parathyroid gland, medullothyroid gland, and pancreas β cells), 1,25-dihydroxyvitamin D, and enzymes are involved (*3, 4*). Moreover, arteriosclerosis and atherosclerosis are calcium-dependent (*3, 5*). In IDD women, the significant negative correlation between Pl-Ca and total cholesterol might be accounted for by the internalization of the calcium or even the cholesterol in the artery wall at a younger age, depending on whether cholesterol or calcium increased in their plasma. Thus, an increase in plasma cholesterol could allow Pl-Ca to penetrate into the artery intima, to form atherosclerotic plaques in patients for whom atherosclerosis is very common and occurs early (*4*). The Bogalusa Heart Study (Framingham Junior survey) has just provided additional proof that cholesterol 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 results raise questions that were not resolved in that study, and suggest that much work remains to be done.

References

1. Speich M. More on the relation between magnesium and calcium in serum and plasma [Letter]. Clin Chem 1986;32:221–2.
2. Speich M. Correlation between calcium and zinc in plasma [Letter]. Clin Chem 1986;32:1427–8.
3. Speich M, Gelot S, Arnaud P, Van Goc N, Robinet N, Pineau A. Multiple and simple correlations between magnesium, calcium, zinc, potassium, total- and HDL-cholesterol in 111 reference subjects. Magnesium-Bull 1984;6:137–41.
4. Speich M, Gelot S, Arnaud P, Murat A. Plasma and erythrocyte magnesium in insulin-dependent and non-insulin-dependent diabetics: correlations between twelve variables. Magnesium-Bull 1985;7:140–5.
5. Speich M, Métayer C, Arnaud P, Nguyen VG, Boiteau HL. Concentrations of lead, magnesium, calcium, zinc and cadmium in twenty rabbit tissues after exposure to low lead doses and atherogenic diet. Ann Nutr Metab 1983;27:531–41.
6. Newman WP, Freedman DS, Voors AW, et al. Relation of serum lipoprotein levels and systolic blood pressure to early atherosclerosis. The Bogalusa Heart Study. N Engl J Med 1986;314:138–44.
7. Broustet JP, Douard H, Mora B. Insuffisance coronaire chez la femme: particularités épidémiologiques, cliniques, ergométriques et thérapeutiques. Rev Prat (Paris) 1986;36:2043–8.

Michelle Speich

Lab. de Biochimie Pharmaceutique
Faculté de Pharmacie
1, rue Gaston Veil
F-44035 Nantes Cedex, France

Problems in Standardization of Digitalis-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 immunoreactive substance(s) (DLIS) in human body fluids (*1–6*), and the putative endogenous substance has been almost partially purified and identified (7), there is no accord on the nomenclature (DLIS, DLF, OLF, Endoxin, Endaline, etc.) and the best procedure(s) of sample collection, storage, and assay. This lack of standardization not only generates confusion, but also can be the cause of discordant results and erroneous findings. The aim of this Letter is to discuss some methodological points of DLIS assay, adding personal contributions, in order to stimulate a more general discussion among the groups interested in studies concerning endogenous cardiac glycosides-like substances.

Sample preparation—Several authors (*8–13*) used deproteinized (by boiling) and concentrated plasma extracts for their studies in animals or humans. We have recently used the extraction with C_{18} reversed-phase cartridges (Sep Pak, Waters Associates) for the concentration and prepurification of biological samples (*5, 14*). This procedure seems preferable when radioreceptor assay (RRA) and (or) transmembrane ion-transport studies are used with the RIA for detection of cardiac glycosides-like compounds. 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 proteins and avoid this kind of interferences 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 extraction and concentration) should be used when plasma samples from newborns, pregnant women, and patients with renal or liver disease are assayed, because of their higher DLIS concentrations in plasma in comparison with

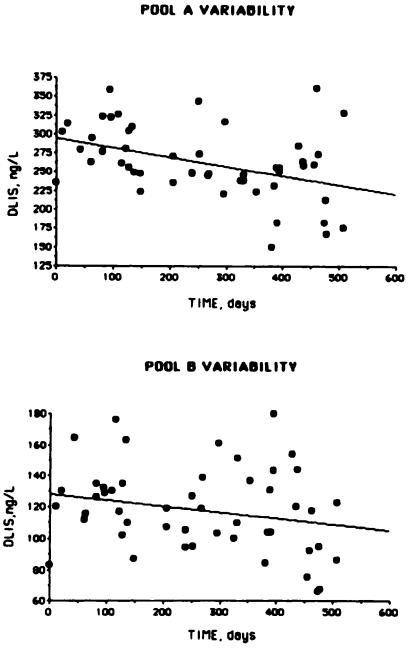

Fig. 1. DLIS stability of two urinary pools collected from newborns in the first day (Pool A) and in the third and fourth days of extrauterine life (Pool B)

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 ($r = 0.405$, $n = 50$, $0.005 < p < 0.0001$, $y = 294.6 - 0.12x$)

normal adult subjects (*2, 3, 5*). In addition, 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 immunoreactivity 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 significant 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., without extraction) assayed, during 10 months, a pool of plasma from blood donors, but the very low DLIS concentration, about 15 ng/L, did not permit a significant statistical analysis because of the high imprecision of the assay

(CV >40%). Pooled plasma from newborns, from pregnant women, or from patients with renal diseases, with higher than normal DLIS concentrations, could be used to test the stability of digitalis-like immunoreactivity in plasma.

Preparation of standard curve—Most authors who have measured DLIS 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 DLIS assay (SPAC Digoxin; Mallinckrodt, Dietzenbach, F.R.G.) we have found that most (>70% in two different assays) plasma samples (direct assay) with DLIS concentrations within the normal range (0–35 ng/L) have a bound radioactivity higher than that of the B_0 standard. Therefore, it is important to test for the presence of significant amounts of DLIS in the serum-basal 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 DLIS is not yet known, it is not possible to perform specific RIA assays for DLIS by use of specific antisera against DLIS, with DLIS as standard. The relatively poor specificity of measurement of DLIS 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 DLIS values obtained by RIA method or radioreceptor assay for 21 chromatographed extracts (made by use of C_{18} 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 DLIS 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-glycosides-like substances in human biological fluids; for confirmation, more specific methods, used after chromatographic purification, are required.

References

1. McCarthy RC. Minimizing the effect of digoxin-like immunoreactive substances in immunoassays for digoxin in neonatal serum [Letter]. Clin Chem 1985;31:1240–1.
2. Valdes R Jr. Endogenous digoxin-like immunoreactive factors: impact on digoxin measurements and potential physiological implications. Clin Chem 1985;31:1525–32.
3. Soldin SJ. Digoxin—issues and controversies [Review]. Clin Chem 1986;32:5–12.
4. Scherrmann JM, Sandoux P, Guedeney X. Specific interaction between antidigoxin antibodies and digoxin-like immunoreactive substances in cord serum. Clin Chem 1986;32:97–100.
5. Clerico A, Ghione S, Balzan S, Del Chicca MG. Digoxin-like immunoreactive substance: non-specific interference or a new hormone? J Nucl Med Allied Sci 1984;28:297–301.
6. De Wardener HE, Clarkson EM. Concept of natriuretic hormone [Review]. Physiol Rev 185;65:658–759.
7. Cloix JF, Crabox M, Wainer IW, et al. High-yield purification of urinary Na^+-pump inhibition. Biochem Biophys Res Comm 1985;131:1234–40.
8. Gruber KA, Buckalew VM Jr. Further characterization and evidence for a precursor in the formation of plasma antinatriferic factor. Proc Soc Exp Biol Med 1978; 159:463–7.
9. Gruber KA, Whitaker JA, Buckalew VM Jr. Endogenous digitalis-like substance in plasma of volume-expanded dogs. Nature (London) 1980;287:1–3.
10. Ghione S, Balzan S, Montali U, et al. Plasma digoxin-like immunoreactivity in essential hypertension: relation to plasma renin activity. J Hypertension 1983;1(suppl 2):142–4.
11. Balzan S, Clerico A, Del Chicca MG, et al. Digoxin-like immunoreactivity in normal human plasma and urine, as detected by a solid-phase radioimmunoassay. Clin Chem 1984;30:450–1.
12. Kelly RA, O'Hara DS, Canessa ML, et al. Characterization of digitalis-like factors in human plasma. Interactions with NaK-ATPase and cross-reactivity with cardiac glycoside-specific antibodies. J Biol Chem 1985;260:11396–405.
13. Valdes R Jr, Graves SW, Becker SL. Protein binding of endogenous digoxin-immunoactive factors in human serum and its variation with clinical condition. J Clin Endocrinol Metab 1985;60:1135–43.
14. Balzan S, Ghione S, Clerico A, Montali U. Correlation between digoxin like immunoreactivity and ^{3}H-ouabain displacement on erythrocyte membranes in extracts of human plasma. Clin Biochem (in press, 1986).
15. Clerico A, Del Chicca MG, Montereggi A, et al. Digoxin-like immunoreactivity in human body fluids. J Nucl Med Allied Sci 1985;29:305–12.
16. Hnatowich M, LaBella F. Endogenous digitalis-like factors: in vitro comparison of biological and immunological activities of peptide and steroid candidates. Eur J Pharmacol 1984;106:567–75.
17. Kramer HJ, Heppe M, Weiler E, et al. Further characterization of the endogenous natriuretic and digoxin-like immunoreacting activities in human urine: effects of change in sodium intake. Renal Physiol 1985;8:80–9.

A. Clerico
S. Ghione
M. G. Del Chicca
S. Balzan

C.N.R. Institute of Clin Physiol.
University of Pisa
Via Savi 8
56100 Pisa, Italy

Measurement of Stable Glycated Hemoglobin

To the Editor:

In view of the concern regarding the effect of the labile Schiff base adduct (pre-Hb A_1) on the true values for Hb A_1 as measured by cation-exchange chromatography (*1, 2*), we report the simple method used routinely in our laboratory to remove pre-Hb A_1.

Hb A_1 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 (*3*). Blood was collected with EDTA as anticoagulant, and samples were variously treated as below before the hemolysates were prepared.

(*a*) Hb A_1 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 fluid was 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 °C for 6 h in a shaking