Digoxin Immunoreactivity in Cord and Maternal Serum and Placental Extracts. Partial Characterization of Immunoreactive Substances by High-Performance Liquid Chromatography and Inhibition of Na$^+$, K$^+$-ATPase

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Digoxin-like immunoreactive substances (DLIS) have been successfully extracted and concentrated from cord serum, mixed (cord and maternal) serum and placentas. Similar substances have also been extracted from normal adult serum, but DLIS in this medium are present in much lower concentrations. Concentrated DLIS have been separated into several immunoreactive fractions by use of reverse-phase high-performance liquid chromatography. Immunoreactive fractions were tested for their ability to inhibit the Na$^+$,K$^+$-ATPase by measuring the ^{86}Rb-uptake of red blood cells in the presence of these fractions. A potent inhibitor was identified in an immunoreactive fraction which also contains progesterone, but the results suggest that the pump inhibitor is not progesterone. Cross-reactivity studies utilising fluorescence polarization immunoassay have shown that cortisone is the most potent immunoreactive substance of cord serum.

Digoxin and other cardiac glycosides have long been used for the management of congestive heart failure and cardiac arrhythmias. Although the exact mode of action of these drugs remains unknown, it is well-established that they inhibit the activity of Na$^+$,K$^+$-ATPase. Digoxin has a low therapeutic index and, consequently, optimum therapy requires measurement of the concentration of the drug in serum. Digoxin is usually measured in body fluids by use of various immunological procedures. The specificity of antibodies used for the measurement of digoxin has recently been questioned (1). Many reports have already appeared in the literature describing digoxin immunoreactivity in tissues and biological fluids of subjects who have never received the drug. Cross-reacting compounds (frequently termed "digoxin-like immunoreactive substances, DLIS") have been found in umbilical cord blood and placental homogenates (2–4), in sera from neonates (4–7) and pregnant women (8), amniotic fluid (5), in sera from patients with renal impairment (4, 9) and even in normal human plasma and urine (10, 11). Others have extracted DLIS from many neonatal tissues and found high levels in bile and meconium (12).

There is experimental evidence of the existence in human serum and urine of a low molecular weight substance (or substances) which acts as an endogenous inhibitor (regulator) of Na$^-$,K$^+$-ATPase. The material(s) also reacts with digoxin antibodies and consequently was named "endoxin". Endoxin, the postulated natriuretic hormone, is believed to play a major role in the pathogenesis of essential hypertension. The enormous literature on this subject has recently been reviewed by experts (13–15). The structure and site of production of endoxin is still unknown. Some workers have isolated substances with digitalis-like activity from mammalian brain (16) and ouabain-like activity in human cerebrospinal fluid (17).

There have been no studies published to show if there is any relationship between DLIS which increase during pregnancy and the postulated natriuretic hormone. The latter reacts with digoxin antibodies, is a Na$^+$,K$^+$-ATPase inhibitor and increases in plasma and urine during salt or water loading. The pregnancy-related DLIS are usually treated as a homogeneous entity although reports do suggest that multiple substances may be involved.

In this paper we describe methods of extraction and concentration of DLIS from normal, cord, maternal serum and placentas. High-performance liquid chromatography of the concentrated material showed that DLIS can be separated into many individual immunoreactive fractions. The biological activity of the fractions was assessed by measuring the Na$^+$,K$^+$-ATPase inhibition by means of inhibition of ^{86}Rb-uptake by red blood cells. A potent inhibitor was isolated in a fraction in which progesterone is eluted. Cross-reactivity studies using fluorescence polarization immunoassay (FPIA) indicate that cortisone is one of the major cross-reacting substances present in cord serum.

Materials and methods

INSTRUMENTATION

The liquid chromatographic system consisted of a model 6000A pump equipped with a solvent select valve which we employed for manual generation of

step gradients, a model U6K injector (all from Waters Scientific Ltd., Mississauga, ON L4V 1M5 Canada), a model 160 absorbance detector set at 254 nm (Beckman Instruments Inc., Brea, CA 92621) and a model 024 recorder (Perkin-Elmer Ltd., Downsview, ON M3N 1X3 Canada). We used a μBondapak C-18 column (30 cm × 3.9 mm, Waters). Disposable C-18 cartridges (Sep-Pak®) were also obtained from Waters.

STEROID SOLUTIONS

Unless otherwise stated, all solutions were prepared in absolute methanol. When needed, methanolic steroid solutions were evaporated to dryness and redissolved in Ringer's solution or digoxin-free serum. All steroids and digoxin were purchased from Sigma Chemical Co., St. Louis, MO 63178.

Digoxigenin, digoxigenin monodigitoxoside and digoxigenin bisdigitoxoside were obtained from Boehringer Mannheim, Indianapolis, IN 46250.

CORD BLOOD, MIXED (CORD AND MATERNAL) BLOOD, PLACENTAS

Placentas, and any blood liberated, were collected in sterile stainless-steel bowls after delivery. Pure cord blood was aspirated with a syringe, just after delivery, from the placental vessels. Mixed blood was collected as follows: Placental vessels were cut and the placenta was squeezed gently so that cord blood was removed from the placenta and drained into a bowl. The blood contained in the bowl (which is a mixture of maternal and cord blood) was collected and centrifuged to provide mixed serum. Serum was stored at 4°C for no longer than a week. Only fresh placentas were used for the extraction of DLIS. Patients' charts were reviewed to exclude the possibility of digoxin therapy.

HPLC METHODS

The mobile phases used were mixtures of methanol or acetonitrile with water. The following phases were employed: Methanol/water (30/70), methanol/water (60/40), methanol 100%, acetonitrile/water (27/73), acetonitrile/water (50/50). The details of each chromatographic run are given in the respective figures. The HPLC fractions were collected manually every 1 minute in 10 mL glass tubes.

DIGOXIN IMMUNOASSAYS

Fluorescence polarization immunoassay (FPIA) for digoxin was performed as recommended by the manufacturer of the TDx® Analyzer (Abbott Diagnostics Division, Abbott Park, North Chicago, IL 60064). The radioimmunoassay procedure (RIA) used was as previously described [1]. For most studies the FPIA was used because of its speed and convenience. HPLC fractions were evaporated to dryness under nitrogen at 60°C and redissolved in 200 μL digoxin-free serum before assay. All other samples analyzed for digoxin immunoreactivity were in a digoxin-free serum matrix.

EXTRACTION PROCEDURE USED PRIOR TO HPLC

(a) Cord, mixed or normal adult serum

Sep-Pak® cartridges were mounted on a 12 mL glass syringe and prewashed with 6 mL of methanol followed by 6 mL of water. Serum was then applied and passed through the Sep-Pak® slowly (1–2 drops/s). Up to 25 mL of serum was passed through a single Sep-Pak®. The cartridge was then washed with 2 mL of water and the substances retained were eluted with 4 mL of methanol. Methanolic extracts from various cartridges (if more than 25 mL serum was processed) were combined and evaporated to dryness at 60°C under nitrogen. The residue was dissolved in 6 mL digoxin-free serum and re-extracted as above with a new Sep-Pak®. This second extraction lengthens the pre-HPLC sample clean-up procedure, but was incorporated as it markedly improved HPLC chromatograms. The methanolic eluant was again evaporated to dryness, the residue was dissolved in 200–300 μL methanol, centrifuged and injected into the HPLC system.

(b) Placentas

Fresh placentas were cut to pieces and weighed. The pieces (about 500 g) were placed in a 2 L glass beaker and approximately 1 L of dichloromethane was added. The placenta was then homogenized for 15 min with a Polytron homogenizer. The homogenate was filtered through glass-wool, the dichloromethane extract was then centrifuged and the clear solvent was separated and evaporated to dryness under an air or nitrogen stream with gentle heating. The residue was dissolved in 50 mL of methanol. The methanolic solution was centrifuged, separated from the residual solids and then evaporated to dryness at 60°C under nitrogen. The residue was dissolved in 10 mL of digoxin-free serum, solids were removed by centrifugation and the serum solution was extracted once with the Sep-Pak® cartridge as described above for serum. The methanolic eluant of the Sep-Pak® was treated exactly as described above, before injection into the HPLC system.

THE ⁸⁶Rb-UPTAKE METHOD

Radioactive rubidium (⁸⁶RbCl, specific activity of 1 mCi/mL) was obtained from Amersham Corporation, Arlington Heights, IL 60005. Working ⁸⁶RbCl solution was prepared by diluting the above solution 100-fold with Ringer's solution.

Potassium-free Ringer's solution [18] was prepared by mixing 1 L of NaCl solution (154 mmol/L), 15 mL of CaCl₂ solution (110 mmol/L), 10 mL of MgSO₄ solution (155 mmol/L) and 210 mL of phosphate buffer pH 7.40 (110 mmol/L). Glucose (1.0 g) was dissolved in the final solution. The final concentrations of sodium, calcium, magnesium, chloride and glucose in this solution were 162.1, 1.34, 1.26, 127.4 and 4.50 mmol/L, respectively.

Fresh blood was obtained in heparinized syringes by venipuncture of healthy individuals not receiving any drugs. The red blood cells were washed three times with Ringer's solution. For the assay 200 μL of packed red

TABLE 1
Effectiveness of Sep-Pak® Extraction of Digoxin Immuno-
reactivity from Serum

Sample	Initially present in serum (nmol/L)		After extraction 1st eluate (nmol/L)		After concentration 2nd eluate (× 10) (nmol/L)	
	FPIA	RIA[2]	FPIA	RIA	FPIA	RIA
Cord serum (3)	0.04	0.20	N.D.	N.D.	0.86	1.12
Cord serum (4)	0.22	0.41	0.07	0.06	2.64	2.44
Mixed serum (3)	0.26	0.30	N.D.	N.D.	1.00	1.13
Digoxin-free serum[3]	N.D.	N.D.	N.D.	N.D.	0.16	0.13
Serum with added digoxin (1, 5)	0.34	—	N.D.	—	1.54	—

[1]In the case of serum with added digoxin, the concentration
was only 4.5 fold. Thus, the recovery of digoxin from Sep-Pak
extraction was 101.0%.

[2]All RIA results are means of quadruplicate analyses.

FPIA results are means of (3) quintuplicate, (4) triplicate
and (5) duplicate assays. N.D. = not detected.

cells, 400 μL of Ringer's solution and 100 μL of sample
or standard are pipetted into 5 mL plastic tubes and
gently mixed. This solution is then incubated for 2 h at
37°C (air-oven) with periodic shaking. At the end of the
incubation 200 μL of the working ^{86}RbCl solution is
added to each tube and the solution is mixed. The latter
is incubated for 1 h at 37°C with periodic shaking. At
the end of the incubation the solution is centrifuged at
4°C and the supernatant discarded. The RBC's are
washed three times with 4 mL of cold Ringer's solution.
Radioactivity in the final RBC pellet was counted using
a Nuclear Enterprises model NE1612 gamma counter
using the ^{57}Co channel. Counting time was 120 s.
Samples tested were HPLC fractions (grouped and de-
scribed in the text) which were evaporated to dryness
and re-dissolved in 1 mL of Ringer's solution. Solutions
of digoxin in Ringer buffers served as standards.

All samples and standards were run in triplicate. For
each run a complete set of digoxin standards was
included. The remaining Na+,K+-ATPase activity
which is proportional to the ^{86}Rb-uptake of the RBC's
was calculated as a percentage assuming that the pump
activity in the presence of Ringer's solution instead of
sample is 100%.

All samples assayed by the ^{86}Rb-uptake method were
dissolved in Ringer's solution and were also tested for
their potassium content by flame photometry. All
samples contained <0.1 mmol/L of potassium.

Results and discussion

DLIS IN CORD AND MIXED SERUM

We measured DLIS in different pools of cord and
mixed serum by the FPIA method. Each pool was com-
posed of serum obtained from 1–3 different subjects.
Values obtained for DLIS (mean ± SD) were 0.27 ±
0.21 nmol/L for cord serum (n = 14) and 0.26 ± 0.15 for

mixed serum (n = 8). Our results are similar to those
obtained by others (4) using the same analytical
technique and confirm that DLIS are consistently found
in both cord and maternal serum.

EXTRACTION OF DLIS FROM SERUM WITH SEP-PAK® CARTRIDGES

We studied the effectiveness of extraction of DLIS
and digoxin from serum by use of the Sep-Pak⁸ dispos-
able cartridges. For this experiment, cord, mixed serum
or digoxin-free serum with or without added digoxin
was first assayed for digoxin by FPIA or RIA to find the
initially present digoxin immunoreactivity. 20.0 mL se-
rum was then passed through the Sep-Pak⁹ to give the
first Sep-Pak® eluate which was assayed again for
digoxin, to find the DLIS which remained in the eluate
after the extraction (i.e. was not retained by the car-
tridge). The cartridge was then eluted with 4 mL of
methanol, the methanol was evaporated to dryness and
the residue redissolved in 2.00 mL of digoxin-free
serum to provide the second Sep-Pak⁸ eluate. This
solution was also assayed for digoxin. The partial
purification procedure described theoretically in-
creases the concentration of DLIS which are initially
present in serum by a factor of 10 (except where in-
dicated otherwise, Table 1). A summary of the results is
presented in Table 1.

The results of Table 1 confirm that DLIS are retained
by the Sep-Pak® cartridge since the amount of DLIS in
serum after the extraction step is either very low or
undetectable. Additionally, DLIS can be eluted from
the cartridge with methanol, concentrated in digoxin-
free serum and measured in the concentrated solution
at a much higher concentration than is initially
present. Quantitative estimation of the recovery of
DLIS from the Sep-Pak® cartridge is not possible
because of the very low concentration of DLIS in the
starting solution which makes the accuracy of mea-
surement of DLIS difficult. Furthermore, the dilution
curves for DLIS as measured by the digoxin immu-
noassay are not linear. The within-run SD for both
FPIA and RIA at the 0.2 nmol/L digoxin concentration
is 0.06 nmol/L.

FRACTIONATION OF DLIS BY HPLC

A three-solvent step gradient system was employed
to separate the injected sample on a C-18 column, in
fractions of relatively high polarity (eluted with
methanol/water, 30/70), intermediate polarity (eluted
with methanol/water 60/40) and low polarity (eluted
with 100% methanol).

Cord, mixed or digoxin-free serum 15–500 mL, or
placenta 200–2,000 g, were concentrated as described
in "methods" and subjected to the three-solvent step-
gradient solvent system for fractionation. Table 2 sum-
marises the retention times of various common steroids
in that system and in two other systems described later.

The chromatographic separation of an extract ob-
tained from 120 mL mixed serum is shown in Figure 1.
Digoxin immunoreactivity is detected in fractions
18–25. A second area of immunoreactivity is found in

TABLE 2

Retention Times of Steroids in Three Different Chromatographic Systems

	Retention time, min		
Steroid	Ternary gradient[1]	Acetonitrile/water 27/73[2]	Acetonitrile/water 50/50[2]
Cortisone	22.5	10.0	—
Cortisol	23.5	10.2	2.8
Corticosterone	26.8	22.2	4.1
11-deoxycortisol	27.4	26.5	3.9
17-hydroxyprogesterone	33.7	>45	6.9
Progesterone	34.6	>45	13.7
Digoxigenin	19.5	—	—
Digoxigenin monodigitoxoside	20.3	—	—
Digoxigenin bisdigitoxoside	24.5	—	—
Digoxin	29.5	16.0	—

[1]The ternary gradient was methanol/water (30/70) (0–10 min), methanol/water (60/40) (10–25 min) and methanol 100% (25–40 min) at a flow rate of 1 mL/min. Times refer to the point of switching the solvent select valve from one solvent to the other. In some chromatograms, slightly different timing was used; this is indicated in the respective figures.

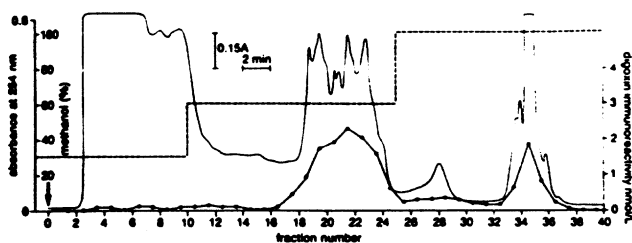[2]Isocratic conditions, flow-rate 1.5 mL/min.

Figure 1 — Gradient separation of a mixed serum extract (120 mL). Flow-rate was 1 mL/min. Immunoreactivity is expressed as digoxin equivalents since the standard curve on the TDx analyzer was constructed with digoxin standards. Change of mobile phases by means of a solvent select valve was done at the points shown. All fractions were evaporated to dryness and redissolved in 200 µL digoxin-free serum before measurement of digoxin immunoreactivity by FPIA (●—●). (---) gradient profile.
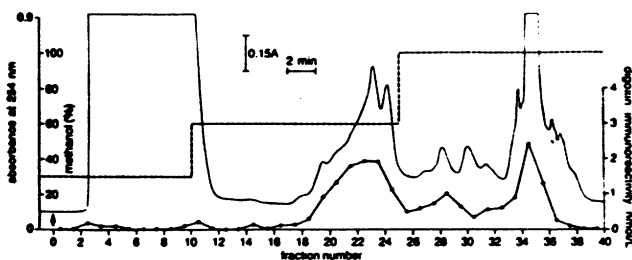

Figure 2 — Gradient separation of a placental extract. All other conditions as in Figure 1.

fractions 34–36. A weak area of immunoreactivity was found in fractions 27–32. This immunoreactivity was detectable only when large amounts of serum were extracted but it was consistently found in placental extracts (Figure 2). This fraction coelutes with digoxin in this chromatographic system as well as in the isocratic system (acetonitrile/water, 27/73) described later. Otherwise, the placental extracts and serum extracts gave similar patterns of immunoreactivity as is shown
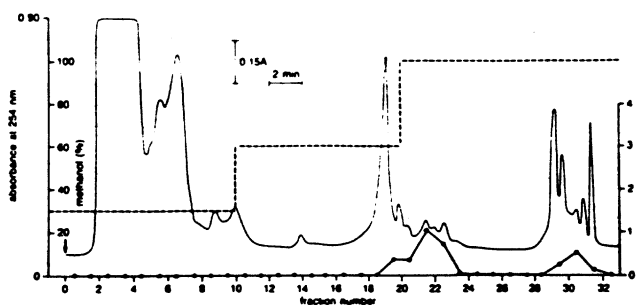

Figure 3 — Gradient separation of a digoxin-free serum extract (150 mL). In this case the flow-rate was 1.5 mL/min and the gradient profile somewhat different from that shown in Figure 1. All other conditions were as in Figure 1.
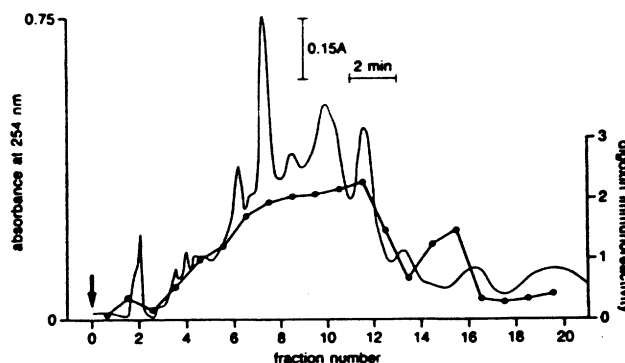

Figure 4 — Isocratic separation of a mixed serum extract (500 mL). This extract was first subjected to gradient separation as shown in Figure 1 and fractions 18–32 were collected, combined, evaporated to dryness, redissolved in methanol and injected. Mobile phase was acetonitrile/water (27/73) and flow-rate 1.5 mL/min. (●—●) Digoxin immunoreactivity by the FPIA.

in Figures 1 and 2.

In Figure 3, the chromatographic separation of an extract derived from 150 mL of digoxin-free serum is presented. Digoxin-free serum is considered a serum
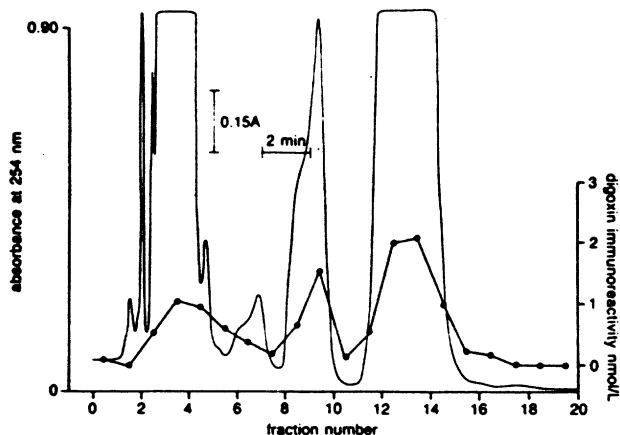
Figure 5 — Isocratic separation of a mixed serum extract (500 mL). The extract was first separated as shown in Figure 1 and fractions 33–37 were collected, combined, evaporated to dryness, redissolved in methanol and injected. Mobile phase was acetonitrile/water (50/50) and flow-rate 1.5 mL/min. (●—●) Digoxin immunoreactivity by FPIA.
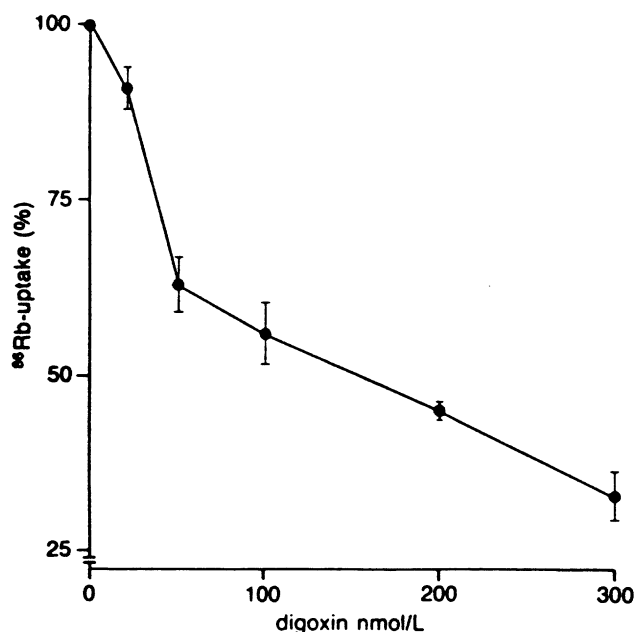

Figure 6 — ^{86}Rb-uptake by red blood cells in the presence of various concentrations of digoxin. Each point is the mean ± SD of three different assays.

obtained from normal adults not receiving digoxin with FPIA and RIA results <0.05 nmol/L. Note that digoxin immunoreactivity is present in 2 of the 3 fractions found in cord or maternal serum. DLIS are present in this serum at much lower concentrations compared to cord or mixed serum.

The fact that DLIS having similar HPLC elution profiles can be extracted from cord serum, mixed serum, placenta and normal serum suggests that these compounds are not specific for the pregnancy period but are present, at much lower concentration, in normal adult sera as well.

Fractions 18–32 of the ternary step-gradient chromatographic system (Figures 1 and 2) were pooled, evaporated to dryness, redissolved in 200 μL methanol

TABLE 3
Results of the ^{86}Rb-Uptake Assay for the Chromatographic Fraction 33–37 (Group V)

Source	Extract dilution	^{86}Rb-Uptake (%)	Digoxin immunoreactivity (nmol/L)[1]
Placenta[2] (i)	Undiluted	24[3]	3.5
	2-fold	66	
(ii)	Undiluted	45	7.5
(iii)	Undiluted	—	1.9
	2-fold	75	
500 mL mixed serum[2]	Undiluted	33[3]	10.8
	2-fold	48[3]	
	5-fold	71	
	11-fold	84	

[1]The digoxin immunoreactivity in the final extract was assessed by measuring the digoxin equivalents at various dilutions of the extract and calculating the initial concentration by arbitrarily using the dilution which gives a value close to 1 nmol/L digoxin. This value is only an approximation since the dilution curves of DLIS are not linear (Figure 7).

[2]Final extract was dissolved in 1 mL Ringer's solution.

[3]Slight hemolysis was observed during the ^{86}Rb-uptake experiments with these samples which may cause artifactual decrease in the ^{86}Rb-uptake result. This problem was overome by testing the samples in dilution as shown.

and re-chromatographed under isocratic conditions (acetonitrile/water (27/73), flow rate 1.5 mL/min) (Figure 4). Digoxin immunoreactivity was present in fractions 5 to 17. Similar results were obtained with placental extracts. These data suggest that the digoxin immunoreactivity in these fractions is due to several substances which elute close to each other and are not resolved if chromatographed for a second time under the conditions described. Efforts to better separate these compounds under isocratic conditions by use of methanol-water or tetrahydrofuran-water mixtures were unsuccessful and results similar to those shown in Figure 4 were obtained. Fractions 15–16 (corresponding to fractions 28–30 on the first HPLC system (Figure 2)) coelute with digoxin (Table 2).

Fractions 33–37 of the ternary step gradient chromatographic system were pooled, evaporated to dryness, redissolved in 200 μL methanol and rechromatographed under isocratic conditions (acetonitrile/water (50/50), flow-rate 1.5 mL/min) (Figure 5). Digoxin immunoreactivity was separated in three peaks which were similar in both mixed serum and placental extracts.

BIOLOGICAL ACTIVITY OF DLIS

Digoxin inhibits the activity of Na+,K+-ATPase. This effect can be demonstrated by measuring the degree of ^{86}Rb-uptake by washed red blood cells in the presence of digoxin. The ^{86}Rb-uptake of RBC exposed to digoxin, expressed as percentage of the uptake in the absence of digoxin, is shown in Figure 6.

We have tested the ability of DLIS to inhibit the RBC Na+,K+-ATPase by performing the ^{86}Rb-uptake assay. Both placental and mixed serum extracts were

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TABLE 4
Results of the ^{86}Rb-Uptake Assay for Some Common Steroids

Steroid	Conc., μmol/L	^{86}Rb-Uptake (%)	Digoxin immunoreactivity (nmol/L) (1)
Progesterone	15.89	98	0.1
	158.9	93	0.6
	1589*	83	4.4
Cortisol	13.79	101	0.1
	137.9	106	0.6
	1379*	85	4.7
17-hydroxyprogesterone	1513*	96	1.3
11-deoxycortisol	1443*	84	3.7
Corticosterone	1443*	64	4.8
Cortisone	138.7*	73	3.5

*The immunoreactivities at these concentrations were calculated as described in Table 3.
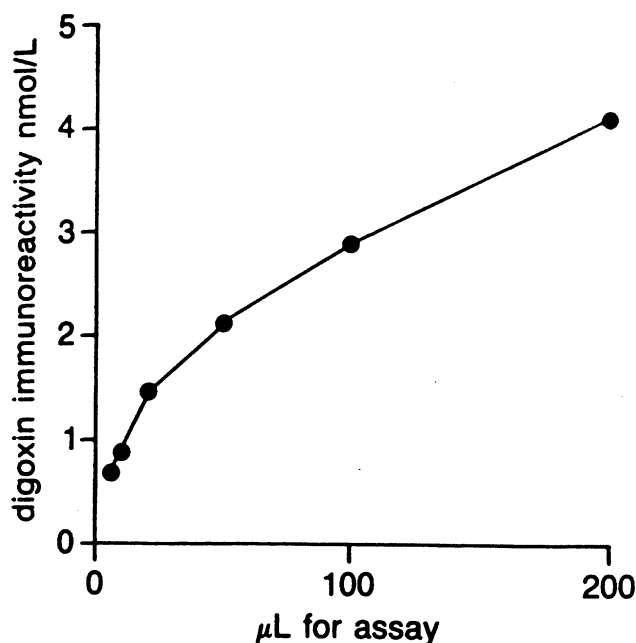

Figure 7 — Assay of a mixed serum extract (fractions 18–32 of Figure 1) at various dilutions, by the FPIA. The dilution curve is not linear.

used. Chromatographic fractions from the ternary step-gradient system (Figures 1 and 2) were pooled into five groups which were then tested separately. Each pool was evaporated to dryness and redissolved in 1 mL of Ringer solution before testing.

Group I (fractions 1–8) exhibited no measurable digoxin immunoreactivity and no significant ^{86}Rb-uptake inhibition. The % uptake of four different experiments (2 placentas, 2 mixed sera, 300 mL each) ranged from 86 to 106% with a mean of 96.5%.

Group II (fractions 9–17) exhibited no measurable digoxin immunoreactivity and the % uptake ranged from 88–98% with a mean of 93.5% (four different experiments as described above).

Group III (fractions 18–26) exhibited significant digoxin immunoreactivity (Figures 1 and 2) and ^{86}Rb-uptake ranging from 74 to 97% with an average of 86% (four experiments as described above). There is slight
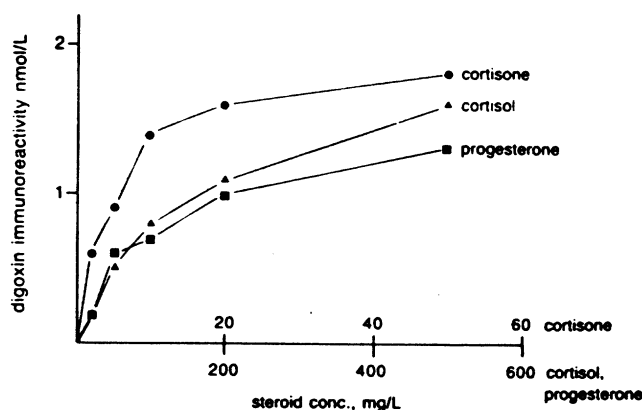

Figure 8 — Cross-reactivity of various concentrations of steroids in the FPIA immunoassay for digoxin.

inhibition of the ^{86}Rb-uptake by substances which elute in this area.

Group IV (fractions 27–32) exhibited weak immunoreactivities (Figures 1 and 2) and ^{86}Rb-uptake ranging from 84–89% with an average of 86% (four experiments, as above).

Group V (fractions 33–37) exhibited immunoreactivities and ^{86}Rb-uptake inhibition as shown in Table 3. This fraction contains a potent Na+,K+-ATPase inhibitor and cross-reacts with digoxin antibodies. While exciting, these data are obviously still preliminary and more material needs to be isolated and purified before the structure and function of this endogenous ATPase inhibitor are firmly established. The equivalent digoxin concentration which causes 45% ^{86}Rb-uptake can be calculated from Figure 6 and is found to be 200 nmol/L.

Group V was rechromatographed as shown in Figure 5 and fractions 1–7, 8–11, 12–16, 17–24, were tested for ^{86}Rb-uptake. Significant ^{86}Rb-uptake inhibition was located in fractions 12–16. Progesterone is eluted in this fraction as well (Table 2).

Table 4 shows digoxin immunoreactivity and ^{86}Rb-uptake for various steroids which are known to be elevated in pregnancy. All steroids tested were initially dissolved in methanol and then evaporated to dryness and redissolved in either digoxin-free serum (for the

FPIA) or in Ringer's solution (for the ^{86}Rb-uptake assay) in a manner identical to that used for the HPLC fractions. Progesterone, cortisol, and 11-deoxycortisol show similar immunoreactivities and results for the ^{86}Rb-uptake assay while 17-hydroxyprogesterone is less reactive in both assays. Corticosterone has similar immunoreactivity to cortisol, but is a more potent inhibitor of Na+,K+-ATPase.

Progesterone concentration in the umbilical vein plasma at birth is about 858 nmol/L and about 382 nmol/L in maternal vein plasma (19). A 50- to 500-fold concentration (see results of Table 3 for mixed serum) with a quantitative recovery would result in an extract containing a maximum of 44 to 445 μmol/L progesterone. This concentration of progesterone does not explain the low ^{86}Rb-uptake obtained in fractions 33–37 (Tables 3 and 4) and suggests that an additional substance is responsible for both the immunoreactivity and the ^{86}Rb-uptake inhibition in that area. LaBella et al. have proposed that certain progesterone derivatives can bind to the digitalis receptor (20).

In Figure 7, the digoxin immunoreactivity of DLIS at various dilutions is shown. In Figure 8 the digoxin immunoreactivity at various concentrations of the three steroids which are present at high concentrations in umbilical and maternal plasma is presented. Valdes calculated the cross-reactivities of pregnancy steroids using an RIA procedure and found very low values, the highest cross-reactivity being observed for progesterone (8). Our results show that cortisol cross-reacts at least as well as progesterone in the FPIA assay and that cortisone is a much more potent cross-reactant in the FPIA procedure as has been previously reported (1). DHEAS does not react even at concentrations of 2.561 mmol/L.

Recent findings suggest that the placenta converts the bulk of the maternal cortisol which reaches the fetus to cortisone (19) and that cortisone levels in umbilical vein plasma are approximately 388 nmol/L. Fifteen mL of cord serum was concentrated 75-fold to provide a cortisone concentration in the region of 28 μmol/L. This concentration provides a FPIA reading equivalent to 1.4 nmol/L of digoxin (from Figure 8). When this extract was chromatographed and the fractions assayed by the FPIA, a value of 1.4 nmol/L of digoxin immunoreactivity was obtained in fraction 22 (see Figures 1 and 2), which suggests that the immunoreactivity in this fraction is primarily due to cortisone which elutes in that area (Table 2).

To further demonstrate the steroid effect on the FPIA and RIA procedures, we prepared solutions of cortisone, cortisol and progesterone in digoxin-free serum and then subjected these to analysis. The digoxin immunoreactivity by the FPIA procedure was undetectable at cortisol and progesterone concentrations up to 3.0 μmol/L. Cortisone gave undetectable readings at 139 nmol/L but 0.14 and 0.17 nmol/L digoxin immunoreactivity at 416 and 832 nmol/L, respectively. It may be concluded that approximately 50% of the cord serum immunoreactivity measured by the FPIA procedure is likely to be due to cortisone alone. Cortisone (up to 832 nmol/L) and cortisol (up to 2.76 μmol/L) gave undetectable results by the RIA methodology

used. Progesterone concentrations of 0.318, 1.590 and 3.18 μmol/L gave 0.16, 0.26 and 0.36 nmol/L digoxin equivalents respectively, indicating that it is one of the major cross-reactants in the RIA method.

In conclusion, we have extracted and concentrated DLIS from cord and mixed serum and separated them into various individual fractions by use of reverse-phase high-performance liquid chromatography. Similar substances were identified at lower concentrations in normal adult serum. A potent Na+,K+-ATPase inhibitor was identified in a chromatographic fraction which contains progesterone but the results suggested that the inhibitor is not progesterone. Cross-reactivity studies have shown that cortisone is a major interferent in the FPIA procedure and that progesterone is a major interferent in the RIA procedure. Part of the digoxin immunoreactivity of cord serum is due to the presence of these substances.

Acknowledgements

References

1. Soldin SJ, Papanastasiou-Diamandi A, Heyes J, Lingwood C, Olley P. Are immunoassays for digoxin reliable? Clin Biochem 1984; 17: 317–20.
2. Besch Jr HR, Hufferd S, Lake M, Hurwida R, Watanabe AM. False elevation of apparent digoxin levels in plasma of premature infants. Clin Chem 1976; 22: 1168.
3. Kelly JG, Naidu RK, O'Malley K. Evidence for a circulating sodium transport inhibitor in essential hypertension. Br Med J 1981; 282: 1241.
4. Yatscoff RW, Desjardins PRE, Dalton JG. Digoxin-like immunoreactivity in the serum of neonates and uremic patients, as measured in the Abbott TDX. Clin Chem 1984; 30: 588.
5. Valdes R, Graves SW, Brown BA, Landt M. Endogenous substances in newborn infants causing false-positive digoxin measurements. J Pediatr 1983; 102: 947–50.
6. Pudek MR, Seccombe DW, Whitfield MF, et al. Digoxin-like immunoreactivity in premature and full-term infants not receiving digoxin therapy. N Engl J Med 1983; 308: 904–5.
7. Pudek MR, Seccombe DW, Jacobson BE, et al. Seven different digoxin immunoassay kits compared with respect to interference by a digoxin-like immunoreactive substance in serum from premature and full-term infants. Clin Chem 1983; 29: 1972–4.
8. Graves SW, Valdes R, Brown BA, Knight AB, Craie HR. Endogenous digoxin immunoreactive substance in human pregnancies. J Clin Endocrinol Metab 1984; 58: 748–51.
9. Graves SW, Brown BA, Valdes R. Endogenous digoxin-like substance in patients with renal impairment. Ann Int Med 1983; 99: 604–8.
10. Balzan S, Clerizo A, Grazia del Chieca M, Montali U, Quione S. Digoxin-like immunoreactivity in normal human plasma and urine, as detected by solid-phase radioimmunoassay. Clin Chem 1984; 30: 450–1.
11. Valdes R, Siegfried B. Digoxin immunoreactivity in urine of persons not taking digoxin. Clin Chem 1983; 29: 1166.
12. Pudek MR, Seccombe DW, Whitfield MF, et al. Perinatal changes and fluid and tissue levels of a digoxin-like immunoreactive substance in neonates. Clin Biochem 1984; 17: 200.

13. DeWardener HE, Clarkson EM. The natriuretic hormone. Recent developments. *Clin Sci* 1982; **63**: 415−20.

14. Buckalew VM, Gruber KA. Natriuretic hormone. *Ann Rev Physiol* 1984; **46**: 343−58.

15. Grantham JJ, Edwards RM. Natriuretic hormones: At last, bottled in bond? *J Lab Clin Med* 1984; **103**: 333−6 (Editorial).

16. Fishman MC. Endogenous digitalis-like activity in mammalian brain. *Proc Natl Acad Sci* 1979; **76**: 4661−3.

17. Halperin J, Schaeffer R, Galver L, Malave S. Ouabain-like activity in human cerebrospinal fluid. *Proc Natl Acad Sci* 1983; **80**: 6101−4.

18. Aronson JK, Grahame-Smith DG, Hallis RF, Hibble A, Wingley F. Monitoring digoxin therapy: I. Plasma concentrations and an *in vitro* assay of tissue response. *Br J Clin Pharmacol* 1977; **4**: 213−21.

19. Sperling MA. Newborn adaptation: Adrenocortical hormones and ACTH. In: Tulchinsky D, Ryan KJ, Eds. *Maternal-fetal endocrinology*. Pp. 387−92. Toronto: Saunders, 1980.

20. Kims RS, Labella FS, Zunza H, Zunza F, Templeton JF. Progesterone derivatives that bind to the digitalis receptor: Structure-activity relationships. *Mol Pharmacol* 1980; **18**: 402−5.