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## Excretion of Digoxin-like Immunoreactivity in Urine of Normal Subjects: Correlations with Excretion of Creatinine and Electrolytes

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To verify whether there is a variation in the 24-h urinary excretion of digoxin-like immunoreactivity (DLIS) in humans, we studied 18 normal adults, who collected their urines for 24-h in several portions. We then measured DLIS (by means of a sensitive RIA method), creatinine, sodium, and potassium concentrations in the urine samples. The mean urinary excretion rate for DLIS in the complete 24-h collection was 84.8 (SD 31.3) pg/min. The mean DLIS urinary excretion rate calculated for overnight collections was significantly lower than those of afternoon collections ( $P < 0.01$ ) and the 24-h collection ( $P < 0.05$ ). Significant positive correlations were found between urinary DLIS and excretion rates for creatinine ( $r = 0.347$ ,  $P = 0.0016$ ),  $\text{Na}^+$  ( $r = 0.232$ ,  $P = 0.038$ ), and  $\text{K}^+$  ( $r = 0.323$ ,  $P = 0.003$ ), respectively. Our data suggest that urinary excretion of DLIS is higher during "active" hours of the day, especially in the afternoon, than at rest, during the night.

**Additional Keyphrases:** radioimmunoassay · digitalis-like substances · cardiac glycosides · renal function

Several studies have documented the presence of an endogenous factor with digoxin-like immunoreactivity (DLIS) in blood of experimental animals and humans, as determined with digoxin RIA or enzyme immunoassay methods for digoxin (for a review, see references 1-5). Experimental studies and theoretical considerations suggest that DLIS might also be an endogenous modulator for  $\text{Na}^+/\text{K}^+$ -ATPase (the receptor of cardiac glycosides) and could play a role in the regulation of fluids and electrolytes, and in the pathogenesis of hypertension (1, 3, 4).

DLIS concentrations in plasma or serum of normal adult subjects are frequently close to the limit of sensitivity of RIA methods (6-8). Therefore, such samples are generally concentrated to improve the precision of the assay in studies of normal adult subjects. On the other hand, because DLIS concentrations in urine samples of adults and newborns are four- to sixfold greater than in plasma samples, direct RIA could be preferable for urine samples (8). However, studies on the variation in 24-h urinary excretion of DLIS in normal subjects have not been reported.

Here we report the urinary excretion rate of DLIS in 18 normal subjects, as measured by a sensitive RIA method in which digoxin added to a buffer solution containing serum albumin serves as the standard. In addition, we investigated whether there are variations of urinary DLIS excretion

throughout the day and if the urinary DLIS values in normal adult subjects are correlated with urinary creatinine and electrolytes, as previously reported for newborns and boys (9, 10).

### Materials and Methods

**DLIS assay.** We measured DLIS by a previously reported solid-phase RIA method (7, 8). In this method, digoxin dissolved in a buffer containing 40 g of human serum albumin per liter is used as standard,  $^{125}\text{I}$ -labeled digoxin is the tracer, and a solid phase (antibody coated test-tubes) is used for separating bound digoxin from free. To improve the sensitivity and the reproducibility of the assay as well as the stability of standard curves, we used an assay buffer containing human serum albumin, 40 g/L. The same albumin-containing buffer was added to unknown urine samples to equalize the influence of albumin in the reaction volume (8). Results are expressed as digoxin equivalents.

We directly assayed 0.2-mL samples of urine. The mean sensitivity obtained in 20 separate experiments, performed during nine months, was 2.98 (SD 1.11) pg per tube. The mean intra-assay CV (precision profile) ranged between 4% and 20% (range of the assay: 50-500 ng/L), as previously reported (8). In addition, the mean between-assay CVs, tested during 10 months ( $n = 25$ ) with two different urine pools, were 12.9% (digoxin equivalent, mean  $\pm$  SD, 281.9  $\pm$  36.4 ng/L) and 19.5% (120.0  $\pm$  23.4 ng/L), respectively. In addition, assaying increasing volumes of one urine pool gave a linear response (from 25 to 250 ng/L), as previously reported (8). The mean DLIS concentration in 62 plasma samples from normal subjects was 15.7 (SD 8.8) ng/L (range 0-32 ng/L).

The antiserum we used cross reacts with digitoxin by 35%, but negligibly (<0.1%) with testosterone, progesterone, cortisone, aldosterone, estradiol, estriol, cholesterol, dehydroepiandrosterone, cortisol, prednisone, prednisolone, spironolactone, or ouabain, as previously reported (7).

**Creatinine and electrolyte assays.** Creatinine was measured colorimetrically with creatinine reagent kit P/N 668306 (Astra System, Beckman Instruments, Inc., Galway, Ireland). Urinary sodium and potassium concentrations were measured with a Model 435 flame photometer (Corning Medical, Halsted, Essex, U.K.).

**Subjects.** We studied 18 normal, healthy subjects (10 women and eight men, ages 22-54 years, mean 29.1, SD 6.7 years), who collected their urines for 24 h, in several portions. The first urine sample on the morning of the day of the 24-h collection (sample 0) was put in a separate container. Subsequently, the complete 24-h urinary collection consisted of: sample 1, from waking to lunch; sample 2, from lunch to dinner; sample 3, from dinner to bedtime;

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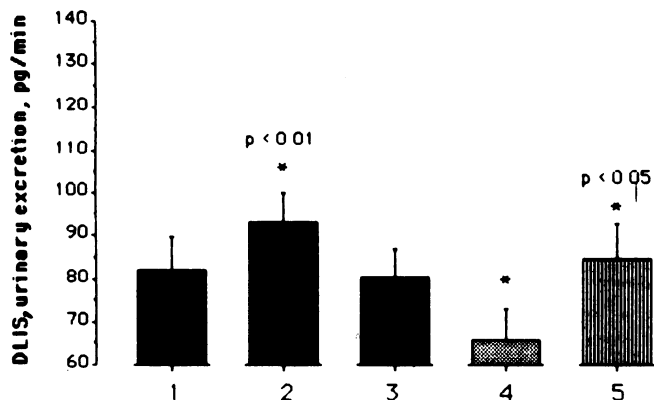


Fig. 1. Mean ( $\pm$ SEM) urinary excretion rate of DLIS in the different portions of 24-h urine collected from the 18 subjects (see Table 1)

**Table 1. DLIS/CR Ratios for the Different Specimens of Urine from 18 Normal Persons**

Specimen	DLIS(ng/L)/CR(g/L) ratio	
	Mean	SD
0. First morning	64.8*	15.7
1. Morning	83.6	28.7
2. Afternoon	92.5	44.1
3. Evening	75.9	30.6
4. Overnight	68.2*	23.7
5. 24 h (1-4)	83.1	29.7

\*Significantly different ( $P < 0.01$ , unpaired *t*-test) from 24-h collection (sample 5).

sample 4, overnight, at rest. The volume of each portion was recorded, as was the time of collection. From each fraction we took three 1-mL aliquots, for assay of DLIS, creatinine, and electrolytes. We then pooled urine fractions 1 to 4, to yield the complete 24-h collection (sample 5). All samples were stored at  $-20^{\circ}\text{C}$  until assay. To minimize analytical error, we used the same lot of standard solutions, coated-tubes, and tracer solution in assaying the urine samples.

DLIS and creatinine concentrations were measured in each portion of urine ( $n = 108$ ); electrolytes were measured in the samples of 16 of the 18 subjects ( $n = 96$ ). Finally, we calculated the urinary excretion rates of DLIS (pg/min), creatinine (mg/min), and electrolytes (mmol/min) on fractions 1 to 4 ( $n = 90$ ), but not on sample 0, which was not timed.

**Statistical analysis.** We calculated all sample values, the index of sensitivity, the precision profile, and the data for quality control of the RIA systems by means of previously described computer programs (11, 12). The interpolation of the dose-response curves was calculated by using a four-parameter logistic function (12).

For statistical analysis for comparison of mean values, and calculation of simple and multiple regression coefficients, we used a Macintosh SE personal computer and the 512+ Stat-View™ program.

## Results

Concentrations of DLIS in all the urine samples ranged between 22.5 and 231.5 ng/L (mean  $\pm$  SD =  $114.1 \pm 43.8$  ng/L,  $n = 108$ ). The mean DLIS excretion rate for our subjects, calculated on the complete 24-h collection, was 84.8 (SD 31.3) pg/min, corresponding to a daily urinary excretion of 122 (SD 45) ng/24 h. The mean DLIS urinary excretion

rates, in samples 1 to 5, differed significantly from portion to portion ( $P < 0.05$ , ANOVA) (Figure 1). The value for the overnight collection (sample 4) was significantly lower than values for samples 2 (afternoon collection,  $P < 0.01$ ) and 5 (24-h collection,  $P < 0.05$ ).

We found a significant positive correlation between urinary DLIS and creatinine concentrations (DLIS =  $107 + 0.33$  creatinine,  $r = 0.601$ ,  $n = 108$ ,  $P < 0.001$ ). Table 1 lists the means of each DLIS/creatinine concentration ratio (DLIS/CR) obtained for the six different portions of 24-h urine (samples 0 to 5). Differences among the mean ratios in the six fractions were significant ( $P < 0.001$ , ANOVA). Moreover, the DLIS/CR ratios found in urine fractions 0 to 4 correlated positively with the value of the complete 24-h collection (fraction 5), with  $r$  ranging between 0.63 and 0.84 ( $n = 18$ ,  $P < 0.001$ ).

Weak positive correlations were found between the rates of urinary excretion of DLIS and of creatinine (DLIS =  $72.7 + 13.3$  creatinine,  $n = 80$ ,  $r = 0.347$ ,  $P = 0.002$ ),  $\text{Na}^+$  (DLIS =  $72.5 + 4.5 \text{Na}^+$ ,  $r = 0.232$ ,  $n = 80$ ,  $P < 0.038$ ), and  $\text{K}^+$  (DLIS =  $72.6 + 146.0 \text{K}^+$ ,  $r = 0.323$ ,  $n = 80$ ,  $P = 0.004$ ). Stepwise multiple-regression analysis among  $\text{K}^+$ ,  $\text{Na}^+$ , and creatinine excretion rates and that of DLIS showed that only  $\text{K}^+$  values significantly ( $P < 0.02$ ) improved the regression between DLIS and creatinine excretion rates: DLIS =  $32.3 + 355.7 \text{K}^+ + 29.1$  creatinine ( $n = 80$ ,  $r = 0.435$ ,  $P < 0.001$ ).

## Discussion

This study confirms that urine from healthy adult subjects contains higher DLIS concentrations than do the corresponding plasma samples. The greater amounts of DLIS in urine samples permit a better precision of assay. In addition, because the DLIS/CR ratio for an untimed morning urinary sample (sample 0 of this study) correlates positively ( $r = 0.819$ ,  $P < 0.001$ ) with the ratio for the complete 24-h collection, one can simply collect the first morning urine and validly use it to estimate the daily excretion of DLIS. However, a complete 24-h collection is necessary for accurate determination of the mean urinary excretion of DLIS during the day.

Here, we measured DLIS values with a very sensitive RIA method specifically developed in our laboratory for assay of digoxin-like immunoreactivity (6-8). As previously reported for plasma extracts from adults, pregnant women, and newborns, the results obtained with this RIA method correlate well with those obtained with a radioreceptor assay involving the binding of [ $^3\text{H}$ ]ouabain to erythrocytes (13). This correlation might suggest that our RIA detects, to some extent, substances with immunological and biological activity similar to cardioactive glycosides.

Our data indicate that urinary excretion of an endogenous factor (or a group of substances) with digoxin-like immunoreactivity is higher during the "active" hours of the day, especially in the afternoon, than at rest during the night. Strenuous physical activity (14), infusion of saline solution (15), and high-sodium diet (16) have been previously reported to increase concentrations of DLIS plasma or urine of normal subjects and hypertensive patients. Significant positive correlations between urinary DLIS and creatinine concentrations (and excretion rates) have been found in our study, as previously reported for samples from newborns and boys (9, 10). Together, these findings suggest that the urinary excretion of DLIS is to some extent dependent on glomerular filtration rate, which in turn accords with the hypothesis that DLIS is an ultrafiltrable substance with a low

molecular mass (1, 3, 5, 17). The increase in the glomerular filtration rate and diuresis may explain a greater urinary excretion of DLIS during the day as compared with at rest, as also previously suggested by other authors (18) who reported a correlation between the presence of digoxin-like immunoreactivity in urine and induced diuresis in normal individuals.

We also found weak, but significant, positive correlations between urinary DLIS excretion rate and the respective rates for  $K^+$  and  $Na^+$ . These data accord well with the findings of Gault et al. (15), who reported that plasma DLIS correlated positively with urinary sodium excretion in 12 hypertensive patients before and after infusion of saline solution, and of Ebara et al. (19), who found a positive correlation between serum DLIS values and the fractional excretion of  $Na^+$  in 53 paired samples of infants. However, in the present study, using a stepwise multiple-regression analysis, only urinary  $K^+$ , not  $Na^+$  values, significantly improved the regression between urinary DLIS and creatinine excretion rates in normal adults. Further studies are needed to elucidate the relationships between urinary DLIS and electrolyte excretions.

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