3.38 ± 0.09</td>
<td>2.57 ± 0.29</td>
<td>0.20 ± 0.019</td>
</tr>
<tr>
<td>7</td>
<td>1.905 ± 0.328</td>
<td>78 ± 10.5</td>
<td>9.2 ± 1.8</td>
<td>1.66 ± 0.08</td>
<td>2.11 ± 1.34</td>
<td>0.19 ± 0.035</td>
</tr>
<tr>
<td>8</td>
<td>1.636 ± 0.225</td>
<td>48 ± 7.5</td>
<td>5.1 ± 0.7</td>
<td>0.99 ± 0.04</td>
<td>1.76 ± 0.19b</td>
<td>0.18 ± 0.022</td>
</tr>
<tr>
<td>9</td>
<td>1.243 ± 0.152</td>
<td>37 ± 6.2</td>
<td>3.6 ± 0.6</td>
<td>0.73 ± 0.03</td>
<td>1.46 ± 0.24b</td>
<td>0.13 ± 0.019</td>
</tr>
<tr>
<td>10</td>
<td>1.330 ± 0.167</td>
<td>25 ± 6.5</td>
<td>2.4 ± 0.6</td>
<td>0.41 ± 0.02</td>
<td>1.47 ± 0.22b</td>
<td>0.13 ± 0.013</td>
</tr>
</tbody>
</table>

* Significantly different from days 1 and 2 by Sheffé’s test.

Fig. 3. Effect of salt intake on ouabain (□) and sodium excretion (□).

<table>
<thead>
<tr>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>Ouabagenin</td>
<td>77.2</td>
<td>40</td>
<td>56.2</td>
<td>NT</td>
<td>72</td>
<td>2.2</td>
</tr>
<tr>
<td>Strophanthidin</td>
<td>70.4</td>
<td>66</td>
<td>45.2</td>
<td>NT</td>
<td>82.2</td>
<td>0.06</td>
</tr>
<tr>
<td>Digoxin</td>
<td>1.25</td>
<td>5.2</td>
<td>4.5</td>
<td>9.2</td>
<td>6.4</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Digitoxin</td>
<td>NT</td>
<td>28</td>
<td>NT</td>
<td>NT</td>
<td>84.6</td>
<td>0.12</td>
</tr>
<tr>
<td>Dihydropuabain</td>
<td>NT</td>
<td>0.16</td>
<td>NT</td>
<td>NT</td>
<td>0.82</td>
<td>0.015</td>
</tr>
<tr>
<td>Sensitivity, nmol/L</td>
<td>0.06</td>
<td>0.1</td>
<td>0.076</td>
<td>0.02</td>
<td>pg/tube</td>
<td>0.03</td>
</tr>
<tr>
<td>Assay method</td>
<td>ELISA</td>
<td>ELISA</td>
<td>RIA</td>
<td>RIA</td>
<td>ELISA</td>
<td>ELISA</td>
</tr>
<tr>
<td>Elution solvent on</td>
<td>CH3CN</td>
<td>CH3CN</td>
<td>CH3CN</td>
<td>C2H5OH</td>
<td>CH3CN</td>
<td>CH3OH</td>
</tr>
</tbody>
</table>

NT, not tested.
superimposable on the ouabain calibration curve. Studies of
digoxin-like factor(s) suggest that there may be conjugated
forms present in urine. [7]. To investigate this in preliminary
studies, we examined the effect of treatment of urine with
glucuronidase on OLS activity and found that there appeared to
be some conjugated OLS (Butt et al.; unpublished observation).
Further studies are in progress to quantify the conjugated form
of OLS. When compared with immunoassays used by other
investigators (Table 4), our anti-ouabain antisem was not the
only most extensively characterized, but also proved to have the
highest degree of specificity for ouabain. The sensitivity of the
assay was comparable with both the ELISA and RIA methods
described by others.

With a variety of techniques, increased concentrations of
sodium transport inhibitor have been found during volume
expansion [21, 22], during high salt intake [5, 23], and in hyper-
tension [24, 25]. As far as we are aware there have been no
studies on the effect of salt intake on excretion of OLS measured
by an immunoassay. Although existing evidence suggests that
there is a relation between sodium and OLS excretion during
salt loading, most of the data were obtained from animal
experiments. The results presented here (Table 3 and Fig. 3)
show that excretion of OLS is increased during high salt intake.
It has been suggested that increased excretion of sodium trans-
port inhibitors could be the result of increased urine volume
[26]. To exclude this possibility we expressed the excretion of
OLS in relation to creatinine (Table 3) and found similar
changes. Furthermore, during acute water loading, the excretion
of OLS did not change significantly [27].

We conclude that our assay is highly specific for ouabain, and
with this assay we show that excretion of OLS is increased by
salt intake.

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