parathyroid gland, medullo-thyroid gland, and parasea β cells), 1,25-dihy-
droxyvitamin D, and enzymes are in-
volved (3, 4). Moreover, arteriosclerosis and atherosclerosis are calcium-dep-
endent (3, 5). In IDD women, the signifi-
cant negative correlation between PI-
Ca and total cholesterol might be ac-
counted for by the internalization of the calcium or even the cholesterol in the
artery wall at a younger age, de-
pending on whether cholesterol or cal-
cium increased in their plasma. Thus,
an increase in plasma cholesterol could
allow PI-Ca to penetrate into the ar-
tery intima, to form atherosclerotic plaques in patients for whom athero-
sclerosis is very common and occurs
early (4). The Bogalusa Heart Study
(Framingham Junior survey) has just
provided additional proof that chole-
sterol is one, if not the major, cause of
atherosclerosis (6). Could calcium be
the missing piece in this puzzle of
atherosclerotic plaque formation?

Once again, biological results are
not the same for both sexes (2–4). The
epidemiologic, clinical, ergometric, and
therapeutic particularities of women
presenting with coronary insufficiency
have just been described (7). Our re-
results raise questions that were not
resolved in that study, and suggest
that much work remains to be done.

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Problems In Standardization of
Digitale-like Substance Assays by
Means of Competitive
Immunological Methods

To the Editor:

Although several studies have been
recently published concerning assay of
endogenous digoxin-like immunoreac-
tive substance(s) (DLIS) in human body
fluids (1–5), and the putative endoge-
nous substance has been almost par-
tially purified and identified (7), there
is no accord on the nomenclature (DLIS,
DLF, OLF, Endoxin, Endaline, etc.) and
the best procedure(s) of sample col-
collection, storage, and assay. This
lack of standardization not only gener-
ates confusion, but also can be the
cause of discordant results and errone-
ous findings. The aim of this Letter is
to discuss some methodological points
of DLIS assay, adding personal contribu-
tions, in order to stimulate a more
general discussion among the groups
interested in studies concerning endog-
nous cardiac glycosides-like subst-
ances.

Sample preparation—Several au-
 thors (8–13) used deproteinized (by
boiling) and concentrated plasma ex-
tracts for their studies in animals or
humans. We have recently used the ex-
traction with C18 reversed-phase
cartridges (Sep Pak, Waters Asso-
 ciates) for the concentration and pre-
purification of biological samples (5,
14). This procedure seems preferable
when radioreceptor assay (ARA) and
(or) transmembrane ion-transport
studies are used with the RIA for de-
tection of cardiac glycosides-like com-
pounds. In fact, by loading the sample
through the column, washing it with
40 mL of distilled water, and eluting
the plasma extracts with methanol it is
possible to remove the ions and pro-
teins and avoid this kind of interfer-
ences in RRA or transmembrane ion-
transport methods (14). This procedure
is rapid and the mean recovery was
82% (14).

On the other hand, we think that a
direct assay (without sample extrac-
tion and concentration) should be used
when plasma samples from newborns,
pregnant women, and patients with
renal or liver disease are assayed, be-
duced by their higher DLIS concen-
trations in plasma in comparison with

Fig. 1. DLIS stability of two urinary pools collect-
ed from newborns in the first day (Pool A) and
in the third and fourth days of extrauterine life
(Pool B).

The two pools were dispensed in several 1-mL
aliquots and stored at −20 °C until RIA. Only pool A
showed a significantly decreased immunoreactivity
during the study (r = 0.405, n = 50, 0.005 < p < 0.0001, y = 294.8 – 0.12x
normal adult subjects (2, 3, 5). In addi-
tion, urine samples from adults and
newborns have DLIS concentrations
four to six times higher than those in
plasma samples, so also in this case the
direct RIA seems preferable, as we
have previously reported (5, 15).

Sample storage—There are no data
in the literature on stability of DLIS
during storage. In our laboratory, two
pools (A and B) of urine from newborns
were stored in various 1-mL aliquots at
−20 °C and assayed by RIA for 17
months. Only pool A demonstrated a
significant decrease in immunoreactiv-
ity during this interval (Figure 1). This
discrepancy was probably ascribable
to the higher imprecision for assay of pool
B (CV about 22% vs 18%). The decay of
digoxin-like immunoreactivity was,
however, very small (about 1% per
month) and, also for pool A, a signifi-
cant decrease of DLIS was not detectable
before eight months of storage at
−20 °C. Evidently DLIS can be validly
measured in urine samples stored for
some months at −20 °C. Unfortunately,
at present we have no definitive
data on the stability of DLIS in serum
(plasma) pools. We directly (i.e., with-
out extraction) assayed, during 10
months, a pool of plasma from blood
donors, but the very low DLIS concen-
tration, about 15 ng/L, did not permit a
significant statistical analysis because
of the high imprecision of the assay
Digoxin values (0.72, obtained to prived tion bound assays) found for digoxin manufacturers. The authors not standard.

**Preparation of standard curve**—Most authors who have measured DLS with RIA or enzyme immunoassay methods have used commercial kits and performed the assay as suggested by the manufacturers. In commercial kits for digoxin assay, digoxin added to human plasma (or serum) is generally used as standard. We have previously demonstrated (5, 11, 15) that, in normal plasma, low but measurable amounts of digoxin-like substance(s) are present. Using a RIA kit with serum standards for DLS assay (SPAC Digoxin; Mallinckrod, Dietzenbach, F.R.G.) we have found that most (>70% in two different assays) plasma samples (direct assay) with DLS concentrations within the normal range (0–35 ng/L) have a bound radioactivity higher than that of the B5 standard. Therefore, it is important to test for the presence of significant amounts of DLS in the serum-based standards used for the calibration curve. To avoid this kind of interference we recommend the use of buffer solutions containing standard digoxin and human serum albumin (11, 15) or low-molecular-mass-deprived human serum as standard for calibration curves.

**Immunological and biological activity**—Because the chemical nature of DLS is not yet known, it is not possible to perform specific RIA assays for DLS by use of specific antisera against DLS, with DLS as standard. The relatively poor specificity of measurement of DLS with digoxin RIA kits makes it possible that substances with biological activity similar to cardiac glycosides are not detected by digoxin RIA methods. For example, ouabain, a compound that strongly inhibits Na/K ATPase activity, does not significantly interfere in our digoxin RIA system (11). On the other hand, it is also possible that several compounds interfering in the RIA system do not share any biological activity (12, 16, 17). However, we have obtained a significant correlation (r = 0.72, n = 21, p <0.001) between DLS values obtained by RIA method or radioreceptor assay for 21 chromatographed extracts (made by use of C18 Sep Pak cartridges) of plasma from normal adults, pregnant women, and neonates (14). Therefore, we think that digoxin RIA or enzyme immunoassay methods can be useful for DLS determination because they generally are more sensitive, precise, and simple than other techniques used to detect endogenous cardiac-glycosides-like compounds. However, immunological methods should be used only as a screening or preliminary test to detect the possible presence of cardiac-glycoside-like substances in human biological fluids; for confirmation, more specific methods, used after chromatographic purification, are required.

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**Measurement of Stable Glycated Hemoglobin**

**To the Editor**

In view of the concern regarding the effect of the labile Schiff base adduct (pre-Hb A1) on the true values for Hb A1 as measured by cation-exchange chromatography (1, 2), we report the simple method used routinely in our laboratory to remove pre-Hb A1.

Hb A1 was measured in one normal subject and eight diabetic patients by use of a medium-sized chromatographic column containing Bio-Rex 70 cation-exchange resin (5). Blood was collected with EDTA as anticoagulant, and samples were variously treated as below before the hemolysates were prepared.

(a) Hb A1 was measured immediately after the blood was drawn.

(b) Whole anticoagulated blood (1.5 mL) was pipetted into 8.5 mL of isotonic saline in a tube, mixed several times with gentle inversion, then centrifuged (10 min, 3000 rpm) and the supernate was discarded. A second 8.5-mL aliquot of isotonic saline was added to the sedimented erythrocytes, mixed by gentle inversion until all the cells were in suspension. The tube was then centrifuged and the supernatant-dialyzed again discarded. We then added 8.5 mL of saline to the washed erythrocytes, mixed well by gentle inversion until all the cells were in suspension, then incubated at 37 ºC for 6 h in a shaking

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