Partial Purification of Endogenous Digitalis-like Compound(s) in Cord Blood
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Increasing evidence indicates the presence of endogenous digitalis-like compound(s) in human body fluids. In this preliminary report, we describe a study of the partial purification by HPLC of these compounds in the plasma of neonates (who have particularly high concentrations of this substance) and adults. Plasma samples from neonates (cord blood) and adults, lyophilized and extracted with methanol, were applied on a 300 × 3.9 mm C18 Nova Pak column and eluted with a mobile phase of acetonitrile/methanol/water (17/17/66 or 14/14/72 by vol) and, after 30 min, with 100% methanol. We assayed eluted fractions for inhibitory activity of 86Rb uptake and for digoxin-like immunoreactivity. The elution profile revealed a first peak of inhibitory activity of 86Rb uptake at the beginning of the chromatography; another peak was eluted with the 100% methanol. The two peaks also cross-reacted with anti-digoxin antibodies. Because the second peak could possibly reflect the nonspecific interference of various lipophilic compounds, we focused our attention on the first peak. For these fractions dose–response curves for 86Rb uptake and for displacement of digoxin were parallel, respectively, to those of ouabain and digoxin, suggesting similarities of digoxin-like immunoreactive substance to cardiac glycosides. Similar chromatographic profiles were also obtained for plasma from adults, suggesting that the endogenous glycoside-like compound(s) in the neonate may be the same as those in the adult.

Additional Keyphrases: 86Rb uptake · erythrocytes · digoxin-like immunoreactivity · chromatography · liquid · pediatric chemistry · cardiac glycosides · radioimmunoassay

Several investigators have reported the presence in human body fluids of endogenous circulating glycoside-like substance(s) that may inhibit the activity of the cell membrane sodium/potassium pump (1–14). Increased circulating concentrations of these inhibitor(s) have been found in pregnancy (3), in neonates (4, 5), in arterial hypertension (6, 7), and in hepatic (8) and cardiac insufficiency (9), which suggests that the compound(s) could have a role in these states. These substances may also interact with several anti-digoxin antisera (1–5). However, neither the structure nor the site of production of these substances is clear. In addition, whether these inhibitors represent various compounds in plasma and (or) to what extent they are the results of the nonspecificity of the assays used is not yet clear. The different methods used for extracting and purifying these substances could partly explain the controversial results in the literature.

We describe a method for partial purification of the substances that cross-react with anti-digoxin antisera and inhibit 86Rb uptake in plasma from neonates (cord blood) and adults.

Materials and Methods

Preparation of samples. Heparinized blood samples, obtained from neonates (cord blood) and from normal adults, were placed on ice, centrifuged within 1 h, and stored at −20 °C. None of the subjects studied had cardiac, hepatic, or renal diseases, and all were normotensive.

Plasma was subsequently treated and assayed within one week according to the following procedure: We lyophilized 10–40 mL of plasma, suspended this in 20 mL of methanol, and centrifuged the sample at 15 000 × g for 5 min at 4 °C. After removing and reserving this supernate, we resuspended the precipitate in 5 mL of methanol and centrifuged it again at 15 000 × g for 5 min. This procedure was repeated three times, and the four supernates were pooled and evaporated under reduced pressure. The extract obtained was then redissolved in 10 mL of doubly distilled water and purified on Sep Pak C18 cartridges (Millipore, Bedford, MA) as previously described (15). We dried the eluate under reduced pressure, reconstituted it in 2–4 mL of the HPLC mobile phase of acetonitrile/methanol/water (17/17/66 or 14/14/72 by vol; see below), centrifuged for 10 min at 10 000 × g, and filtered the supernate through 0.5-μm (pore size) filters (Water, Milford, MA).

HPLC chromatography. We injected 100-μL samples, obtained as described above, onto a 300 × 3.9 mm C18 Nova Pak column (Waters) coupled to an HPLC instrument fitted with a variable-wavelength detector set at 220 nm (UVIECC 100-V, Jasco International Co., Tokyo, Japan).

Chromatographic runs were performed under isocratic conditions, with a mobile phase of acetonitrile/methanol/water (17/17/66 by vol) at a flow rate of 1 mL/min. For each run, we collected 16 fractions, then changed the mobile phase to 100% methanol, and collected five subsequent fractions. In a further set of experiments, the mobile phase was changed slightly (14/14/72 by vol) for better resolution of the early fractions. In some cases, to increase the material for the assays, we pooled the fractions from two or more chromatographic runs that had the same absorbance profile and the same retention time. The fractions were dried under reduced pressure and stored at −20 °C for the assays.

Erythrocyte uptake of 86Rb. We used the method of

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Aronson et al. (16) with some modifications (17). We obtained erythrocytes immediately before the experiments from fresh blood samples from healthy donors not receiving any drugs. The blood samples were transferred to an ice-water bath and, after separation from plasma by centrifugation at 4 °C (3000 × g), were washed three times by centrifugation with MgCl₂ (110 mmol/L) at 4 °C, resuspended in Ringer solution (containing, per liter, 9 g of NaCl, 0.24 g CaCl₂, and 0.2 g of NaHCO₃, pH 7.4) to give a hematocrit of 50%, and added in 25-μL aliquots to the HPLC-eluted fractions reconstituted in 100 μL of Ringer solution. All cell suspensions were pre-incubated for 4 h at 37 °C in a shaking water bath. At the end of the pre-incubation, we added 20 μL of ⁸⁶Rb solution, containing 1–2 μCi of ⁸⁶RbCl (Amersham International, Bucks, U.K.; 1 Ci/L, 1 Ci/g), to each tube. The incubation was continued for 1 h and stopped by washing the cells at 4 °C (except for the vials to be used for determining total radioactivity) three times with 2 mL of isotonic saline (NaCl, 154 mmol/L). The radioactive rubidium taken up by the erythrocytes was determined with a gamma counter, and inhibition of the ⁸⁶Rb uptake in erythrocytes was calculated as a percentage, assuming as 100% the pump activity in the presence of the Ringer solution devoid of column-fraction samples.

Dose–response curves were set up for ouabain (concentrations ranging from 0.1 nmol/L to 1 μmol/L) and for the early HPLC peak (mobile phase: acetonitrile/methanol/water, 14/14/72 by vol) obtained by drying and diluting the fractions pooled from several elutions of extracts from cord blood and corresponding to a range of 0.16–5.7 mL of the original pre-extraction plasma.

RIA. HPLC fractions from cord blood and adult plasma, reconstituted with 400 μL of phosphate buffer (pH 7.4, 0.15 mol/L), were assayed for endogenous digoxin-like immunoreactive substance (DLIS) by RIA as previously described (18). For our radioimmunoassay, we used a solid-phase system (antibody-coated test tubes) for separation of the free/bound moiety and ¹²⁵I-labeled digoxin as tracer ("Spak" Digoxin RIA kit; BYK Gulden, the Netherlands). The antibody was prepared by injecting a conjugate of digoxin and bovine serum albumin into goats. This antiserum cross-reacts with digitoxin by 35% but negligibly (<0.01%) with testosterone, progesterone, cortisone, and aldosterone (19).

For preparing the standard curve, we dissolved pure digoxin (Wellcome Laboratories, Pomezia, Italy) in pyridine, then diluted this with phosphate buffer (pH 7.4, 0.15 mol/L) containing 40 g of human serum albumin and 20 mmol of sodium azide per liter. We incubated 0.4 mL of standard solution or reconstituted fractions with 0.5 mL of tracer solution in the test tubes for 12 h at 4 °C. After incubation, we aspirated the supernate and counted for 1–2 min the radioactivity remaining in each tube, using a well-type gamma counter. The mean sensitivity of our RIA was 5.16 (SD 1.1) pg/0.4 mL.

Dose–response curves were set up similarly to that for ouabain (see above). Digoxin concentrations ranged from 7.8 to 1000 pg/0.4 mL and the volumes of original neonate plasma from 1.6 to 33 mL.

Results

Figure 1 shows the HPLC elution profile of a cord blood extract (corresponding to 1 mL of original cord blood) chromatographed with the acetonitrile/methanol/water (17/17/66 by vol) mobile phase, and then 100% methanol. The ⁸⁶Rb uptake revealed two significant peaks of inhibitory activity: the first in fraction 2 (55% inhibition) and the second in fraction 19 (64% inhibition), when the mobile phase was changed to 100% methanol. Fractions 2 and 19 also cross-reacted with anti-digoxin antibodies, and another significant immunoreactive peak was present in fraction 8.

When the corresponding individual fractions, obtained by HPLC elutions of extracts from cord blood, were pooled to increase the concentration of the active substances up to fourfold, only fraction 2 was associated with increasing inhibition of ⁸⁶Rb uptake. On the other hand, when the active fraction eluted with 100% methanol was used, we observed hemolysis when the concentration was increased by >1.5 times. Using the other fractions, we observed no clear trend but rather a high variability of inhibitions, between −30% and +30%.

To obtain a resolution of the early fractions, we chromatographed an extracted sample (corresponding to 1 mL of original cord blood), using a mobile phase of acetonitrile/methanol/water (14/14/72 by vol), and collected 15 fractions of 1 mL (Figure 2). Both profiles of immunoreactivity and inhibitory activity of ⁸⁶Rb uptake revealed the presence of a single peak, eluted with a retention time of 4.7 min, that showed a dose-dependent inhibition of ⁸⁶Rb uptake and of ¹²⁵I-labeled digoxin displacement from antiserum. The dose curves
were parallel to those of ouabain for $^{86}$Rb uptake (Figure 3) and of digoxin for radioimmunoassay (Figure 4).

Chromatographies with adult plasma extracts (corresponding to 1.5 mL of original plasma), performed as described above, gave inhibitory profiles similar to those for cord blood (data not shown). The inhibitory values of the two peaks were respectively 40% and 55%.

**Discussion**

Several laboratories are currently engaged in the study of endogenous cardiac glycoside-like compounds that may inhibit the activity of the cell membrane sodium/potassium pump. Although different groups of molecules—e.g., steroid-like compound(s), some peptides, fatty acids, and phospholipids—have been claimed to have digitalis-like activity, no unique substance has, at present, been completely purified, chemically characterized, and identified (10–14) except for a steroidal dienolide in toad skin (20). Here we describe our preliminary data on the purification of endogenous digitalis-like factor(s) in neonatal plasma, which represents an enriched source of these compounds. In fact, particularly high values of a factor with potential digitalis-like activity have been reported by us and others in samples from neonates (4, 15, 17). Because of the high and continuous ingestion of sodium from the amniotic fluid, an inhibitor of the sodium pump may be of physiological importance in newborns to maintain high sodium excretion by reducing tubular sodium reabsorption (4, 22).

Several studies on HPLC separation of endogenous digitalis-like factors in the plasma and urine of adults have identified partially purified inhibitors of the sodium pump as a series of unsaturated fatty acids and lysophospholipids (2, 11, 23) or as a polar digitalis-like factor, resembling ouabain in polarity, nonpeptidic nature, and molecular mass (24–27). On the other hand, few studies of HPLC separation of endogenous digitalis-like factors in newborns have been done (5, 28, 29).

In our studies we observed the presence of (at least) two digitalis-like activity fractions, corresponding to one or more polar (peak I) and nonpolar (peak II)
substances.

Gault et al. (28), in agreement with our results, also reported the presence of a polar digoxin-like immunoreactive peak (appearing in the first two fractions of HPLC procedure) in cord blood. Seccombe et al. (29) recently reported the presence of several immunoreactive peaks obtained by preparative HPLC fractionation of cord blood but, in contrast to our results, were unable to demonstrate significant inhibition of 86Rb uptake.

Whether the activity present in the first peak reflects one or more substances is uncertain. The relatively broad peak obtained by the second HPLC step could suggest that more than one substance is present in this peak. On the other hand, for the first peak the dose–response curve, both for 86Rb uptake and for digoxin displacement, paralleled respectively that of ouabain and digoxin. This finding, albeit not definite proof, suggests the presence of a compound that shares similarities to cardiac glycosides.

It is unclear whether the second peak represents a truly interacting compound (e.g., a nonpolar steroid) or rather reflects the sum of minor nonspecific effects of several lipophilic compounds eluted from the column with 100% organic phase, which could exert an inhibitory action on 86Rb uptake through a detergent effect on the cells and interfere with the RIA assay by sequestration of the label. Further studies are needed to clarify this point.

Our method, in view of the relatively small volume of plasma required to detect inhibition peaks, could be used for the partial purification of endogenous glycoside-like compound(s) in several human fluids. In fact, the extraction procedure we used, compared with the dialyzation techniques used by others (23–26), could allow a higher recovery of endogenous glycoside-like compound(s) by detaching the moeity bound to proteins, because this substance reportedly is predominantly (90%) bound to plasma proteins (30). However, the high variability of our 86Rb method hinders detection of other fractions with smaller but nonetheless real inhibition.

Whether digitalis-like factor(s) in the plasma of neonates is identical to that in adults remains uncertain and cannot be established until these compounds are unequivocally identified. However, our preliminary finding of similar elution profiles supports this hypothesis.

References
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Osteocalcin Concentrations in Plasma Prepared with Different Anticoagulants

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We investigated the effects on plasma osteocalcin concentrations of different anticoagulants used to collect the blood samples. Plasma osteocalcin concentrations measured by enzyme immunoassay and radioimmunoassay are influenced by the nature of the anticoagulants used. The most significant difference between concentrations found in plasma and serum was seen with oxalate/fluoride anticoagulant, which reduced osteocalcin concentrations to 37.3% of serum values. This is probably related to increased hemolysis with this anticoagulant compared with osteocalcin concentrations in plasma prepared with other anticoagulants. Samples prepared with sodium citrate (0.105 mol/L) or lithium heparin gave values 92.4% and 83.6% of those obtained with matched serum samples. Osteocalcin concentrations were relatively stable in plasma and serum at −20°C for two freeze/thaw cycles. In blood from 100 patients there was a good correlation between osteocalcin concentrations in serum and plasma (lithium heparin) (r² = 0.831); the slope and intercept (±SE) were 0.924 ± 0.04 and 4.92 ± 1.25 μg/L, respectively. However, in 10 patients, serum osteocalcin concentrations were twofold to threefold higher than those in matched plasma samples.

Additional Keyphrases: calcium-binding protein • osteoporosis • bone gla protein • metabolic bone disease • radioimmunoassay • enzyme immunoassay

Osteocalcin is a bone-specific protein consisting of 49 amino acids, three of which are γ-carboxylglutamic acid (1). Synthesized by osteoblasts, osteocalcin enters the blood (2–4), where its concentration in plasma reflects the rate of bone formation rather than the rate of bone resorption (5). Measurements of plasma osteocalcin are used in various clinical situations, including metabolic bone diseases, hyperthyroidism, liver cirrhosis, renal disorders, and diseases related to excessive glucocorticoids (6–12). Among the published immunoassays of osteocalcin (13–17) is one from this laboratory, a relatively rapid enzymoimmunoassay (EIA) involving use of a monoclonal antibody (18).

Published ranges of plasma osteocalcin concentrations in controls' and patients' samples vary widely (19). One reason for this is the use of various polyclonal antisera, which differ in the extent to which they may recognize some of the multiple forms of osteocalcin in the circulation. In addition to intact osteocalcin, other immunoreactive forms in plasma are a high-molecular-mass form (100 kDa) (19, 20) and peptides of lower molecular mass (1000–1500 Da) (21–23).

Here we show that anticoagulants used to prepare plasma samples influence the immunoreactivity of osteocalcin and may represent another reason for the wide range of published osteocalcin values.

Materials and Methods

Subjects: Plasma and serum were prepared from the same blood samples from two groups of patients (A and B), selected without conscious bias in the outpatient department of University College Hospital. Group A consisted of 20 women, ages 30–60 years, who had no clinical history or symptoms of bone-related disorders or conditions known to influence bone metabolism; one patient with anorexia nervosa was under investigation for osteoporosis. Samples from these subjects were used for the studies described in Tables 1–3. Group B consisted of 100 subjects, both men and women: 40, ages 18–38 years, had no clinical history or symptoms of bone-related disorders; 60, ages 50–90 years, included patients with hyperparathyroidism, Paget's disease, osteoporosis, and renal disease. We used 36 samples from these 120 subjects, Groups A and B, selected without conscious bias, for the studies of the stability of osteocalcin.

Serum and plasma: Immediately after collection, portions of each blood sample were put into tubes that contained various anticoagulants or no anticoagulant (for serum preparation). Samples were kept at 4°C until serum and plasma were separated—usually after ~24 h, but some samples were separated after 3 h. The following anticoagulants were used in Vacutainer Tubes (Becton-Dickinson, Meylan Cédex, France): EDTA (75 g/L), lithium heparin (14 300 USP units/L), potassium oxalate/sodium fluoride (10 and 12.5 mg, respectively, per 5-mL tube, i.e., 2.0 and 2.5 g/L), and